EBV Symposium 2004

20th – 25th September
Regensburg, Germany

Tumour Associated Herpesviruses

The 11th Biennial Conference of The International Association for Research on Epstein-Barr Virus and Associated Diseases

Welcome to Regensburg

You will find the venue close to the old town of Regensburg, one of the oldest townships of Germany with still visible portions of arches and walls from Roman times (100 – 200 AD) as integral part of city houses and a nice medieval mediterranean styled inner city. Many palaces, convention halls, monasteries and churches invite you to take a stroll in the city which was home of the first form of a German parliament from 1663 to 1806.

In contrast to the old town, where the very well known scientists Albertus Magnus and Johannes Kepler worked, the University campus is quite new and operates since 1962 for the main campus and since 1992 for the Medical Faculty and University hospitals.

Regensburg cannot be seen without its enjoyable surroundings, the largest natural forest area of central Europe (Bavarian Forest) linking to equally preserved forest areas in the Czech Republic and Austria, the Franconian Jurassic park and the agricultural area flanking the Danube. During the meeting, we have arranged for a trip to Munich to the "Wiesn" (“Münchener Oktoberfest”), the largest public festival in the world.

The organisation of the Symposium has been supported by a very helpful local committee and a wonderful and ever available international board of advisers. Also corporate donors receive our thanks and gratitude. Without their help the meeting would have been more expensive for the participants. Most of all, the submitters of abstracts for talks and posters contribute to the quality and style of the conference and deserve our respect and thanks.

This Symposium shall continue to advance our knowledge on tumor associated herpesviruses. On behalf of the Organizing Committee I welcome you to Regensburg, and trust that everyone enjoys the meeting and the town very much.

With kind regards,
Prof. Dr. Hans Wolf

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11th Biennial Symposium of the International Association for Research on Epstein-Barr Virus and Associated Diseases

would like to thank the following sponsors for their generous financial support

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General Information

Registration desk, lectures, poster sessions, media check room, zip pool, and cafeteria are all located at or around the auditorium maximum of the university campus, the banquet takes place in the old town.

Addresses

Chairman
Prof. Dr. Hans Wolf
Institute of Medical Microbiology and Hygiene
University of Regensburg
Franz-Josef-Strauss-Allee 11
D-93053 Regensburg, Germany
hans.wolf@ebv2004.com

Contact and Messages:
phone: +49 941 943 2530
fax: +49 941 943 2529
e-mail: office@ebv2004.com

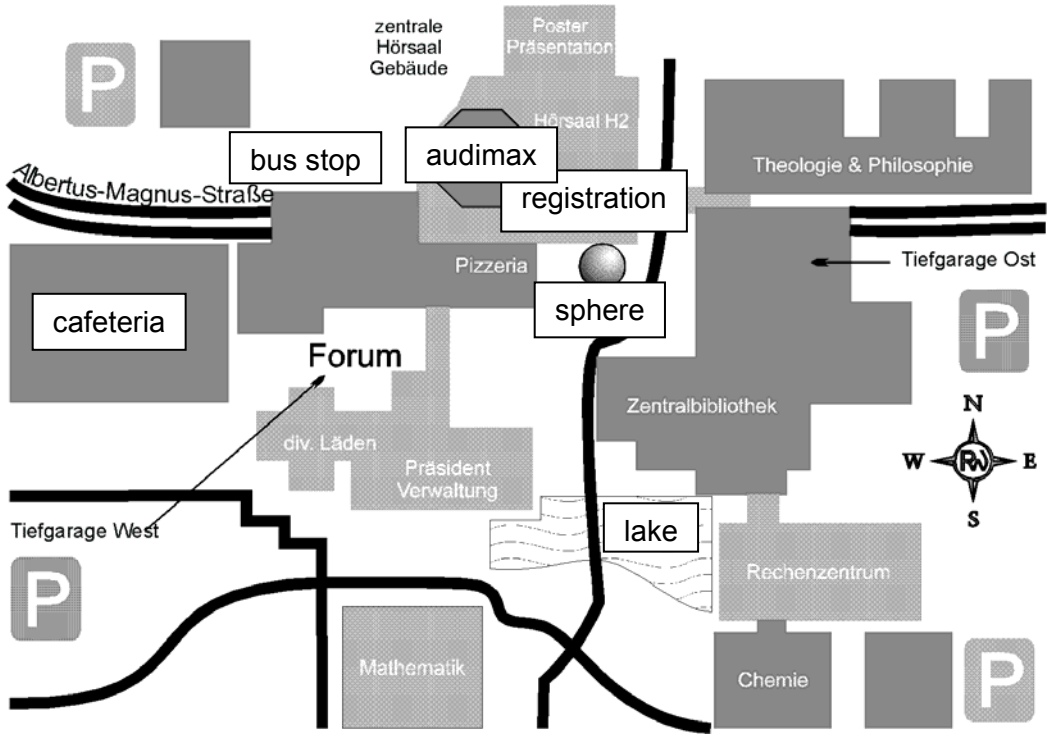
Congress Secretariat
Dr. Hans Helmut Niller
hans-helmut.niller@ebv2004.com
Dr. Fritz Schwarzmann
fritz.schwarzmann@ebv2004.com

Assistant
Ms Berit Brüdigam
phone: +49 941 944 6440
fax: +49 941 944 6402

EBV Association
International Association for Research on Epstein-Barr Virus & Associated Diseases
http://ebvassociation.qimr.edu.au

Registration and Information
COCS – Congress Organisation C. Schäfer
Franz-Joseph-Straße 38
D-80801 Munich
info@cocs.de, phone +49 89 307 1011
fax +49 89 307 1021

Conference Venue
University of Regensburg
Audimax
Universitätsstrasse 31
D-93053 Regensburg



Program at a Glance

	Monday September 20	Tuesday September 21	Wednesday September 22	Thursday September 23	Friday September 24	Saturday September 25
8:00			Oncology	Oncology	Pathomechanisms	
8:15		Welcome				Clinical Aspects
8:30		Pathomechanisms	5 Hodgkin´s Disease	9 Other Malignancies and PTLD		
9:00					10 Oncogenesis and Cell Cycle	13 Diagnostics
9:30		1 Signal Transduction	6 Nasopharyngeal Carcinoma			
10:30		Morning Coffee	Morning Coffee		Morning Coffee	Morning Coffee
11:00		2 Immune Mechanisms	6 Nasopharyngeal Carcinoma		11 Infection and Reactivation	15 Therapy
12:00						
12:30		Lunch	Lunch		Lunch	Closing Remarks
13:00			EBV Board Meeting			Lunch
14:00		Life Cycle	7 Burkitt´s Lymphoma		12 Epigenetics	
15:00	Registration	3 Primary and Persistent Infection		Oktoberfest		
15:30		Afternoon Coffee	Afternoon Coffee			
15:45					Henle Lecture	
16:00		4 Viral Replication	Life Cycle 8 Latency and Gene Regulation			
16:45					Poster Talks	
17:00						
17:30					Free Time	
18:00						
18:30		Dinner	Dinner			
19:00	Reception				Banquet	
20:00		Poster Session 1 Sessions 1 - 7	Poster Session 2 Sessions 8 - 15			
21:00						

08:30 - 10:30 **Session 1: Signal Transduction**
Chairs: Yu-Sun Chang
Martin Rowe

08:30 - 09:00 **01.01** **Opening Lecture**
Kieff E

09:00 - 09:12 **01.02** **Regulation of p55alpha PI3 Kinase subunit by Epstein-Barr Virus**
Spender LC, Bilancio A, Vanhaesebroeck B, Farrell PJ

09:12 - 09:24 **01.03** **LMP-1 signaling to IRF-7, a role for RIP and noncanonical IKKs**
Huye LE, Maier C, Pagano JS

09:24 - 09:36 **01.04** **Characterization of Epstein-Barr Virus Latent Membrane Protein 1 Mediated NFκB Activation**
Luftig M, Yasui T, Soni V, Cahir-McFarland E, Kang MS, Kieff E

09:36 - 09:48 **01.05** **The unique type of TRADD binding site determines the non-apoptotic phenotype of LMP1-induced TRADD signaling**
Neugebauer J, Briseno C, Liefold N, Schneider F, Kieser A

09:48 - 10:00 **01.06** **Epstein-Barr Virus Encoded Latent Membrane Protein 1 Forms a New Heterodimer between c-Jun and Jun B Proteins and Modulates p16 Directly**
Xin S, Tao Y, Deng X, Cao Y

10:00 - 10:12 **01.07** **LMP-1 Inhibits the Expression of SH2D1A/SAP and Induces T Cell Activation: An Alternative Mechanism for EBV-associated Hemophagocytic Syndrome**
Su IJ, Chuang HC, Lay JD, Hsieh WC

10:12 - 10:24 **01.08** **Expression of Latent Membrane Protein Two B Augments Localization of B-cell Proteins: Implication of LMP2B Mediation of Cell Signaling**
Tomaszewski MJ, Rowe DT

10:24 - 10:36 **01.09** **EBV-encoded LMP2A augments cellular proliferation through activation of the mTOR pathway**
Moody CA, Scott RS, Nathan CA, Sixbey JW

10:40 - 11:00 **Morning Coffee**

11:00 - 12:30 **Session 2: Immune Mechanisms**
Chairs: Maria Masucci
Pierre Busson

11:00 - 11:12 **02.01** **Dendritic cells initiate immune control against Epstein Barr virus transformation of B lymphocytes in vitro**
Bickham K, Goodman K, Paludan C, Nikiforow S, Tsang ML, Steinman RM, Munz C

11:12 - 11:24 **02.02** **An Endogenous Superantigen and Disease: Role of HERV-K18 Env in EBV Lymphomagenesis**
Sutkowski N, Huber BT

11:24 - 11:36 **02.03** **Characterization of cytotoxic cord blood-derived CD4+ T cells reactive to Epstein-Barr virus**
MacArthur GJ, Wilson AD, Morgan AJ

11:36 - 11:48 **02.04** **The recognition of Epstein-Barr virus (EBV)-transformed B cell lines by CD4+ T cell clones to EBV latent cycle antigens**
Taylor GS, Long H, Haigh TA, Gudgeon NH, Tsang CW, Leen A, Brooks J, Landais E, Houssaint E, Rickinson AB

11:48 - 12:00 **02.05** **Evasion from HLA class II-restricted T cell immunity during lytic EBV infection**
Ressing ME, van Leeuwen D, Verreck F, Keating S, Gomez R, Schumacher T, Hutt-Fletcher L, Rowe M, Wiertz EJHJ

12:00 - 12:12 **02.06** **Can one cytotoxic T cell bank be used to treat most EBV-positive lymphomas?**
Haque T, Wilkie G, Jones M, Wingate P, McAulay K, Crawford DH

12:12 - 12:24 **02.07** **The Temporal Relationship Between the Onset of EBV Antibodies and Lupus Autoimmunity Supports a Role for EBV in the Development of SLE**
Heinlen LD, McClain MT, Dennis GJ, Harley JB, James JA

12:30 - 14:00 **Lunch**

14:00 - 15:30 **Session 3: Primary & Persistent Infection**
Chairs: Jen-Yang Chen
Takeshi Sairenji

14:00 - 14:12 **03.01** **Initiation of latency in infected B-lymphocytes is the third phase of EBV's life cycle**
Altmann M, Hammerschmidt W

14:12 - 14:24 **03.02** **Epstein-Barr virus can establish a persistent infection in the absence of a classical memory B cell population**
Conacher M, Callard R, McAulay K, Chapel H, Webster D, Kumararatne D, Spickett G, Crawford DH

14:24 - 14:36 **03.03** **EBNA2 and LMP1 heteroduplex analysis of EBV strains in infectious mononucleosis patients : ex vivo material versus in vitro-rescued virus isolates**
Tierney R, Croom-Carter D, Roy S, Edwards R, Sitki-Green D, Raab-Traub N, Rickinson AB

14:36 - 14:48 **03.04** **Epstein-Barr virus structural genomics**
Burmeister WP, Buisson M, Tarbouriech N, Geoui T, Morand P, Cusack S

14:48 - 15:00 **03.05** **High Epstein-Barr-Virus DNA-Load, Multiple Infection and Long-Term Persistence of Infectious Virions in Saliva after Infectious Mononucleosis: A Longitudinal Prospective Study**
Fafi-Kremer S, Morand P, Brion JP, Pavese P, Baccard M, Germi R, Genoulaz O, Nicod S, Ruigrok R, Stahl JP, Seigneurin JM

15:00 - 15:12 **03.06** **Present EBV genome in blood and absent anti-EBV antibody is a novel one-point early diagnostic criterion for EBV primary infection**
Dohno S, Maeda A, Ishiura Y, Imai S, Wakiguchi H

15:12 - 15:24 **03.07** **EBV infection of oropharyngeal mucosal epithelium**
Tugizov S, Herrera R, Xiao J, Berline J, Veluppillai P, Greenspan D, Palefsky JM

15:30 - 16:00 **Afternoon Coffee**

16:00 - 18:30 **Session 4: Viral Replication**
Chairs: Janet Mertz
Bill Sugden

16:00 - 16:12 **04.01** **EBP2 is Important for the Attachment of EBNA1 to Human Mitotic Chromosomes and is Regulated by Ipl1/Aurora Kinases**
Kapoor P, Lavoie BD, Frappier L

16:12 - 16:24 **04.02** **Interactions of EBV capsid and capsid associated proteins with gB and the p38/BFLF2 complex**
Ahuja M, Lake C, Hutt-Fletcher L

16:24 - 16:36 **04.03** **Proteins Associated with Purified Epstein Barr Virus**
Johannsen E, Luftig M, Chase M, Weicksel S, Illanes D, Cahir-McFarland E, Sarracino D, Kieff E

16:36 - 16:48 **04.04** **Viral transforming protein LMP-1 plays a critical role in virus production**
Ahsan MN, Kanda T, Takada K

16:48 - 17:00 **04.05** **Gene regulation in Epstein-Barr virus lytic cycle**
Amon W, Binne UK, Farrell PJ

17:00 - 17:12 **04.06** **Epstein-Barr Virus Rta Response Elements (RREs) Vary Markedly in their Capacity to Bind and to be Activated by Rta**
Chen LW, Chang PJ, Miller G

17:12 - 17:24 **04.07** **Signaling Pathways Affect Expression of Epstein-Barr Virus Latent-Lytic Switch BZLF1 Gene Promoter in Part via ZEB**
Kraus R, Yu X, Perrigoue E, Mertz J

17:24 - 17:36 **04.08** **Phosphorylation of Epstein-Barr Virus ZEBRA Protein at its Casein Kinase 2 Sites Mediates its Ability to Repress Rta Activation of a Viral Lytic Cycle Late Gene by Rta**
El-Guindy AS, Miller G

17:36 - 17:48 **04.09** **Implications for Pathogenesis: Post-translational Control of Epstein-Barr Virus Lytic Cycle Gene BZLF1 is impaired in a Strain Isolated from a Case of Chronic Active Infection.**
Jager M, Konig U, Niller HH, Larcher C, Huemer H, Mitterer M, Wolf H, Schwarzmann F

17:48 - 18:00 **04.10** **Interaction of the Epstein-Barr virus (EBV) mRNA export factor EB2 with a novel human Spen protein, OTT3, suggests a link between Spen proteins and mRNA splicing and export**
Hiriart E, Keppler S, Mikaelian I, Buisson M, Meresse P, Mercher T, Bernard O, Sergeant A, Manet E

18:00 - 18:12 **04.11** **Characterization of the minimal replicator of Kaposi's sarcoma-associated herpesvirus**
Hu JH, Renne R

18:30 - 20:00 **Dinner**

20:00 – 22:00 **Poster Session 1:** Sessions 1 to 7

08:00 - 09:06 **Session 5: Hodgkin's Disease**
Chairs: Riccardo Dolcetti
Hans Wolf

08:00 - 08:30 **05.01** **Molecular Biology of Hodgkin's Lymphoma**
Kueppers R

08:30 - 08:42 **05.02** **The viral etiology of Hodgkin's lymphoma: Epidemiologic evidence for a role of EBV in both EBV(+) and EBV(-) HD**
Mueller NE,Chang E, Levin L, Ambinder R, Lennette E, Zhang T

08:42 - 08:54 **05.03** **Induction of Autotaxin by the Epstein-Barr Virus is specific to Hodgkin's Lymphoma Cells and Promotes their Growth and Survival through the Generation of Lysophosphatidic Acid**
Baumforth KR, Flavell JR, Reynolds GM, Davies GL, Pettit T, Wei W, Morgan SL, Nowakova M, Stankovic T, Pratt G, Aoki J, Wakelam MJO, Young LS, Murray PG

08:54 - 09:06 **05.04** **LMP1 and LMP2 specific T-cell stimulation in EBV+ Hodgkin's disease**
Lauterslager TGM, Klarenbeek JB, Bloemena E, Middeldorp JM

09:06 - 10:30 **Session 6: Nasopharyngeal Carcinoma**
Chairs: Riccardo Dolcetti
Hans Wolf

09:06 - 09:18 **06.01** **Molecular Signatures and Classification of Nasopharyngeal Carcinoma's**
Pegtel DM, Ramaswamy A, Tsai CH, Golub TR, Thorley-Lawson DA

09:18 - 09:30 **06.02** **Alteration of the Global Gene Expression in Nasopharyngeal Carcinoma by EBV Infection**
Lin CT

09:30 - 09:42 **06.03** **Characterisation of the transcriptional and phenotypic consequences of EBV infection of nasopharyngeal carcinoma cells identifies novel cellular targets of EBV**
Waites ER, Stewart SE, Arrand JR, Wei W, Dawson C, Laverick L, Takada K, Young LS, Murray PG

09:42 - 09:54 **06.04** **The role of ID1 expression in immortalization and transformation of nasopharyngeal epithelial cells**
Tsao SW, Li HM, Man C, Yip YL, Young LS, Huang DP

09:54 - 10:06 **06.05** **Expression of (active) caspase 3 in tumour cells relates to clinical outcome in patients with nasopharyngeal carcinoma (NPC)**
Harijadi A, Hariwiyanto B, Haryana SM, Oudejans JJ, Middeldorp JM

10:06 - 10:18 **06.06** **Epstein-Barr virus RK-BARF0 protein inhibits i-MFA-mediated repression of WNT signaling pathway by disruption of I-MFA-TCF/LEF-1 complex formation**
Kusano S, Raab-Traub N

10:18 - 10:30 **06.07** **Expression of the Epstein-Barr virus (EBV)-Encoded Latent Membrane Protein 2A (LMP2A) in EBV-Associated Nasopharyngeal Carcinoma**
Heussinger N, Buttner M, Ott G, Brachtel E, Pilch BZ, Kremmer E, Niedobitek G

10:30 - 11:00 **Morning Coffee**

11:00 - 12:30 **Session 6 continued: Nasopharyngeal Carcinoma**
Chairs: Nancy Raab-Traub
Yi-Xin Zeng

11:00 - 11:30 **06.08** **What do we learn from EB virus encoded LMP1 story? (II) The Role of Signaling Pathways Activated by EBV-Encoded LMP1 in the Carcinogenesis of NPC**
Cao Y

11:30 - 11:42 **06.09** **Dissecting the role of LMP1 signalling in epithelial cells in vivo**
Charalambous C, Wilson JB

11:42 - 11:54 **06.10** **Epstein-Barr Virus Encoded Latent Membrane Protein 1 Modulates Nuclear Translocation of Telomerase Reverse Transcriptase Protein through Activating NF-kB p65 in Human Nasopharyngeal Carcinoma Cells**
Ding L, Yang J, Li L, Tao Y, Ye M, Shi Y, Gong J, Cao Y

11:54 - 12:06 **06.11** **Involvement of cross-talk between c-Jun and Ets-1 in EBV-LMP1 regulating expression of MMP9 in nasopharyngeal carcinoma cells**
Zeng L, Tao Y, Song X , Liu YP, Yi W, Li W, Cao Y

12:06 - 12:18 **06.12** **Evidence of LMP1 interaction with galectin 9 in lipid rafts of malignant NPC cells**
Busson P, Durieu C, Keryer C, Nishi N, Faigle W, Middeldorp JM, Loew D

12:18 - 12:30 **06.13** **EBV is colinear with the immunoglobulin gene loci**
Niller HH, Salamon D, Rahmann S, Koroknai A, Banati F, Ilg K, Schwarzmann F, Wolf H, Minarovits J

12:30 - 14:00 **Lunch**
EBV Board Meeting

14:00 - 15:30 **Session 7: Burkitt's Lymphoma**
Chairs: Georg W. Bornkamm
Lawrence S. Young

14:00 - 14:12 **07.01** **Clonal analysis of Burkitt's Lymphoma cell lines carrying EBNA2-deleted and wild type Epstein-Barr virus genomes**
Hutchings IA, Kelly GL, Bell A, Rickinson AB

14:12 - 14:24 **07.02** **EBV induces co-expression of CD40 and CD40L for host cell survival and transformation**
Shirakata M, Imadome K

14:24 - 14:36 **07.03** **Downregulation of a polyamine regulator, spermidine/spermine N1-acetyltransferase (SSAT), is associated with the EBV-induced tumor phenotype**
Scott RS, Shi M, Davis T, Gan YJ, Su T, Sixbey JW

14:36 - 14:48 **07.04** **The EBERs: Small but Influential?**
Repellin CE, Wilson JB

14:48 - 15:00 **07.05** **Comparison of EBV-specific IFN-γ responses in children with Burkitt's lymphoma to healthy children living in malaria holoendemic and sporadic regions of Kenya.**
Moormann AM, Chelimo K, Tisch DJ, Sumba OP, Kazura JW, Rochford R

15:00 - 15:12 **07.06** **Does Epstein-Barr virus co-infection influence the outcome of Plasmodium falciparum malaria in African children?**
Yone CLRP, Kube D, Kremsner PG, Luty AJF

15:12 - 15:24 **07.07** **Production of high-titer EBV recombinants derived from Akata cells by using a bacterial artificial chromosome system**
Kanda T, Yajima M, Ahsan N, Tanaka M, Takada K

15:30 - 16:00 **Afternoon Coffee**

16:00 - 18:30 **Session 8: Latency & Gene Regulation**
Chairs: Bettina Kempkes
Martin J. Allday

16:00 - 16:30 **08.01** **EBV persistence in vivo - closing the circle**
Thorley-Lawson DA

16:30 - 16:42 **08.02** **Non proliferating EBV-B lymphocyte interactions**
Klein E, Kis LL, Liu A, Nagy N

16:42 - 16:54 **08.03** **EBV Infection Induces the Expression of specific Splice Variants of IRF-5**
Martin H, Kwon HC, Dong SM, Park JH, Hayward SD, Lee JM

16:54 - 17:06 **08.04** **Cell cycle-associated chromatin modifications regulate DNA replication at OriP**
Zhou J, Chau CM, Deng Z, Shiekhattar R, Lieberman PM

17:06 - 17:18 **08.05** **The EBNA1-dependent promoter-enhancer element FR in oriP is regulated by oct-proteins and their co-factors: implications for a viral switch**
Almqvist J, Zou J, Ernberg I

17:18 - 17:30 **08.06** **EBNA-1 Enhances Transcription Through Ying-Yang 1 (YY1)**
Perrigoue J, Nanbo A, Sugden B

17:30 - 17:42 **08.07** **Transcriptional Activation of EBER Expression**
Arrand JR, Chen X, Dawson CW, Felton-Edkins Z, Murray PG, O'Neil JD, Reynolds GA, Wei W, White RJ, Wood VHJ, Yao Y, Young LS

17:42 - 17:54 **08.08** **Epstein-Barr virus latent membrane protein 2A regulates viral and cellular gene expression via modulation of the STAT and NF-κB transcription factor pathways**
Stewart SE, Dawson CW, Takada K, Moody CA, Sixbey JW, Young LS

17:54 - 18:06 **08.09** **Epstein-Barr virus Protein Kinase Phosphorylates EBNA2 and Suppresses EBNA2 Transactivation of the LMP1 Promoter**
Yue W, Gershburg E, Pagano JS

18:06 - 18:18 **08.10** **EBNA-LP preferentially co-activates EBNA2-mediated stimulation of latent membrane proteins expressed from the viral divergent promoter**
Ling P

18:18 - 18:30 **08.11** **The atypical bZIP domain of EBNA 3C forms oligomers not homodimers but has a novel role in maintaining the RBP-Jk interaction**
West MJ, Webb HM, Sinclair AJ, Woolfson DN

18:30 - 20:00 **Dinner**

20:00 - 22:00 **Poster Session 2:** Sessions 8 to 15

08:00 - 09:30 **Session 9: Other Malignancies & PTLD**
Chairs: Irene Joab
Axel zur Hausen

08:00 - 08:12 **09.01** **LMP1 expression induced by cytokines in EBV positive NK malignant cell lines**
Takahara M, Kis LL, Nagy N, Liu A, Klein E

08:12 - 08:24 **09.02** **Distinct Subsets of Primary Effusion Lymphoma Can Be Identified Based on Their Cellular Gene Expression Profile and Viral Association**
Fan W, Bubman D,Chadburn A, Harrington WJ, Knowles DM, Cesarman E

08:24 - 08:36 **09.03** **Expression Of MHC Class I And II In Gastric Carcinomas N.O.S. In Relation To T-Cell Infiltrate: Differences Between Epstein Barr Virus Positive And Negative Tumours**
van Beek J, Bloemena E, Snel A, Vos W, van de Velde CJ, Kranenbarg EK, Meijer CJLM, Middeldorp JM

08:36 - 08:48 **09.04** **Loss of EBV-specific CD4+T cells during progression to EBV-related AIDS non-Hodgkin Lymphoma: restoration by highly active antiretroviral therapy (HAART)**
Piriou E, van Dort K, Nanlohy N, van Oers M, Miedema F, van Baarle D

08:48 - 09:00 **09.05** **The Frequency and Characterization of Ig-null CD19+ B Cells in Pediatric Solid Organ Transplant Recipients**
Schauer E, Green M, Webber S, Rowe DT

09:00 - 09:12 **09.06** **Mechanisms underlying the antiproliferative effects of retinoic acid in EBV-immortalized B lymphocytes: further support for a therapeutic role in the management of EBV-related lymphoproliferations**
Dolcetti R, Zancai P, Guidoboni M, Dal Col J, Cariati R, Rizzo S, Boiocchi M

09:12 - 09:24 **09.07** **Monitoring of Epstein-Barr virus (EBV) DNA load after hematopoietic stem cell transplantation for prevention, early diagnosis as well as antiviral and immune therapy of EBV-associated lymphoproliferative diseases**
Meerbach A, Gruhn B, Hafer R, Zintl F, Wutzler P

9:30 – 19:00 **Oktoberfest**

08:00 - 10:30 **Session 10: Oncogenesis & Cell Cycle**
Chairs: Friedrich Grasser
Tadamasa Ooka

08:00 - 08:30 **10.01** **Tumor suppressor function of RASSF1A in nasopharyngeal carcinoma**
Lo KW

08:30 - 08:42 **10.02** **Characterization of BARF1 protein: its mitogenic activity**
Sall A, Caserta S, Jolicoeur P, Franqueville L, de Turenne-Tessier M, Ooka T

08:42 - 08:54 **10.03** **EBV LMP-1 Half-Life and Signaling in Epithelial Cells is Down-Regulated by lyLMP-1**
Pandya J, Walling DM

08:54 - 09:06 **10.04** **Induction of id1 and id3 by latent membrane protein 1 of Epstein-Barr virus and regulation of p27/kip and cyclin dependent kinase 2 in rodent fibroblast transformation**
Everly DN, Mainou BA, Raab-Traub N

09:06 - 09:18 **10.05** **What do we learn from EB virus encoded LMP1 story? (I) Effect of LMP1 on the Cell Cycle**
Cao Y

09:18 - 09:30 **10.06** **EBV Nuclear Antigen 3C Modulates Cyclin A-Associated Kinase Activity**
Knight J, Robertson E

09:30 - 09:42 **10.07** **The EBNA-3 gene family proteins disrupt the G2/M checkpoint**
Sculley T, Krauer K, Burgess A, Buck M, Flanagan J, Gabrielli B

09:42 - 09:54 **10.08** **The Epstein-Barr virus immediate early gene BRLF1 influences the in-vitro and in-vivo growth of lymphoblastoid cell lines.**
Hong GK, Kenney SC

09:54 - 10:06 **10.09** **Cooperation of exogenous and endogenous viruses: Human endogenous retrovirus K (HERV-K) Np9 protein downregulates Epstein-Barr nuclear antigen (EBNA2) activated promoters**
Spurk A, Pfuhl T, Armbruster V, Sauter M, Muller-Lantzsch N, Grasser FA

10:06 - 10:18 **10.10** **Dominant negative derivatives of EBNA1 inhibit survival of EBV(+) and KSHV(+) PEL-derived cells**
Mack AA, Sugden B

10:18 - 10:30 **10.11** **Interaction of Marek's Disease Virus Meq protein with CtBP in the pathogenesis of Marek's disease: lessons from and for EBV**
Brown A, Hickabottom M, Nair V, Allday MJ

10:30 - 11:00 **Morning Coffee**

11:00 - 12:30 **Session 11: Infection & Reactivation**
Chairs: Ingemar Ernberg
Fred Wang

11:00 - 11:12 **11.01** **Epstein-Barr virus infection of naive and memory B cells in vitro**
Begue Pastor N, Baldwin G, Rickinson AB, Delecluse HJ

11:12 - 11:24 **11.02** **Characterization of EBV gH residues important for B cell and epithelial cell fusion**
Omerovic J, Longnecker R

11:24 - 11:36 **11.03** **Mutational Analysis of EBV gp42 Reveals New Binding Domain Essential for Membrane Fusion**
Silva A, Omerovic J, Kirschner A, Jardetzky T, Longnecker R

11:36 - 11:48 **11.04** **Herpesvirus Reactivation in the JSC-1 Lymphoma Cell Line and the Effects on Cellular and Viral Gene Expression**
Bagni RK, Marshall VA, Yuan C, Hines-Boykin R, Dittmer D, Vahrson W, Whitby D

11:48 - 12:00 **11.05** **Transfer Infection: a novel and efficient method of epithelial cell infection**
Shannon-Lowe C, Rickinson AB, Delecluse HJ

12:00 - 12:12 **11.06** **Novel Roles For The Basic Region Of Zta for both Transactivation And Viral Latency Reactivation Revealed by Mutation Of C189**
Valencia S, Schelcher C, Delecluse HJ, Sinclair AJ

12:12 - 12:24 **11.07** **EBV and multiple sclerosis**
Ascherio A, Munger K

12:30 - 14:00 **Lunch**

14:00 - 16:00 **Session 12: Epigenetics**
Chairs: Janos Minarovits
Qian Tao

14:00 - 14:30 **12.01** **The Human Epigenome Project**
Beck S

14:30 - 14:42 **12.02** **TSLC1 involvement in nasopharyngeal carcinoma**
Lung M, Lung HL, Xie D, Guan XY,Murakami Y

14:42 - 14:54 **12.03** **Genome-wide screening for candidate tumor suppressor genes in nasopharyngeal carcinoma (NPC) using epigenetic/genetic approaches**
Tao Q, Ying J, Qiu GH, Tan J, Liu D

14:54 - 15:06 **12.04** **Hyperacetylation of histones H3 and H4 at Zp and Rp does not result in activation of the EBV lytic cycle**
Countryman J, Gradoville L, Heston L, Miller G

15:06 - 15:18 **12.05** **The EBV lytic switch protein, Z, activates the methylated viral genome through a novel mechanism**
Bhende PM, Seaman WT, Delecluse HJ, Kenney SC

15:18 - 15:30 **12.06** **Dynamic Chromatin Boundaries Delineate a Latency Control Region of Epstein-Barr Virus**
Chau CM, Lieberman PM

15:30 - 15:42 **12.07** **High resolution methylation analysis and in vivo protein-DNA interactions at latent Epstein-Barr virus promoters Cp and Qp in the nasopharyngeal carcinoma cell line C666**
Minarovits J, Bakos A, Banati F, Takacs M, Salamon D, Schwarzmann F, Wolf H, Niller HH

15:45 - 16:45 Chair: Paul J. Farrell

HENLE LECTURE
Alan B Rickinson

16:45 - 17:30 Chairs: Hans Helmut Niller
Fritz Schwarzmann

Poster Talks

17:30 - 19:00 **Free Time**

19:00 - 22:00 **Banquet**

08:15 - 09:45 **Session 13: Diagnostics**
Chairs: Andrew Morgan
Denis Moss

08:15 - 08:45 **13.01** **Improving survival, expansion and persistence of adoptively-transferred tumor-specific CTLS**
Rooney CM, Huls MH, Bollard C, Straathof KC, Okamura T, Gottschalk S, Brenner MK, Heslop HE

08:45 - 08:57 **13.02** **Non-invasive diagnosis of nasopharyngeal carcinoma by detection of EBV DNA load plus BARF1 and EBNA1 mRNA in Nasopharyngeal brushings**
Stevens SJC, Verkuijlen SAWM, Hariwiyanto B, Harijadi H, Fachiroh J, Paramita DK, Tan IB, Haryana SM, Middeldorp JM

08:57 - 09:09 **13.03** **The role of Epstein-Barr virus DNA measurement in plasma in the clinical management of Dutch nasopharyngeal carcinoma (NPC) patients**
Kroes ACM, Kalpoe JS, Douwes Dekker PB, Claas ECJ, van Krieken JHJM, Baatenburg de Jong RJ

09:09 - 09:21 **13.04** **EBV serology: stochastics and basic patterns**
Bauer G

09:21 - 09:33 **13.05** **Induction of MHC class-I and -II restricted epitope presentation by urea-adjuvated soluble proteins: A novel technology for the simultaneous restimulation of various populations of antigen-specific T-cells**
Barabas S, Bauer T, Weinberger B, Pullmann K, Lindner J, Wagner R, Jilg W, Wolf H, Deml L

09:33 - 09:45 **13.06** **Cancer Cell's Secreted Proteome as a Basis for Searching Potential Tumor Markers - Nasopharyngeal Carcinoma as a Model**
Wu CC, Chien KY, Tsang NM, Chang KP, Hao SP, Tsao CH, Huang WC, Chang YS, Yu JS

09:45 - 10:30 **Session 14: Vaccines**
Chairs: Andrew Morgan
Denis Moss

09:45 - 09:57 **14.01** **Vaccination against Epstein-Barr virus (EBV): report of phase ii studies using recombinant viral glycoprotein gp350 in healthy adults**
Denis M, Haumont H, Bollen A

09:57 - 10:09 **14.02** **Pre-clinical studies to assess the efficacy of a polyepitope vaccine for nasopharyngeal carcinoma**
Duraiswamy J, Tellam J, Connolly G, Cooper L, Davis J, Tscharke D, Corban M, Smith M, Busson P, Khanna R, Moss DM

10:09 - 10:21 **14.03** **An Epstein-Barr virus (EBV) BDLF2 knock-out mutant lacking expression of LMP1, EBNA1, and EBNA2 and failing in immortalization of B-Lymphocytes as a potential EBV life vaccine aiming at sterile immunity**
Kleines M, Schellenberg K, Ritter K, Scheithauer S

10:30 - 11:00 **Morning Coffee**

11:00 - 12:30 **Session 15: Therapy**
Chairs: Sofia M. Haryana
Stephen Gottschalk

11:00 - 11:12 **15.01** **Retargeting EBV-specific CTL to ErbB2 positive tumors for the adoptive immunotherapy of breast cancer**
Gottschalk S, Ratnayake M, Brenner MK, Rooney CM

11:12 - 11:24 **15.02** **Fas down-modulation in EBV-specific Cytotoxic T-Lymphocytes (CTLs) reduces their sensitivity to Fas/FasL-induced apoptosis**
Savoldo B, Pule M, Straathof KC, Biagi E,Yvon E,Vigouroux S, Brenner MK, Rooney CM, Dotti G

11:24 - 11:36 **15.03** **Identification of LMP2-epitopes in cytotoxic T cell lines (CTL) from patients with nasopharyngeal carcinoma (NPC) and Hodgkin's disease (HD)**
Straathof KC, Bollard C, Leen AM, Heslop HE, Rooney CM

11:36 - 11:48 **15.04** **Production of T cell populations for immuonotherapy of EBV malignancy by the transfer of LMP2-specific T cell receptor sequences**
Jurgens L, Khanna R, Weber J, Orentas R

11:48 - 12:00 **15.05** **Peptides encoding conformational epitopes and generating high affinity anti-LMPs antibodies for immunotherapy of EBV-associated diseases**
Tranchand-Bunel D

12:00 - 12:12 **15.06** **Methotrexate induces reactivation of latent Epstein-Barr virus (EBV): a potential contributor to methotrexate-associated lymphomas**
Feng W, Cohen J, Fischer S, Li L, Sneller M, Goldbach-Mansky R, Raab-Traub N, Delecluse HJ, Kenney SC

12:12 - 12:24 **15.07** **NF-kB is essential for progression of KSHV- and EBV-infected lymphomas in vivo**
Keller SA, Hernandez-Hopkins D, Schattner E, Cesarman E

12:30 - 13:00 **Closing Remarks**

13:00 - 14:00 **Lunch**

Regulation of p55alpha PI3 Kinase subunit by Epstein-Barr Virus

Spender LC, Bilancio A, Vanhaesebroeck B, Farrell PJ

Contact:
Dr. Lindsay Spender
Ludwig Institute for Cancer Research
Imperial College School of Medicine, St Mary's Campus (London, UK)
Email: l.spender@ic.ac.uk

Abstract:
Objective: Signalling via the phosphoinositide-3-kinase (PI3K) pathway is essential for the proliferation of lymphoblastoid cell lines. We previously reported that RNA for a regulatory subunit of the PI3K pathway is induced by EBNA-2. Our aim was to study the PI3K pathway during immortalisation and to investigate the role of the regulatory subunit induced by EBNA-2.

Methods: EREB2.5 cells containing EBNA-2 were used to study early PI3K signalling during EBV-induced proliferation. The components and substrates of the signalling pathway were analysed by western blotting and immunoprecipitation and the sensitivity of cells to PI3K inhibitors was tested by proliferation assay.

Results: PI3K activation was essential for EBNA-2 induced proliferation since treatment of cells with LY294002 (an inhibitor of PI3K catalytic subunits) prevented EREB2.5 proliferation. Signalling through PI3K, assayed by detection of phosphorylated AKT, occurred within 2 hours of estrogen addition. The EBNA-2 target gene LMP-2A, a protein known to constitutively activate AKT, was produced later, suggesting that EBNA-2 may have an earlier function involved in PI3K signalling. Constitutive LMP-1 expression in EREB2.5 cells also had no effect on this early PI3K signalling.
Analysis of the PI3KR1 family of splice variants revealed that the p55alpha isoform was induced by EBNA-2, not p85alpha which is the usual regulatory subunit. p55alpha specific RNA was induced in the presence of protein synthesis inhibitors. Following induction, the p55alpha regulatory subunit associated with the p110delta catalytic subunit present in EREB2.5 cells. The importance of signalling through p110delta was confirmed by use of a specific inhibitor D000 that prevented cell proliferation. Analysis of a wide range of LCLs revealed that expression of p55alpha varied with cell background and virus strain.

Conclusions: PIKR1 p55alpha, a regulatory subunit of the PI3K signalling pathway, is a direct target of EBNA-2 and associates with catalytic components crucial to EBNA-2 driven cell proliferation. The expression of p55alpha in LCLs varies with cell background and virus strain. This provides a unique opportunity to study the function of PI3KR1 isoforms in human B cells and their relationship to biological properties of different EBV strains.

LMP-1 signaling to IRF-7, a role for RIP and noncanonical IKKs

Huye LE, Maier C, Pagano JS

Contact:
Dr. Leslie Huye
University of North Carolina at Chapel Hill
Lineberger Comprehensive Cancer Center (Chapel Hill, USA)
Email: leslie_huye@med.unc.edu

Abstract:
Objective: The major EBV oncoprotein, LMP-1, and interferon regulatory factor 7 (IRF-7) are intimately associated. LMP-1 both induces IRF-7 gene expression and activates IRF-7 protein, which is an essential mediator of the type I interferon response, and IRF-7 likely plays an important role in LMP-1 signaling. Here, we have studied the LMP-1 signaling pathway that activates IRF-7.

Methods: LMP-1 activation of IRF-7 was analyzed in 293 cells using IRF-7 responsive promoter-reporter constructs as readouts of IRF-7 activity. LMP-1 mutants were used to determine which domains are necessary for activation of IRF-7. We inhibited the expression of RIP, IKKi, and TBK1 using RNAi in 293 cells and analyzed the ability of LMP-1 to activate IRF-7 in RIP-deficient Jurkat cells.

Results: LMP-1 enhancement of IRF-7 activity is significantly reduced (50-70% or more) by LMP-1 mutants with deletions of CTAR2 or mutations in the YYD motif. Overexpression of RIP, a serine/threonine kinase that binds LMP-1 CTAR2 through the YYD motif, by itself enhanced IRF-7 activity in a dose-dependent manner, and disrupting RIP expression by two different methods, RNAi and cellular mutation, resulted in a decrease (~50%) in LMP-1 activation of IRF-7, correlating with the 50-70% reduction in activity observed with the LMP-1 YYD mutant. In addition, knocking down IKKi or TBK1 expression with RNAi resulted in a ~50% reduction in LMP-1-induced IRF-7 activity as well as a reduction in RIP activation of IRF-7.

Conclusions: These results point to a novel function for RIP in LMP-1 activation of IRF-7 and indicate that IKKi and TBK1, two noncanonical IKKs that have not been linked with LMP-1 signaling, are components of the signaling pathway that activates IRF-7. Moreover, these data suggest that IKKi and TBK1 may be downstream of RIP in the activation pathway.

Characterization of Epstein-Barr Virus Latent Membrane Protein 1 Mediated NFκB Activation

Luftig M, Yasui T, Soni V, Cahir-McFarland E, Kang MS, Kieff E

Contact:
Dr. Micah Luftig
IRBM P. Angeletti (Pomezia, Italy)
Email: micahluftig@merck.com

Abstract:
Epstein-Barr virus latent membrane protein 1 mimics a constitutively active TNF receptor in activation of NFκB, JNK, and p38 signal transduction pathways. The activation of NFκB is required for the survival of EBV-transformed B lymphocytes and thus represents a model system for studying the contribution of LMP1 in the molecular pathogenesis of EBV-associated malignancies. In this study, we have analyzed the contributions of signaling molecules in LMP1 mediated NFκB activation using knockout murine embryonic fibroblasts and somatic cell mutants. We have found that LMP1 mediated NFκB-dependent transcription surprisingly requires TRAF6, but not TRAFs 2 and 5. Further, IKKb, but not IKKa or IKKg are required for LMP1 mediated NFκB activation. The deletion of the essential TIR signaling molecule IRAK4 did not effect LMP1 mediated NFkB activation, where the TRAF6-associated IRAK1 was critical. Thus, LMP1 induces IKKb-dependent, IKKa/IKKg-independent NFκB activation in a unique TRAF6/IRAK1-dependent, but IRAK4-independent manner.
The signal-induced processing of the p100/NFkB2 precursor to p52 downstream of LTbR, BAFF-R, TLRs, and CD40 depends on a novel IKKb/g-independent, NIK/IKKa-dependent activity referred to as non-canonical NFkB activation. The constitutive expression of the mature p52/NFkB2 subunit in EBV-transformed lymphoblastoid cell lines and LMP1 expressing epithelial cells led to the investigation of the genetic requirements for LMP1 mediated p100 processing. The C-terminus of NIK interacts with both TRAFs and IKKs and the mutation of a single amino acid in this region rendered NIK non-functional in mice. Here we showed that this mutation resulted in the inability of NIK to associate with IKKa, while retaining its ability to bind to TRAFs. Further, the specific nature of this C-terminal mutation in the disruption of NIK-IKKa complexes was used to demonstrate the importance of the NIK-IKKa interaction in LMP1 mediated p100 processing. As expected, IKKb and IKKg were not required for this pathway. Analysis of endogenous gene expression downstream of LMP1 in IKK KO MEFs indicated the existence of three pathways; i) canonical IKKb/IKKg-dependent, ii) non-canonical IKKa-dependent, and iii) atypical IKKb-dependent/IKKg-independent.
While the activation of NFκB-dependent reporter activity by LMP1 mutants lacking each of its transformation effector sites (TES1 or 2) was 50% of wild-type activity, the induction of p100 processing was predominantly mediated through the TRAF-binding site, TES1, suggesting a general mechanism for the initiation of non-canonical NFkB activation.

The unique type of TRADD binding site determines the non-apoptotic phenotype of LMP1-induced TRADD signaling

Neugebauer J, Briseno C, Liefold N, Schneider F, Kieser A

Contact:
Dr. Arnd Kieser
GSF-National Research Center for Environment and Health
Dept. of Gene Vectors (München, Germany)
Email: a.kieser@gsf.de

Abstract:
The LMP1 oncoprotein of EBV acts as a constitutively active receptor-like molecule. LMP1 can immortalize and transform cells by recruiting the TNF-receptor-associated death domain protein (TRADD) through a unique TRADD binding site in its CTAR2 domain. In TNF-receptor 1 (TNFR1) signaling, TRADD binds to the receptor's death domain to induce apoptosis. We want to understand the molecular basis for the counterintuitive role of TRADD in LMP1-induced cell transformation. Here, we performed domain swapping experiments between the signaling domains of LMP1 and TNFR1 to reveal whether the phenotypical readout of TRADD signaling is encoded by the type of TRADD binding site or the receptor context. The death domains of a constitutively active LMP1-TNFR1 fusion protein and wildtype TNFR1 were replaced by the 17 C-terminal amino acids (pos. 370 to 386) of LMP1. The resulting chimeras, LMP1-TNFR1-CTAR2 and TNFR1-CTAR2 were further analysed. Amino acids 370 to 386 of LMP1 were not only essential but also sufficient to recruit TRADD into membrane lipid rafts and to induce JNK1 and NF-kappaB also in the TNFR1 context. Thus, these 17 amino acids must encompass the complete functional TRADD binding domain of LMP1. Moreover, the LMP1-specific type of signaling was co-transferred with amino acids 370 to 386 to TNF-receptor 1. NF-kappaB and JNK1 activation by amino acids 370-386 of LMP1 was dependent on TRAF6 in the context of both receptors. LMP1 amino acids 370 to 386 did not require TRAF2 for JNK1 activation, irrespective of the receptor context. A novel type of transient cell death assay was developed to study the chimera's apoptotic potential in B-cells. In contrast to the TNFR1 death domain, the TRADD binding site of LMP1 did not induce apoptosis in the TNFR1 context. From these data we conclude that the type of TRADD binding site intrinsically determines a specific signaling network and the biological readout of TRADD signaling. EBV found a unique way to exploit the cellular pro-apoptotic TRADD protein to cause immortalization and cell transformation.

Epstein-Barr Virus Encoded Latent Membrane Protein 1 Forms a New Heterodimer between c-Jun and Jun B Proteins and Modulates p16 Directly

Xin S, Tao Y, Deng X, Cao Y

Contact:
Dr. Song Xin
Cancer Research Institute, Xiangya School of Medicine
Central South of University (Changsha, China)
Email: xsongcn@hotmail.com

Abstract:
Epstein-Barr virus (EBV) encoded latent membrane protein 1 (LMP1) is considered to be the major oncogenic protein of EBV encoded proteins which could transactivate many transcription factors including activator protein 1 (AP-1). We confirmed that p16 was a putative target gene of AP-1 using bioinformatics. We found that LMP1 could mediate a new heterodimers form of c-Jun and Jun B in a time-course dependent manner, and phosphorylation of c-Jun ser63 and ser73 involved in the heterodimer formation of c-Jun and JunB. The new heterodimer mediated by LMP1 could bind to the p16 promoter region and downregulated both the promoter activity of p16 and p16 expression, and accel erated the G1/S stage of cell cycle progression. These findings establish a new direct connection between AP1 signal pathway and cell cycle, and provide a new model for the carcinogenesis mechanism.

LMP-1 Inhibits the Expression of SH2D1A/SAP and Induces T Cell Activation: An Alternative Mechanism for EBV-associated Hemophagocytic Syndrome

Su IJ, Chuang HC, Lay JD, Hsieh WC

Contact:
Prof. Ih-Jen Su
National Health Researth Institute
Division of Clinical Research (Tainan, Taiwan)
Email: suihjen@nhri.org.tw

Abstract:
Epstein-Barr virus (EBV) has been associated with Duncan's disease or X-linked lymphoproliferative disease (XLP), a childhood hemophagocytic syndrome characterized by hyperimmune T cell response, cytokine storm, macrophage activation and hemophagocytosis. Genomic study has identified the specific mutations of the SH2D1A/SAP gene which is involved in SLAM/SAP signaling pathway. SAP protein respresents a suppressor molecule of SLAM signaling pathway. The mutation of SAP will lead to an overimmune T cell response. In Taiwan and Japan, there is a prevalence of EBV-associated childhood hemophagocytic syndrome (EBVAHS). However, no evidence of X-linked phenomenon is observed. The molecular basis and pathogenesis of these 'sporadic cases' of EBVAHS remains to be clarified. In this study, we investigated the genomic sequence of SAP gene on 11 cases of childhood EBVAHS and no mutation of SAP gene was detected, indicating that these sporadic cases of EBVAHS did not belong to X-linked XLP in Taiwan. We next studied whether LMP-1 may suppress the function of SAP gene and behave in a similar way like that in XLP. Construction of SAP plasmids and expression of SAP and LMP-1 were studied on a series of B cells and T cells. We demonstrated that LMP-1 can effectively suppress the expression of SAP in T cells, and the suppression of SAP can be reverted by TRAF-2,5. The suppression of SAP by LMP-1 further led to T cell activation as evidenced by the enhanced secretion of cytokine TNF-alpha and phosphorylation of a few kinases. Our studies provide an alternative role of SAP in the pathogenesis of EBVAHS.

Expression of Latent Membrane Protein Two B Augments Localization of B-cell Proteins: Implication of LMP2B Mediation of Cell Signaling

Tomaszewski MJ, Rowe DT

Contact:
Monica Tomaszewski
University of Pittsburgh
Department of Infectious Disease and Microbiology (Pittsburgh, USA)
Email: mjt7@pitt.edu

Abstract:
Objective: The Epstein-Barr Virus (EBV) Latent Membrane Protein Two (LMP2) gene is expressed in immortalized cells and in many EBV-associated disease states. It has been proposed to have a role in the establishment and maintenance of latency. Two mRNAs produce nearly identical protein isoforms. The LMP2a protein isoform provides a ligand-independent signal similar to the B cell receptor while the LMP2b isoform, which is structurally similar to LMP2a but lacks the cytoplasmic signaling domain, is postulated to be a negative regulator of LMP2a. We have determined which parts of the transmembrane (TM) domains of LMP2b are required for localization to perinuclear compartments and for interaction with both viral and cellular encoded proteins.

Methods: A panel of fluorescent and epitope tagged LMP2b truncation mutants were constructed. Progressive truncations were made from the N and the C terminus removing 2 TM segments at a time. These mutants were used to assess the localization and aggregation properties of these proteins in comparison with wild type LMP2b. In addition epitope loop-tagging allowed detection of the orientation and surface expression of LMP2b to be determined in live cells. Stable cell lines of LMP2b mutants were used to assess the interaction of the truncation mutants with virally encoded proteins and with endogenously produced cellular proteins (CD19 and B cell tetraspanins CD9 and CD81) using flow cytometry and fluorescence microscopy.

Results: LMP2b is uniquely and specifically able to sequester proteins involved in B cell signaling. The integrity of the transmembrane region of LMP2b is critical to intracellular localization and protein-protein interaction. LMP2b looses the ability to interact with itself or cellular proteins upon truncation. The effects of these alterations on the phosporylation state of signaling proteins were determined.

Conclusions: The localization of LMP2b to the trans-Golgi network appears to be critical to its function. This study demonstrates that there are exclusive LMP2b protein-protein interactions that are not shared by LMP2a. These data suggest that LMP2b can alter cell signaling pathways and that it is not merely a simple (and negative) regulator of LMP2a signaling.

EBV-encoded LMP2A augments cellular proliferation through activation of the mTOR pathway

Moody CA, Scott RS, Nathan CA, Sixbey JW

Contact:
Cary Moody
Louisiana State University Health Sciences Center-Shreveport
Microbiology and Immunology, Center for Molecular and Tumor Virology (Shreveport, USA)
Email: cmoody@lsuhsc.edu

Abstract:
Epstein-Barr virus (EBV) is sporadically linked to an increasing array of human cancers. The ubiquitous, life-long infection characteristic of EBV raises the possibility that, rather than acting as tumor initiator in all virally linked cancers, EBV may enter pre-existing neoplastic lesions to drive malignant progression. In carcinoma cells where EBV genes were experimentally introduced by infection or transfection, the EBV latency protein LMP2A activated an essential mediator of cellular growth signals, the mammalian target of rapamycin (mTOR) that is downstream of PI3-kinase/Akt. Activated mTOR signaled through its translational effectors eIF4E and 4E-BP1 to augment protein synthesis of growth-related genes as well as cell proliferation, each of which was suppressed by treatment with the inhibitor of mTOR, rapamycin, or the PI3-kinase inhibitor, wortmannin. Signaling through mTOR was proportionate to LMP2A expression levels, and both varied inversely to reiterations of EBV terminal repeats. LMP2A intervention in mTOR-mediated protein translation may be insufficient for tumorigenesis. However in a context of viral opportunism where reactivation of endogenous EBV leads to infection of cancers, it may advance disease progression.

EBV downregulates components of the toll-like receptor (TLR) signal transduction pathway

Broderick P, Hubank M, Sinclair AJ

Contact:
Peter Broderick
University of Sussex
Life Sciences (Brighton, UK)
Email: p.m.broderick@sussex.ac.uk

Abstract:
Objective: Identify host genes expressed differentially by EBV

Methods: Gene expression was compared between the EBV Burkitt's lymphoma cell-line Akata and AK31 an EBV-negative Akata cell-line. Cell gene expression was profiled in presence and absence of stimulation through surface IgG. Comparisons were made using Affymetrix HG-U95Av2 GeneChips (each condition was undertaken in triplicate) and analysis was carried out using Affymetrix Microarray Suite and Silicon Genetics GeneSpring. Flow cytometry, western blotting and cell survival/cell cycle experiments were used to investigate selected genes further.

Results: Gene expression of the EBV positive Burkitt's lymphoma cell Akata was compared to the EBV-negative Akata cell-line AK31. Selection of differentially expressed genes was made on the basis of GeneChip detection call, fold change and significance of change (P value). For an initial analysis only those genes that were differentially expressed between Akata and AK31 both in the presence and absence of surface IgG stimulation were considered. This produced a list of 18 genes, of these 9 were signalling related, 2 were transcription related, 1 was apoptosis related, two were known tumour suppressors and four had other functions. Three of the EBV regulated genes, Bruton tyrosine kinase (Btk), Sab and MyD88 were involved in antigen detection signalling. All three were down-regulated. Btk is involved in B cell receptor and toll-like receptor (TLR) signalling, Sab interacts with Btk and MyD88 is an important adaptor molecule linking TLR and interleukin1 receptor (IL-1R) signalling. The relevance of the regulation of these genes by EBV was further investigated. We found that 3 TLR proteins are expressed by Akata and AK31: TLR3, TLR9 and RP105 (a TLR related receptor). We are currently using these 3 TLRs to question the relevance of Btk, Sab and MyD88 down-regulation by comparing survival/proliferation in TLR-ligand stimulated cells.

Conclusions: The effect of EBV on gene expression was analysed using Affymetrix GeneChips. 18 genes were found to be differentially expressed in the presence and absence of surface IgG activation. Of these 18 genes, 3 (Btk, Sab and MyD88) are components of the TLR pathway.

The rhesus homologue of Epstein-Barr virus LMP2A activates β-catenin signaling in epithelial cells

Siler C, Wang F, Raab-Traub N

Contact:
Catherine Siler
University of North Carolina-Chapel Hill
Lineberger Comprehensive Cancer Center (Chapel Hill, USA)
Email: csiler@med.unc.edu

Abstract:
Objective: Rhesus lymphocryptovirus (LCV) is a γ-herpesvirus closely related to Epstein-Barr virus. The LCV latent membrane protein 2A (LMP2A) is highly homologous to EBV LMP2A as the transmembrane domains and the cytoplasmic domain associated with signaling are well conserved. EBV LMP2A has been shown to activate the phosphatidylinositol 3-kinase (PI3K) and β-catenin signaling pathways in epithelial cells and affect epithelial cell differentiation. This study was to characterize the biochemical and biologic properties of LCV LMP2A in epithelial cells.

Methods: Telomerase immortalized human foreskin keratinocyte (HFK) stable cell lines expressing LCV LMP2A or vector alone were generated using a retroviral vector. Whole cell lysates were generated, subjected to SDS-PAGE, and Western blot analysis performed to detect constituents of the PI3K and β-catenin signaling pathways. Cellular fractionations were performed and analyzed by Western blot to ascertain cytoplasmic and nuclear levels of β-catenin. Stable cell lines were also used in a cellular differentiation assay and analyzed by Western blot.

Results: The expression of LCV LMP2A in HFK cells induced the activation and phosphorylation of Akt. The phosphorylated Akt was functional as demonstrated by the subsequent phosphorylation of the Akt targets, glycogen synthase kinase (GSK3b) and Forkhead transcription factor (FKHR). Cellular fractionation experiments revealed increased levels of cytoplasmic and nuclear β-catenin in LCV LMP2A expressing cells. In methylcellulose-induced differentiation assays, expression of cellular differentiation markers were reduced in LCV LMP2A expressing epithelial cells.

Conclusions: The expression of LCV LMP2A in epithelial cells induces Akt activation, GSK3b inactivation and accumulation of β-catenin in both the cytoplasm and the nucleus. The expression of LCV LMP2A in epithelial cells impairs their ability to differentiate. These data suggest that similarly to EBV LMP2A, the LCV homologue also affects epithelial cell growth. These similarities underscore the similarity of LCV to EBV and its suitability as an animal model for studying EBV pathogenesis in epithelial cells.

Expression of Tumour Necrosis Factor Receptor-Associated Factor 1 (TRAF1) in Nasopharyngeal Carcinoma: Possible Up-Regulation by Epstein-Barr Virus Latent Membrane Protein 1

Siegler G, Meyer B, Dawson C, Brachtel E, Lennerz J, Koch C, Kremmer E, Niedobitek E, Gonnella R, Pilch BZ, Young LS, Niedobitek G

Contact:
Prof. Dr. Gerald Niedobitek
Friedrich-Alexander-Universität
Pathologisches Institut (Erlangen, Germany)
Email: gerald.niedobitek@patho.imed.uni-erlangen.de

Abstract:
Objective: Epstein-Barr virus (EBV) infection is associated with virtually all cases of undifferentiated nasopharyngeal carcinoma (NPC) and the EBV-encoded latent membrane protein 1 (LMP1) is expressed in a proportion of cases. LMP1 has transforming functions similar to members of the tumour necrosis factor (TNF) receptor family and activates intracellular signalling cascades through interaction with TNF receptor-associated factors (TRAF). In B-cells, expression of TRAF1 is in turn up-regulated by LMP1. Recent work has indicated that LMP1 signalling in epithelial cells may be affected by the presence or absence of TRAF1. The purpose of this study was to examine primary NPC biopsies for evidence of TRAF1 expression and to investigate the possibility of an association with LMP1 expression.

Methods: Biopsy specimens of 42 NPCs were studied using immunohistochemistry and antibodies specific for TRAF1 and LMP1. Expression of TRAFs was examined in three NPC-derived cell lines (C15, C17, C19) by RNase Protection Assay (RPA). Furthermore, an LMP1-transfected keratinocyte line, RHEK1, was studied by immunocytochemistry, RPA, and Western blotting.

Results: By immunohistochemistry, we have detected TRAF1 expression in 17 of 42 (40%) EBV-positive undifferentiated NPC. All 7 LMP1-positive NPC biopsies were also TRAF1-positive. Using RPA, high level TRAF1 expression was detected in an LMP1-expressing NPC-derived cell lines (C15) whereas expression was weaker in two LMP1-negative cell lines (C17, C19). Finally, LMP1 up-regulated TRAF1 expression in an EBV-negative keratinocyte cell line at the RNA and protein levels.

Conclusions: Our results demonstrate that TRAF1 is expressed in NPC tumour cells in vivo and suggest that TRAF1 expression may be up-regulated by LMP1 in NPC. An anti-apoptotic function has been proposed for TRAF1 and this may be relevant for the pathogenesis of NPC.

Identifying the components in the LMP-1 mediated signaling pathway that induces phosphorylation of eIF2α

Lee DY, Sugden B

Contact:
Dong Yun Lee
University of Wisconsin-Madison
Oncology, McArdle Laboratory for Cancer Research (Madison, USA)
Email: dongyunlee@wisc.edu

Abstract:
EBV's ability to induce proliferation of infected cells is dependent on the viral membrane protein LMP1 (Kaye et al., 1993) and its expression is essential for continued proliferation (Kilger et al., 1998). The level of LMP1 expression in individual cells within a clone of EBV-infected lymphoblasts ranges over 100-fold (Lam et al., 2004). LMP1 not only induces and maintains proliferation but also elicits an opposing response when expressed at high, physiological levels. Cells expressing such levels induce a cytostatic state and inhibit general protein synthesis by inducing phosphorylation of eIF2α through its transmembrane domain (Floettmann et al., 1996; Kaykas and Sugden 2000, Lam et al., 2004). This LMP1-mediated inhibition occurs normally in EBV-infected cells. There are four different kinases known to phosphorylate eIF2α: GCN2, PKR, HRI, and PERK (Dever et al., 1993, Berry et al., 1985, Chen and London 1995, Harding et al., 1999). PERK appeared a likely candidate kinase because of its membrane localization shared with LMP1. However, a dominant negative derivative of PERK failed to inhibit the phosphorylation of eIF2α induced upon conditional expression of LMP's six membrane-spanning domains. Conditional expression of LMP1 in both 293 and BJAB cells did activate PKR as measure by its autophosphorylation, which also correlated with increased phosphorylation of eIF2α. PKR is thus the kinase likely to be activated by high physiological levels of LMP1 to inhibit protein synthesis. We now are testing this possibility using a dominant negative derivate of PKR.

Heterodimer formation between c-Jun and Jun B proteins mediated by Epstein-Barr virus encoded latent membrane protein 1

Xin S, Tao Y, Deng X, Cao Y

Contact:
Dr. Song Xin
Cancer Research Institute, Xiangya School of Medicine
Central South of University (Changsha, China)
Email: xsongcn@hotmail.com

Abstract:
Epstein-Barr virus (EBV) encoded latent membrane protein 1 (LMP1) is essential for the immortalization of human B cells and is linked etiologically to several human tumors. LMP1 is an integral membrane protein which acts like a constitutively active receptor. It binds tumor necrosis factor (TNF)-receptor-associated factors (TRAFs), activates NF-kB and triggers the transcription factor activating protein-1 (AP-1) via the c-Jun N-terminal kinase (JNK) cascade, but its specific contribution to AP-1 has not been elucidated fully. Members of AP-1 family, the Jun and fos related protein, have been shown to directly interact and form heterodimeric complexes. In this report, using a Tet-on LMP1 HNE2 cell line which is a dual-stable LMP1 integrated nasopharyngeal carcinoma (NPC) cell line and the expression of LMP1 in which could be regulated by Tet-on system, we show that Jun B can efficiently form a new heterodimeric complex with the c-Jun protein under the regulation of LMP1, phosphorylation of c-Jun (ser63, ser73) and Jun B involved in the process of the new heterodimeric form. We also find that this heterodimeric form can bind to the AP-1 consensus sequence. Transfection studies suggest that JNK interaction protein (JIP) could inhibit the heterodimer form of c-Jun and Jun B through blocking the AP-1 signaling pathway triggered by LMP1. The interaction and function between c-Jun protein and Jun B protein increase the repertoire of possible regulatory complexes by LMP1 that could play an important role in the regulation of transcription of specific cellular genes in the process of genesis of nasopharyngeal carcinoma.

Latent membrane protein 1 encoded by Epstein-Barr virus modulates cyclin D1 by c-Jun/Jun B heterodimer

Xin S, Tao Y, Xiyun D, Cao Y

Contact:
Dr. Song Xin
Cancer Research Institute, Xiangya School of Medicine
Central South of University (Changsha, China)
Email: xsongcn@hotmail.com

Abstract:
Based on recent study on LMP1 encoded by EBV accelerating active c-Jun/Jun B heterodimers formation, it was investigated that c-Jun/Jun B heterodimers regulated cyclin D1 by laser scanning confocal influorescence microscope, western blot, luciferase activity assay, super-EMSA and flow cytometry in the Tet-on-LMP1 HNE2 cell line in which LMP1 expression is regulated by Tet-on system. Results indicated that c-Jun/Jun B heterodimers induced by LMP1 upregulated cyclin D1 promoter activity and expression. Cyclin D1 overexpression accelerated progression of cell cycle.

Both cyclinD1 and cylin E as the direct targets for the nucleus of EGFR, and acceleration of G1/S transition by transcription factor EGFR mediated by LMP1

Tao Y, Song X, Deng X, Li W, Li L, Cao Y

Contact:
Dr. Yongguang Tao
Cancer Research Insitute
Central South University (Changsha, China)
Email: ygtao2000@hotmail.com

Abstract:
Both Cyclin D1 and cyclin E are essential regulators for cell cycle at G1/S boundary. Previous studies hint that epidermal growth factor receptor (EGFR) has an indirect connection with cell cycle through signaling cascade. Our findings suggest that LMP1 mediated the nuclear translocation of EGFR. But the function of EGFR in the nucleus remains to be unknown.
In this study, we showed that EGFR could bind to the cyclinD1 and cyclinE promoters in vivo under the control of LMP1 using chromatin immunoprecipitation assay and EMSA. We also found that EGFR-DNA binding activity in the proximal region of cyclinD1 promoter was stronger than that of the distance region of cyclinD1 promoter; meanwhile the EGFR-DNA binding activity in the proximal of cyclinE promoter was weaker than that of the distance region of cyclinE promoter via super-EMSA. Using reporter gene assay, data showed that LMP1 increased the transcription of cyclinD1 7.5 folds, while the increase of cyclin E promoter is 4.6 folds.
We also found that LMP1 accelerated the G1/S transition after the effects of EGFR on the expression levels of cyclinD1, cyclinE, E2F1-Rb-CDK4 and CDK2. Data demonstrated that p53 and cdc2 (Thr15), which indicated the G2/M arrest of cell cycle by LMP1, were also enhanced. Cell sorting analysis showed that the levels of cyclin D1, cyclin E, CDK4 and E2F1 in G1 stage cells were increased, while CDK2 and p53 were decreased. When LMP1 accelerated the cells into the S phase, the changes of cylinE, CDK2, Cdc2 (Thr15) and CDK4 were enhanced greatly, but the expression of p53 still decreased. After LMP1 triggered the cells arresting in G2/M phases, the levels of Cdc2 (Thr 15) and p53 greatly increased, and the expression of CDK4 still increased while the levels of CDK2 decreased greatly. Taken together, these findings suggest that EGFR in the nucleus triggered by LMP1 could modulate the key regulators of cell cycle including cyclinD1, cyclinE, and E2F1 as a new transcription factor, resulting in the acceleration of G1/S transition.

Elucidation of the NF-κB pathway mediated by EBV-encoded latent membrane protein 1 (LMP1).

Wu L, Mak TW, Wu Z

Contact:
Liming Wu
The Hong Kong University of Science and Technology
Department of Biochemistry (Hong Kong SAR, Hong Kong)
Email: limwu@ust.hk

Abstract:
Objective: Elucidation of the NF-κB pathway mediated by EBV-encoded latent membrane protein 1 (LMP1).

Methods: To facilitate dissection of the LMP1-mediated the NF-κB pathway, we transfected LMP1 into HEK293T cells. IKK kinase assays, NF-κB-luciferase reporter assays, electrophorectic mobility shift assays (EMSA), and I-kappaB Western blot were used to study NF-κB activation by LMP1. To implicate key signaling molecules, small interfering RNA (siRNA) and mouse embryonic fibroblast (MEF) derived from knockout mice were used.

Results: In agreement with previous results, transient transfection of LMP1 into HEK293T cells can induce significant NF-κB activation by luciferase reporter assays and IKK kinase assays. Both CTAR1 and CTAR2 contributed to NF-κB activation by LMP1. When TAK1 was knocked down by siRNA, LMP1-mediated IKK activation was completely abolished. In TRAF6 knockout MEF cells, LMP1-mediated IKK activity also significantly decreased and could be rescued when TRAF6 was put back into this MEF cell.

Conclusions: Expression of LMP1 in host cells activates both the JNK and NF- kB pathways. In contrast to the LMP1-mediated JNK pathway, Both CTAR1 and CTAR2 contribute to the LMP1-mediated NF-κB pathway. While TAB2, TRADD and MEKK1 are not required, TAK1, TRAF2/5 and TRAF6 are critically involved in the LMP1-mediated NF-kappaB pathway.

EBV-encoded LMP1 promotes stress-induced apoptosis upstream of caspase-2-dependent mitochondrial perturbation

Zhang X, Uthaisang W, Hu LF, Fadeel B, Ernberg I

Contact:
Xiangning Zhang
Karolinska Institutet
Microbiology and Tumor Biology Center (Stockholm, Sweden)
Email: xiangning.zhang@mtc.ki.se

Abstract:
Objective: LMP1 was found to inhibit tumor necrosis factor (TNF)-α -induced apoptosis, while potentiates apoptosis induced by the chemotherapeutic agent, etoposide. In order to further dissect the modulation of etoposide-induced apoptosis by LMP1, we have focused on the activation of caspase-2, an important initiator caspase, as well as on mitochondrial perturbation, following etoposide and cisplatin treatment. Methods:

Methods: We measured mitochondrial membrane potential with FACS-based tetramethylrhodamine ethyl ester (TMRE)staining, determined the degree of caspase-2 activity in LMP1-expressing and control cells, using a fluorescent peptide substrate, VDVAD-AMC, and Western blot techniques.

Results: With Hela cells with regulatable LMP1 expression system, we observed that the dissipation of mitochondrial membrane potential was greater in cells expressing LMP1, when exposed to etoposide. LMP1 was found to potentiate etoposide-induced caspase-2 activation in this model.

Conclusions: LMP1-mediated potentiation of stress-induced apoptosis in HeLa cells occurs at an early step in the apoptosis cascade, upstream of caspase-2 activation and mitochondrial alterations, and appears to involve the two C-terminal domains of LMP1.

The viral oncoprotein Latent Membrane Protein 1 regulates cellular genes like IP-10 affecting their mRNA half-life.

Kube D, Pinkert D, Smola S, Michels A, Ransohoff RM, Tesch H, Vockerodt M

Contact:
Dr. Dieter Kube
Georg-August-Universitaet Fachbereich Humanmedizin
Zentrum fuer Innere Medizin, Abt. Haematologie und Onkologie (Goettingen, Germany)
Email: dkube@gwdg.de

Abstract:
The latent membrane protein 1 (LMP1) of Epstein-Barr Virus (EBV) is the main inducer of immuno-modulatory molecules affecting growth and survival of EBV infected cells. However, the network of signalling pathways involved remains to be elucidated. Here we show that LMP1 may regulate cellular genes like IFN-gamma-inducible protein-10 (IP-10) not only through transcriptional but also post-transcriptional mechanisms. LMP1-mediated IP-10 expression is independent from IFN-gamma, TNF-alpha or IL-18. Transcriptional activation of IP-10 by LMP1 or CD40 stimulation depends on a NF-kappaB motif within the proximal 435bp fragment. Carboxy-terminal activating regions 1 or 2 of LMP1 are sufficient to direct IP-10 promoter activation. IP-10 induction is inhibited by blockade of p38SAPK2 with SB 202190, which results in decreased IP-10 mRNA half-life wihout affecting IP-10 promoter activity. Thus, LMP1-mediated p38SAPK2 activation regulates transcript stability. This new mechanism of gene regulation demonstrates the potential of the oncoprotein LMP1 to orchestrate a network of signalling pathways at different regulatory levels including mRNA stability.

Identification of a new in-vivo phosphorylation site of EBV-LMP1 by mass spectrometry
Chi LM, Chien KY, Chang YS

Contact:
Dr. Lang-Ming Chi
Yuanpei University of Science and Technology
Medical Technology (Hsinchu, Taiwan)
Email: langming@mail.yust.edu.tw

Abstract:
Latent membrane protein 1 (LMP1), an Epstein-Barr virus encoded oncoprotein, has been detected frequently in nasopharyngeal carcinoma. It has the ability to transform the rodent cells and to promote the cell migration activity. The ability of LMP-1 to transform Rat-1 fibroblasts and the degradation of LMP1 protein can be regulated by its phosphorylation status. Carboxyl-terminus of LMP1 has been determined as the phosphorylation region by in vivo 32P-labeling. However, the phosphorylation sites and the kinases responsible for LMP1 phosphorylation are unclear.
For identifying the kinases and the sites of LMP1 phosphorylation, GST- and Flag-LMP1 fusion gene are constructed and expressed in bacteria and mammalian cell line, respectively. Using in-vitro kinase assay, casein kinase 2 (CK2) was found to be a potent kinase of LMP1. According to the consensus sequence analysis, S211, S309, S369 and S383 are the candidate phosphorylation sites of B95-8 LMP1 by CK2. Since, CK2 is a constitutively active kinase in eukaryotic cells. It is possible that CK2 mediates the phosphorylation of LMP1 in vivo. The mass spectrometry provides a powerful tool to monitor the phosphorylation status of protein without radioisotope labeling. Using LC/MS and MALDI/TOF/TOF MS analyses, we found a new in-vivo phosphorylation site located within amino acid 211 to 215 of B95-8 LMP1. The exact phosphorylation site will be characterized by LC/MS spectrometry combined with site-directed mutagenesis. By the way, the phosphorylation status of this site affects the biological functions of LMP1 will be further studied.

The genetic knockout of TRADD in human B-cells defines the functions of TRADD in signaling of LMP1 and TNF-receptor 1

Schneider F, Kutz H, Kaiser C, Briseno C, Kieser A

Contact:
Dr. Arnd Kieser
GSF-National Research Center for Environment and Health
Dept. of Gene Vectors (München, Germany)
Email: a.kieser@gsf.de

Abstract:
The tumor necrosis factor-receptor-associated death domain protein (TRADD) is an important signaling molecule of the LMP1 oncogene of EBV and the cellular TNF-receptor 1. TRADD is involved in the regulation of cell survival and apoptosis by both receptors. However, important questions regarding its role in distinct signal transduction pathways and in regulating the balance between survival and apoptosis remain to be answered. So far, no TRADD knockout system has been available to adress these questions. Here, we have cloned and sequenced the complete human TRADD gene locus from a genomic library of human blood cells. The human TRADD gene locus comprises 5 exons. Exons two to five encode the TRADD protein. Based on this genomic clone, we constructed two different knockout vectors allowing the specific and consecutive gene targeting of both TRADD alleles in human cell lines by homologous recombination. Using these constructs, we deleted all genomic TRADD sequences from human DG75 B-cells. Subsequently, signal transduction of LMP1 and TNF-receptor 1 was characterized in DG75-TRADD-/- cells versus DG75 wildtype cells. TRADD is essential for NF-kappaB activation specifically triggered by the CTAR2 domain of LMP1. Accordingly, LMP1 activation of IKK-beta, mediating the CTAR2 -> NF-kappaB pathway, is also dependent on TRADD. In contrast, TRADD is dispensable for JNK1 activation by LMP1. Additional data will be presented with respect to the functions of TRADD in signal transduction and the signaling complex of TNF-receptor 1. For the first time, we could generate a genetic TRADD-null system allowing to adress unresolved aspects of molecular TRADD functions.

LMP-1 activates the beta-catenin/Tcf pathway by inhibition of p53-dependent beta-catenin ubiquitination.

Jang KL, Shackelford J, Pagano JS

Contact:
Dr. Julia Shackelford
Lineberger Comprehensive Cancer Center
UNC at Chapel Hill (Chapel Hill, USA)
Email: shackelfordj@yahoo.com

Abstract:
LMP-1 is the principal EBV oncoprotein and is required for transformation of B-lymphocytes. LMP-1 promotes cell survival, adhesion, and invasive potential through induction of numerous cellular genes, using different signaling pathways. We found that LMP-1 induces beta-catenin accumulation and increases beta-catenin/Tcf transcriptional activity in B-cells. There are two currently known pathways for beta-catenin ubiquitination and proteasomal degradation: GSK3beta-mediated phosphorylation-dependent and GSK3beta-independent. In the presence of LiCl - an inhibitor of GSK3beta - both the activity and protein level of endogenous beta-catenin were significantly increased, indicating that the phosphorylation-dependent pathway of beta-catenin regulation is active in B-cells. Interestingly, however, LMP-1 was able to increase beta-catenin/Tcf activity further in the presence of LiCl and, moreover, the activity of a non-phosphorylatable mutant of beta-catenin as well. Therefore, we suggest that LMP-1 activates the beta-catenin/Tcf pathway by a mechanism different from GSK3beta-dependent beta-catenin phosphorylation and degradation. Phosphorylation-independent ubiquitination and degradation of beta-catenin is mediated by the ubiquitin-ligase complex, Siah-SIP-Skp1-Ebi. Siah is a p53-inducible gene, the expression of which increases p53-dependent beta-catenin ubiquitination and degradation. Expression of p53 was found to decrease activation of the beta-catenin/Tcf pathway itself, indicating that a p53-dependent pathway of beta-catenin regulation exists in B-cells. In the presence of LMP-1 results are very similar: overexpression of p53 inhibits LMP-1-mediated activation of the beta-catenin/Tcf pathway in a dose-dependent manner.
These data indicate that LMP-1 activates beta-catenin through a new, GSK3beta-independent, mechanism that involves p53. Since p53 down-regulates beta-catenin protein levels through the induction of Siah ubiquitin-ligase-dependent ubiquitination and degradation, we hypothesize that LMP-1 activates beta-catenin by dysregulation of this pathway in B-cells. These findings not only indicate a new pathway for stabilization of beta-catenin, but also suggest that the principal EBV oncoproteins may negatively regulate the p53 pathway.

The Role of NF-κ B p50 Homodimer/Bcl-3 Complexes in LMP1 Induction of EGFR Expression

Thornburg N, Raab-Traub N

Contact:
Natalie Thornburg
University of North Carolina at Chapel Hill
Microbiology and Immunology (Chapel Hill, USA)
Email: nthornbu@med.unc.edu

Abstract:
Objective: To determine if the NF-κ B p50 homodimer / Bcl-3 complex contributes to LMP1 mediated signaling in epithelial cells

Methods: Western blotting, co-immunoprecipitation, quantitative reverse transcription PCR, eletrophoretic mobility shift assay (EMSA), siRNA knockdown, and chromatin immunoprecipitation (ChIP)

Results: LMP1 has profound effects on gene expression and induces multiple forms of NF-κB. The LMP1 CTAR1 domain specifically activates formation of p50 homodimers that are not induced by LMP1 CTAR2. The CTAR1 domain is also uniquely responsible for induction of epidermal growth factor receptor (EGFR) expression in C33A cells. In LMP1 expressing C33A, p50 and Bcl-3 co-immunoprecipitated and could be immunopreciptated with the NF-κB sites within the EGFR promoter by ChIP. Transient expression of NF-κB p50, Bcl-3 or a combination increased levels of EGFR by immunoblotting and by quantitative reverse transcription PCR. LMP1 stable cells transiently transfected with a dominant negative IKK Beta had decreased levels of nuclear p50 homodimers by EMSA, which correlated with lower levels of EGFR protein. Transfection of NF-κB1 siRNA into C33A cells stably expressing vector control or LMP1 greatly decreased NF-κB p50 and its precursor p105. While knocking down p105/p50 facilitated an increase in EGFR levels by western blot and quantitative reverse transcription PCR, complex formation between residual p50 and Bcl-3 was enhanced by knockdown of p105/p50.

Conclusions: These data indicate that p50/Bcl-3 complexes contribute to the regulation of EGFR expression. The p50/Bcl-3 complex is bound to the EGFR promoter and overexpression of p50 or Bcl-3 increases EGFR independently of LMP1. A decrease in p105 and p50 expression by siRNA results in increased amounts of p50/Bcl-3 complexes with increased EGFR expression. These data not only confirm the role of p50/Bcl-3 in the regulation of EGFR, but also suggest that the p50/Bcl-3 complex is stable and is not affected by a decrease in the intracellular pool of the p50 precursor and binding partner, p105.

Regulation of LMP-1 signaling by lyLMP-1 and TRAF molecules
Geiger T, Coffin W, Erickson K, Martin J

Contact:
Dr. Jennifer Martin
University of Colorado,Boulder
Molecular, Cellular and Developmental Biology (Boulder, USA)
Email: jm@colorado.edu

Abstract:
LMP-1 activates signaling via interaction of TRAF molecules with its cytoplasmic C-terminus. C-terminal LMP-1 signaling triggers activation of NFkB and JNK pathways in B cells. Expression of a naturally occurring, amino-terminally truncated form of LMP-1, called lyLMP-1 due to the restriction of its expression to EBV's lytic cycle, inhibits LMP-1 activation of NFKB (Erickson and Martin, 2000).Here we explore further the mechanism by which LMP-1 signaling is subject to regulation by the intracellular signaling environment. We show that LMP-1 activation of JNK is inhibited by lyLMP-1 expression.We explore the contribution of TRAF binding to lyLMP-1, TRAF protein levels and their relationship to LMP-1 mediated NFkB activation, and the relationship between lyLMP-1 expression and TRAF protein accumulation in LMP-1 expressing cells.Our results are consistent with a model in which lyLMP-1 signaling results in accumulation of TRAF proteins to levels consistent with inhibition of LMP-1's activation of NFkB. Inhibition of LMP-1 signaling upon lytic cycle induction, by upregulation of lyLMP-1, is consistent with the finding that LMP-1's activation of NFkB plays a role in preventing lytic cycle activation.Our results suggest that like LMP-1, lyLMP-1 has signaling activity, but that the signaling activity associated with lyLMP-1 is distinct from the known signaling activities associated with LMP-1.

W98 of Transmembrane domain 3 & 4 of Latent Membrane Protein 1 (LMP1) is important in intermolecular interactions

Soni V, Yasui T, Kieff E

Contact:
Dr. Vishal Soni
Harvard Medical School
Department of Microbiology & Molecular Genetics Channing Lab
Brigham & Women's Hospital Harvard Medical School (Boston, USA)
Email: vsoni@rics.bwh.harvard.edu

Abstract:
Epstein-Barr virus latent membrane protein 1 (LMP1) activation of NF-kB is critical for Epstein-Barr virus infected B lymphocyte survival. LMP1 has six transmembrane domains that cause homotypic aggregation in the plasma membrane and enable constitutive NF-kB activation through its two cytoplasmic transformation effector sites (TES1 and TES2). Yasui et al. have shown that amino acids FWLY of latent infection membrane protein transmembrane domain 1 are critical for intermolecular interaction, raft localization and signaling. Yasui et al. have also shown that transmembrane domain 1 & 2 (TM 1 & 2) if mutated for FWLY amino acids does not interact with transmembrane domain 3 & 4 (TM 3 & 4) of the other LMP1 molecule. All these results suggest that FWLY of TM 1 & 2 of one LMP1 molecule interact with TM 3 & 4 of the other molecule. In this study, TM 3 & 4 aromatic amino acids, which are also near the external surface of the plasma membrane, were mutated. W98 and Y106, of TM 3 & 4 were mutated to alanine and their intermolecular interaction, membrane localization, raft localization and signaling was checked. The Y106A mutant is wild type in all assays, whereas W98A mutant loses intermolecular interaction with TM 1 & 2 and did not form a visible patch on the membrane. The amount of protein in the raft fractions was also comparatively less. However, this mutation alone did not reduce NF-kB activation.

Dendritic cells initiate immune control against Epstein Barr virus transformation of B lymphocytes in vitro

Bickham K, Goodman K, Paludan C, Nikiforow S, Tsang ML, Steinman RM, Munz C

Contact:
Prof. Dr. Christian Münz
The Rockefeller University
Laboratory of Viral Immunobiology (New York, USA)
Email: munzc@rockefeller.edu

Abstract:
The initiation of cell-mediated immunity to Epstein-Barr virus (EBV) has been analyzed with cells from EBV seronegative blood donors in culture. The addition of dendritic cells (DCs) is essential to prime naïve T cells that recognize EBV latent antigens in ELISPOT assays by Interferon-gamma secretion and eradicate transformed B cells in regression assays. In contrast, DCs are not required to control the outgrowth of EBV-transformed B lymphocytes from seropositive donors. Enriched CD4+ and CD8+ T cells mediate regression of EBV transformed cells in seronegative and seropositive donors, but the kinetics of T cell-dependent regression resemble a secondary immune response in seropositive and a primary immune response in seronegative cultures. EBV infection of DCs cannot be detected by RT-PCR with primers specific for EBNA1 mRNA. Instead, DCs capture B cell debris and then generate T cells specific for EBV latency antigens. We suggest that the cross-presentation of EBV latent antigens from infected B cells by DCs is required for the initiation of EBV specific immune control in vivo and that future EBV vaccine strategies should target viral antigens to DCs.

An Endogenous Superantigen and Disease: Role of HERV-K18 Env in EBV Lymphomagenesis

Sutkowski N, Huber BT

Contact:
Dr. Natalie Sutkowski
Tufts University School of Medicine
Department of Pathology (Boston, USA)
Email: natalie.sutkowski@tufts.edu

Abstract:
Objective: The Hu PBL-SCID mouse is a well-defined animal model for EBV lymphomagenesis. In these mice, the generation of B cell lymphomas is dependent upon T cells, which supply a form of help to the growing tumor. We have recently shown that infection with Epstein-Barr virus (EBV) activates a host-encoded superantigen that strongly activates T cells. The EBV latent membrane proteins are sufficient for transactivation of this superantigen, which is encoded by the env gene of human endogenous retrovirus HERV-K18. We speculate that superantigen activated T cells contribute to oncogenesis, we thus looked for evidence of HERV-K18 expression in Hu PBL-SCID lymphomas.

Methods: In order to confirm that T cells play a role in oncogenesis, we injected 4 groups of SCIDbeige mice with purified human B cells (n=8); purified human CD4 T cells (negative control, n=4); unseparated human PBMC (n=9); and purified human CD4 T cells plus purified B cells from the same donor (n=8). All donors were EBV seropositive. Mice were checked for survival over 8 weeks. Weekly serum samples were tested by ELISA for human immunoglobulin. Tumors were diagnosed by histology, in situ hybridization for EBERs, and ex vivo growth. RT PCR was performed for EBV LMP-1, LMP-2A and HERV-K18 env transcripts. Lymphomas were tested ex vivo for functional superantigen expression.

Results: B cell expansion and production of human IgM and IgG were found in mice injected with both B and T cells, but not in mice injected with purified B cells or purified CD4 T cells. High grade EBER+ B cell lymphomas developed in 50 - 80% of mice injected with unseparated PBMC or CD4 T cells plus B cells within 6 weeks. No mouse injected with CD4 T cells only or purified B cells only developed lymphoma over 8 weeks. Lymphomas strongly expressed LMP-1, LMP-2A and HERV-K18 env transcripts, while low level transcripts were infrequently detected in mice injected with purified B cells. Lymphomas functionally expressed superantigen activity, and preferentially activated TCRBV13 T cell hybridomas. This activity was inhibited with antisera specific for HERV-K18 Env peptide.

Conclusions: We confirmed that T cells are requisite for the generation of EBV lymphomas in Hu PBL-SCID mice. We propose that the T cell stimulation elicited by the HERV-K18 superantigen facilitates the generation of EBV lymphomas in the mice.

Characterization of cytotoxic cord blood-derived CD4+ T cells reactive to Epstein-Barr virus

MacArthur GJ, Wilson AD, Morgan AJ

Contact:
Dr. Andrew Morgan
University of Bristol
Pathology and Microbiology (Bristol, UK)
Email: andy.morgan@bristol.ac.uk

Abstract:
Objective: Previous studies have demonstrated that Epstein-Barr Virus (EBV)-specific CD4+ T cells can be cytotoxic towards EBV transformed lymphoblastoid cell lines (LCL) but can also enhance outgrowth of LCL colonies, in vitro. The main focus has been the role of CD4+ T cells derived from seropositive individuals, whilst the primary response of cord blood-derived CD4+ T cells has been less extensively studied. We aimed to investigate whether the cytotoxic capacity of cord blood derived CD4+ T cells was influenced by the conditions present during their differentiation and the mechanisms involved.

Methods: CD4+ T cell lines were generated by stimulation of cord blood lymphocytes or purified CD4+ T cells and monocytes with autologous LCL. Certain lines were Th2-polarised during generation by treatment with IL-4 and anti-IFN-γ. Cytotoxicity was assessed using a standard 16h Chromium-51 release assay in the presence or absence of concanamycin A or antibodies to FasL and TRAIL. Cytokine secretion and cell surface marker expression were determined by intracellular or cell surface staining and flow cytometry.

Results: We have generated CD4+ T cell lines from cord blood that are cytotoxic towards LCL. Cytotoxicity was contact-dependent and mediated via the perforin/granzyme pathway. Cytotoxic cells secreted IFN-g and IL-4 at a ratio suggestive of a Th1-polarised response following polyclonal stimulation (n=5/6). By manipulating culture conditions during the generation of CD4+ T cell lines from naïve precursors, it was also found that CD4+ T cells which were Th2-polarised during differentiation did not inhibit LCL proliferation and displayed low cytotoxicity towards LCL (n=3). We determined the expression of CD3, CD69, CD25, CD38, CD28 and CTLA-4 on resting and LCL-stimulated CD4+ T cells to investigate whether altered expression patterns might correlate with cytotoxic activity. Resting non-cytotoxic CD4+ T cells expressed lower levels of CD3 compared to cytotoxic lines and lacked the upregulation of CD25 observed on cytotoxic CD4+ T cells following LCL stimulation.

Conclusions: These results confirm the established cytotoxic capacity of CD4+ T cells for LCL. The results also show that Th2-polarised CD4+ T cells are not cytotoxic for LCL and might facilitate the persistence and amplification of EBV-infected B cells.

The recognition of Epstein-Barr virus (EBV)-transformed B cell lines by CD4+ T cell clones to EBV latent cycle antigens

Taylor GS, Long H, Haigh TA, Gudgeon NH, Tsang CW, Leen A, Brooks J, Landais E, Houssaint E, Rickinson AB

Contact:
Dr. Graham Taylor
University of Birmingham
CRUK Institute for Cancer Studies (Birmingham, UK)
Email: grahamt@cancer.bham.ac.uk

Abstract:
EBV latent antigen-specific CD8+ T cell responses are thought to be important in controlling EBV-driven lymphoproliferative disease. The role of HLA class II restricted CD4+ T cell responses is less well understood; most interesting is the possibility that latent antigen-specific CD4+ T cells may act as effector cells in their own right, directly recognising latently-infected HLA class II-positive B cells.
CD4+ T cell clones to 12 representative epitopes from EBNAs 1, 2, 3A and 3C were established in vitro and their function analysed by interferon-gamma release. Clones specific for the same epitope had similar avidities in peptide titration assays (irrespective of their method of establishment) and behaved similarly in assays of lymphoblastoid cell line (LCL) recognition. However there were marked differences between clones specific for different epitopes, with levels of unmanipulated LCL recognition ranged between 0-35% of the maximum seen on peptide-loaded targets. Short-term cytotoxicity and longer-term outgrowth inhibition assays on LCL targets gave similar patterns of results. These differences between individual epitope responses could not be explained by differences in the functional avidity of the T cell clones.

Where CD4+ T cell recognition of LCLs did occur, the target antigen did not appear to be accessing the HLA class II pathway directly within cells but only after intercellular transfer and uptake as exogenous protein. Thus, while LCL recognition could be greatly enhanced by over-expressing an HLA class II-directed form of the antigen in cells from Modified Vaccinia Ankara vectors, endogenous over-expression of the native antigen had no detectable effect. Furthermore cell mixing experiments showed that epitope-negative 'recipient' B cell lines of the appropriate HLA type could be sensitised to CD4+ T cell recognition by co-cultivation with epitope-positive HLA-mismatched 'donor' LCLs; indeed recipient cells could be sensitised by exposure to donor LCL supernatant via a chloroquine-sensitive processing step. The sensitivity of EBV positive lymphomas to CD4+ T cell mediated immune control in vivo may likewise depend upon the degree of antigen cross-feeding within the lesion and the capacity of tumour cells to process exogenous antigen.

Evasion from HLA class II-restricted T cell immunity during lytic EBV infection

Ressing ME, van Leeuwen D, Verreck F, Keating S, Gomez R, Schumacher T, Hutt-Fletcher L, Rowe M, Wiertz EJHJ

Contact:
Prof. Dr. Emmanuel Wiertz
Leiden University Medical Center
Medical Microbiology (Leiden, The Netherlands)
Email: wiertz@lumc.nl

Abstract:
Objective: During EBV replicative cycle, up to 100 viral proteins are expressed, which creates a wide variety of targets for detection and destruction by the immune system. Thus, for EBV to spread, it must have evolved effective ways to evade immune recognition. The EBV lytic-phase protein gp42 serves at least two functions: gp42 acts as a coreceptor for viral entry into B cells and hampers T cell recognition via HLA class II molecules. Previously, we have shown that gp42 blocks T cell receptor - HLA class II interactions (PNAS 2003, 100: 11583). Here, we studied the biosynthesis, posttranslational modifications and trafficking of gp42, and its intracellular interactions with HLA class II.

Methods: Biosynthesis and intracellular transport of gp42, and its association with HLA class II was analyzed in a temporal fashion in cells stably expressing gp42. The results were then confirmed in cells supporting natural replicative infection; we obtained almost pure populations of cells in lytic cycle using a novel approach to positively select for Akata cells expressing a lytic cycle reporter (see Ressing et al., abstract 04.22). Pulse/chase experiments were used to examine gp42 and interactions of gp42 with HLA class II complexes at various maturation stages. Immunomodulation was evaluated in experiments assaying T cell proliferation and HLA class II tetramer binding.

Results: EBV gp42 associates with class II molecules at their various stages of maturation, including immature α β Ii heterotrimers and mature α β peptide complexes. We found that gp42 occurs in two forms: a full-length type II membrane protein and a truncated soluble form. The soluble form, s-gp42, is generated by proteolytic cleavage in the ER and is secreted. S-gp42 is sufficient to inhibit HLA class II-restricted antigen presentation to T cells. In Burkitt's lymphoma cells in lytic EBV cycle, both transmembrane and soluble forms of gp42 are detected.

Conclusions: We show that EBV gp42 is posttranslationally modified to produce soluble truncated s-gp42 that can mediate HLA class II immune evasion. Our results implicate that s-gp42 is generated during lytic EBV infection and could contribute to undetected virus production by mediating evasion from HLA class II-restricted T cell immunity.

Can one cytotoxic T cell bank be used to treat most EBV-positive lymphomas?
Haque T, Wilkie G, Jones M, Wingate P, McAulay K, Crawford DH

Contact:
Dr. Tanzina Haque
University of Edinburgh
Laboratory for Clinical and Molecular Virology (Edinburgh, UK)
Email: t.haque@ed.ac.uk

Abstract:
Epstein-Barr virus (EBV) is associated with a variety of tumours in humans. Adoptive transfer of cytotoxic T lymphocytes (CTL) has been used to treat EBV-associated tumours, particularly post transplant lymphoproliferative disease (PTLD) as a model to assess the efficacy of this form of therapy. However whereas others have used autologous or donor derived CTL ([1], [2]), we have established a bank of EBV-specific CTL lines generated from around 100 HLA-typed healthy blood donors ([3]). We select CTL lines for patients with EBV-positive tumours on the basis of best HLA-match and specific cytotoxicity in in vitro assays.
We have infused multiple doses (1 to 7 doses of 2x106/kgm body weight) of partially HLA-matched allogeneic CTL into 24 patients without any immediate or long-term adverse effects or graft-versus-host disease. Of the 20 PTLD patients treated, we can assess outcome in 15 (the rest are on-going). Three patients died rapidly due to complications of PTLD before the effects of CTL infusion could be assessed and 12 patients completed the treatment. Of these 12 patients, 6 attained complete remission, 1 showed a partial response and 5 patients did not respond. Tumour responses were primarily seen in those with early, localised disease and when PTLD developed following primary EBV infection.
We have also treated 4 non-transplant EBV-positive lymphoma patients (ulcerative colitis, chronic lymphocytic leukaemia, haemophagocytic syndrome and primary immunodeficiency) and CTL infusions were well tolerated in all. EBV-positive lymphoma in the brain of one patient with primary immunodeficiency regressed completely after 7 infusions of allogeneic CTL and the patient remains well and tumour-free after 5 months indicating that these CTL can be effective against CNS lymphoma.
Our bank bypasses the need to grow CTL for individual patients and provides immediate access to fully characterised CTL lines for a large number of patients with EBV-positive lymphomas. We have shown that these partially HLA matched allogeneic CTL are safe and effective. This approach can now be utilised to treat other infectious and malignant diseases.

[1] Rooney et al. Lancet 1995; 345: 9-13.
[2] Khanna et al. PNAS 1996; 96: 10391-10396
[3] Haque et al. Lancet 2002; 360: 436-442

The Temporal Relationship Between the Onset of EBV Antibodies and Lupus Autoimmunity Supports a Role for EBV in the Development of SLE

Heinlen LD, McClain MT, Dennis GJ, Harley JB, James JA

Contact:
Latisha Heinlen
Oklahoma Medical Research Foundation
Arthritis and Immunology (Oklahoma City, USA)
Email: latisha-heinlen@ouhsc.edu

Abstract:
Objective: The potential for a link between the Epstein-Barr virus (EBV) and SLE has long been debated. To better understand this correlation, we analyzed the onset and progression of various herpes viral immune responses beginning years before the onset of clinical lupus.

Methods: Using the U. S. Department of Defense Serum Repository, 130 cases with stored serum samples from before SLE diagnosis and 520 matched controls were identified. These samples were tested for IgG antibodies to EBV viral capsid antigen (VCA), CMV, HSV1, HSV2, EBV nuclear antigen-1 (EBNA-1), and common lupus autoantibodies by standard ELISA.

Results: Anti-EBV VCA antibody levels are significantly higher in SLE patients compared to controls while no differences were detected with CMV, HSV1 or HSV2 antibody titers ($p>0.05$). Specifically, international standardized ratio (ISR) values for anti-EBV VCA antibody levels were 3.65 in SLE patient sera compared to 2.82 for control sera ($p<0.005$). When evaluated by time period prior to lupus onset, VCA ISR values for SLE gradually increase from their first detectable levels years prior to the onset of SLE up until diagnosis. However, titers in controls and titers of lupus patients to other viruses over the same period do not significantly increase. This increase in anti-viral titers closely parallels published data demonstrating the increase in autoantibodies up to the time of diagnosis. Antibodies targeting EBNA-1 were significantly more common in lupus patients (98%) than in normal, matched controls (88%, $p<0.05$). Furthermore, lupus patients produced significantly higher levels of anti-EBNA-1 antibodies compared to normal individuals ($p<0.01$). In the population studied, anti-EBNA-1 antibodies preceded or appeared concurrently with the development of anti-Sm and anti-Ro in every instance These results are consistent with the latest data demonstrating that cross-reactive mechanisms may serve as a direct link between EBNA-1 and early epitopes of Sm B and 60kD Ro.

Conclusions: The observation that EBV VCA and EBNA-1 antibodies are increased and precede the development of autoantibodies in lupus patients is consistent with the possibility of an early role for EBV immunity in SLE. These data also suggest that the increase is EBV specific and thus further support the hypothesis that EBV plays a role in the onset or pathogenesis of SLE.

Association of Epstein-Barr virus infection with multiple sclerosis in pediatric patients

Wagner HJ, Kollmann S, Bucsky P, Hanefeld F, Pohl D

Contact:
Dr. Hans-Joachim Wagner
University of Lübeck
Department of Pediatrics (Lübeck, Germany)
Email: hans-jwagner@web.de

Abstract:
Background and Objective: Multiple sclerosis (MS) is an inflammatory, demyelinating disorder of the central nervous system of an as yet unknown etiology. Epidemiological studies suggest an association between Epstein-Barr virus infections and risk of multiple sclerosis in adults. MS is rare in children and adolescents, who had limited contacts to infectious agents in previous life. Therefore these individuals with early-onset MS are optimal candidates for examining microorganism as co-factors for the development of MS. Aim of our study was to determine the prevalence and reactivity of anti-Epstein-Barr virus antibodies in children and adolescents with early-onset MS.

Methods: 74 pediatric patients with early-onset MS (48 girls and 22 boys, median of age 15 years, range 8-23 years) were enrolled in this cross-sectional study. Blood samples were taken at diagnosis of MS and were analysed for anti-EBNA1-IgG, anti-EA-IgG, -IgM and -IgA antibodies in comparison to 78 age- and sex-matched healthy controls.

Results: All but one pediatric MS patients were EBV-seropositive (73/74 patients or 98.6% anti-EBNA1-IgG-positive), which is a significant difference to healthy children and adolescents being EBV-seropositve in only 70.5% (55/78 controls anti-EBNA1-IgG-positive) (Chi-square = 22.6; $p < 0.001$). No primary EBV-infections were found in MS patients. Anti-EBNA1-IgG antibody titers were significantly higher in the group of pediatric MS patients compared with healthy controls ($p < 0.001$). No differences in the prevalence or reactivity of anti-EA-IgG, -IgM or -IgA antibodies were found between both groups.

Conclusions: The nearly 100 % EBV-seropositivity of children and adolescents with early onset-MS and the lack of primary infections suggest that MS patients are infected with EBV before the development of MS. These results support a role of EBV in the etiology of MS.

T cells inhibit EBV infected B cell growth in cord blood mononuclear cells when activated by the cytokine products of PSK and Trx80 stimulated monocyte

Liu A, Holmgren A, Klein G, Klein E

Contact:
Dr. Anquan Liu
Karolinska Institute
Microbiology and Tumorbiology Center (Stockholm, Sweden)
Email: Anquan.Liu@mtc.ki.se

Abstract:
Objective: EBV specific immunity is not transferred from mother to child. Therefore, in cord blood mononuclear cell (CBMC) population the cellular interactions in primary infection can be studied. In contrast to adult blood derived lymphocytes EBV can transform B cells in cord blood culture without the necessity to remove T lymphocytes. We investigated whether the immunopotentiators added to the EBV infected cord blood lymphocytes can generate immune response against the infected B cells. We used PSK (polysaccharide K) and Trx80 which is present in normal human plasma.

Methods: Thymidine incorporation assay to monitor the B cell outgrowth induced by EBV; SAP expression in immunoblot for detection of T cell activation; Flow cytometry to detect intracellular cytokines; ELISA to analyze the cytokine content in the culture media; chromium release assay to detect cytotoxic function of T and NK cells

Results: PSK and Trx80 potentiated T cell activation in the EBV infected cultures as detected by the intensity of the SAP band. B cell transformation was inhibited in these cultures. The effect of PSK or Trx80 was abrogated by antibodies against IL15 or IL12. The effect of PSK and Trx80 was mediated by monocytes through IL15 or IL12 production, respectively. EBV-specific CD4 positive cytotoxic T cells could be generated in the EBV infected cultures.

Conclusions: NK and T cells could recognize the EBV infected B cells in cord blood cell cultures and inhibited their proliferation when the immunopotentiators PSK or Trx80 were added. The details of the experiment suggested the following steps: in the EBV infected cell population, B cells were activated. These were recognised by NK and T cells which entered in a primed state, in which they expressed CD40L and IL2 receptor. They could respond to the cytokines produced by the immunopotentiator PSK and Trx80 stimulated monocytes. PSK induced IL-15 while Trx80 induced IL-12. Restimulation of the cultures with autologous EBV transformed B cells generated specific cytotoxic activity.

The capacity of Epstein-Barr virus to infect monocytes and inhibit their development into dendritic cells is affected by the cell type supporting virus replication

Guerreiro-Cacais AO, Li LQ, Donati D, Bejarano MT, Morgan A, Masucci MG, Hutt-Fletcher L, Levitsky V

Contact:
Andre Ortlieb Guerreiro Cacais,
Karolinska Institutet
Microbiology and Tumor Biology Center (Stockholm, Sweden)
Email: andre.ortlieb@mtc.ki.se

Abstract:
Epstein-Barr virus (EBV) is a ubiquitous human herpes virus involved in the pathogenesis of a wide spectrum of malignant and non-malignant diseases. Strong evidence implicates T-lymphocytes in the control of EBV replication and tumorogenesis but cellular components of the innate immune system are poorly characterized in terms of their function in the development of EBV-specific immunity or interaction with the virus. In this study we demonstrate that EBV virions produced in epithelial cells surpass their B-cell derived counterparts in the capacity to enter monocytes and inhibit their development into dendritic cells (DCs). The differences in EBV tropism are primarily accounted for by differences in the relative contents of the glycoproteins gp42 and gH in the virus envelope. We show that EBV enters DCs at different stages of their differentiation. Efficient binding of purified gH/gL heterodimer to monocytes and DCs, but not to B-cells, suggesting that high levels of expression of a putative binding partner for gH contribute to the entry of the virus, which takes place despite very low or undetectable expression of CD21, the canonical EBV receptor. Our results indicate that the tropism of EBV for monocytes and DCs alternates upon virus replication in either B-cells or epithelial cells thereby changing its immunomodulating capacity that may have important implications for the regulation of virus/host interactions during primary and chronic EBV infection.

Analysis of EBV specific CD8+ T cell Vbeta repertoire: Acute through chronic infection
Prince CK, Sullivan JL, Brody R, Luzuriaga K

Contact:
Dr. Kenya Prince Cohane
University of Massachusetts Medical School
Department of Pediatrics and Program in Immunology and Virology (Worcester, USA)
Email: kenya.princecohane@umassmed.edu

Abstract:
EBV infection induces strong CD8+ T cell immune responses to both lytic and latent viral epitopes. To better understand the evolution of the EBV-specific TCR repertoire, we studied a cohort (N=13) of individuals from presentation with symptoms of acute infectious mononucleosis (AIM) through convalescence. Blood taken from HLA-A0201individuals (N=13) was stained ex vivo with tetramers representing 2 lytic (A2BMLF1 and A2BRLF1) and 1 latent (A2LMP2 426) EBV specific CD8+ T cell epitopes, along with Vb antibodies. At presentation of symptoms, tetramer frequencies ranged from 0.2% to 7.8% (Median 3%) for A2BRLF1 and 0.3% to 10% (Median =1.5%) for A2BMLF1 of total CD3+/CD8+ T cells. These populations were still detectable at 6 to 12 months post entry (range: 0.07-1.4% and 0.05-0.9%, respectively). The distribution of Vbeta usage within these tetramer positive populations varied from individual to individual. In many cases, one or two Vbetas dominated the tetramer positive population specific for lytic or latent epitope over the course of infection. This trend was observed more often in the A2BMLF1 population (N=8) than the A2BRLF1 (N=5) and A2LMP2 426 (N=3) populations. Though there was no single dominant TCR, several Vbeta subfamilies were well represented within the cohort. Vbetas that commonly represented > 20% of the tetramer positive CD8+ T cells included Vb2 (N=8), Vb9 (N=5) and Vb14 (N=3). At 6-12 months post presentation, the original dominant Vbetas continued to dominate the response in each individual. These data are among the first to characterize the TCR Vbeta repertoire of EBV epitope-specific CD8+ T cells during acute infection and the stability of the repertoire into chronic infection. Characterization of Vbeta subpopulations by multi-parameter flow and functional analyses are in progress.

Differential segregation of Epstein-Barr virus-specific CD8+ T cells in blood versus tonsillar lymphoid tissue: contrasting patterns in acute primary infection and long term virus carriage.

Hislop AD, Kuo M, Drake-Lee A, Bergler W, Akbar AN, Leese AM, Rickinson AB

Contact:
Dr. Andrew Hislop
University of Birmingham
Institute for Cancer Studies (Birmingham, UK)
Email: andrewh@cancer.bham.ac.uk

Abstract:
Objective:To compare the distribution and cellular phenotype of EBV-specific CD8 T cells in the blood and tonsillar lymphoid tissue of individuals that are either undergoing acute primary EBV infection (Infectious mononucleosis (IM)) or are healthy carriers of EBV.

Methods: Blood and tonsil specimens were collected from patients either undergoing routine tonsillectomy or from IM patients.Single cell suspensions of both types of specimens were examined by HLA class I tetramer staining analysis and costained with cell surface specific antibodies. As well EBV DNA loads were estimated by quantitative PCR analysis.

Results: In IM tonsils, unexpectedly the percentage representation of lytic antigen-specific T cells in the CD8+ T cell pool is markedly lower than in the blood; a similar but much less marked trend is also seen for the smaller latent epitope responses.All these primary effectors both in tonsil and blood are highly activated CD45RO+, CD38+ cells which lack the CCR7 and CD62-l migration markers normally associated with recirculation through lymphoid tissue, and also lack the CD103 marker associated with homing to epithelium. By contrast, in long-term healthy virus carriers, there is a four-fold enrichment of EBV lytic epitope-specific cells in the tonsillar CD8+ T cell pool versus that seen in the blood, and an even more marked ten fold enhancement of latent epitope specificities.These tonsillar populations are again CD45RO+ (even in cases where the lytic epitope response in blood shows some switching to a CD45RA+ phenotype) but are negative for the CD38 activation marker. Interestingly these EBV-specific cells here have lower CCR7 and CD62-l expression than equivalent tetramer-staining cells in blood, but are highly positive for expression of the CD103 epithelial homing marker.

Conclusions: These findings indicate that studies of peripheral blood alone do not give an accurate overall picture of the size and phenotype of EBV-specific CD8+ T cell responses and that the situation in tonsillar lymphoid tissue can be quite distinct.

T cell-mediated regression of EBV-induced B cell transformation in vitro: the relative importance of CD4+ and CD8+ effector cells

Gudgeon NH, Taylor GS, Long H, Haigh TA, Pudney VA, Rickinson AB

Contact:
Dr. Nancy Gudgeon
University of Birmingham
CRUK Institute for Cancer Studies (Birmingham, UK)
Email: gudgeonnh@cancer.bham.ac.uk

Abstract:
Host T cell memory to Epstein-Barr virus (EBV) infection was first demonstrated through the T cell-mediated regression of EBV-induced B cell transformation to lymphoblastoid cell lines (LCLs) in infected peripheral blood mononuclear cell (PBMC) cultures. Here we re-examine recent reports that the effector cells mediating regression are CD4+, and not as previously assumed CD8+, T cells specific for EBV latent cycle antigens.
We studied EBV-immune donors with known CD4+ and CD8+ memory T cell responses to EBV latent cycle epitopes. In EBV-infected cultures of undepleted PBMCs, there was marked expansion of CD8+ T cells specific for such epitopes within the first 14 days post-infection, coincident with the reversal of EBV-infected CD23+ B cell proliferation; at the same time there was no expansion of latent epitope-specific CD4+ T cells. Epitope-specific CD8+ T cell clones derived from regressing cultures reproduced regression when co-cultivated with EBV-infected autologous B cells. Regression was also observed, with expansion of latent epitope-specific CD8+ effector cells, in cultures of CD4+ T cell-depleted PBMCs but was less efficient. The data are consistent with a major effector role for epitope-specific CD8+ T cells in mediating regression and a support role for CD4+T cell help in expanding the CD8+ T cell response.

However, with certain EBV-immune donors, we also observed late regression in CD8-depleted PBMC cultures though this was never associated with any detectable expansion of pre-existing CD4+ T cell memory to EBV latent cycle epitopes. CD4+ T cell clones derived from such cultures were operationally 'LCL-specific' in interferon-a release assays but did not map to any of the known EBV latent cycle proteins or derived peptides. Some of these 'LCL-specific' CD4+ T cell clones were also able to kill LCL targets in conventional cytotoxicity assays and only the cytotoxic clones reproduced regression when co-cultured with autologous EBV-infected B cells. These novel effectors may be specific for target antigens of cellular rather than viral origin.

Comparison of MYC- and EBV-driven growth programs: mRNA expression profiles provide insights into molecular mechanisms determining growth pattern and immunogenicity

Schlee M, Holzel M, Laux G, Rosenwald A, Staudt LM, Mailhammer R, Fagard R, Feuillard J, Bornkamm GW

Contact:
Dr. Martin Schlee
GSF-National Research Center for Environment and Health
Institute of Clinical Molecular Biology and Tumor Genetics (Munich, Germany)
Email: schlee@gsf.de

Abstract:
Burkitt's lymphoma (BL) cells and EBV-immortalized cells are human B cell lines that differ dramatically in their growth pattern, immunogenicity and phenotype. To gain insight into the changes imposed by different EBV genes and the cellular oncogene c-myc we have established in vitro model systems that recapitulate important features of the pathogenicity of BL. We have generated first a conditionally EBV-immortalized cell line (EREB2) that expresses a hormone-regulatable EBNA2 gene, and secondly two cell lines by transfection of EREB2 cells with a constitutively active (A1) and a tetracycline-regulatable c-myc gene (P493-6) that proliferate in the absence of estrogen and adopt the growth pattern, immunogenicity and phenotype of BL cells. To better understand the molecular mechanisms determining growth pattern, immunogenicity and phenotype, we have compared the expression profile of four EBV-positive group I BL cell lines; the c-myc-driven B-cell lines A1 and P493-6 (proliferating and arrested after tetracycline treatment); proliferating, arrested (estrogen-deprived) and re-stimulated EREB2 cells (1, 2, 3, 6, 8 hours after addition of estrogen); and three EBV-immortalized cell lines (LCL). Genes were classified mainly into three large categories. The first group (genes upregulated in BL, A1, LCLs and proliferating EREB2 and P493-6 cells, and downregulated in arrested cells) comprises primarily genes involved in cell cycle progression and cell division and includes direct and indirect c-myc target genes. The second group (genes downregulated in BL and upregulated in EREB2 and LCLs) represents to a large extent NF-kB-target genes involved in protection from apoptosis, cell activation, and interaction of B and T cells like e. g. cell surface molecules and T-Helper cell specific chemokines. A third group (genes highly expressed in resting and EBV-immortalized cells) contains IFN-regulated genes including the key players of the IFN response STAT1, STAT2 and IRF9. Promoter activity studies in EREB2 and P493-6 cells using the IFNbeta-, STAT1-and the Mx-promoter confirmed that the interferon pathway is activated in EBV-immortalized B cells. We conclude that the divergently regulated genes mediate susceptibility versus resistance to apoptosis as well as homo- and heterotypic cell interactions that render EBV-immortalized cells highly immunogenic.

Understanding immunodominance of EBV lytic antigen-specific CD8 T cell responses and their selection from primary to persistent phases of EBV infection

Sauce D, Pudney VA, Leese AM, Hislop AD, Rickinson AB

Contact:
Dr. Delphine Sauce
University of Birmingham
Institute for Cancer Studies (Birmingham, UK)
Email: d.sauce@cancer.bham.ac.uk

Abstract:
Objective: To examine the immunodominance hierarchy of EBV lytic cycle-specific CD8 T cell responses and the factors affecting selection of these specificities from primary into memory responses.

Methods & Results: By generating CD8 T cell clones from the blood of infectious mononucleosis (IM) patients, we find the epitope specificities of EBV-reactive T cells generated are biased towards immediate early and early gene products but away from late gene products. We have shown previously that not all of these expanded specificities present in IM patients survive to the persistent phase of the response. Comparison of the efficiency of recognition of lytic antigen expressing B-LCLs by CD8 T cell specificities that either do or do not enter memory shows that the efficiency of recognition of LCLs by such T cell clones is equivalent and the functional avidities of these responses are similar. We are now extending our analysis of selection into memory by examination of the EBV-specific CD8 response for the expression of cytokine receptors associated with the induction and maintenance of memory namely: IL-7Ra and IL-15Ra. Our preliminary studies show that (i) in IM patients, during the acute phase there is little or no expression of these receptors on EBV-specific cells and that (ii) after resolution of symptoms there is acquisition of these two receptors with IL-7Ra expressed at earlier time points than IL-15Ra. In responses present in healthy carriers all specificities express the IL-15Ra. Interestingly in these donors there is differential expression of the IL-7Ra on EBV-latent and -lytic specificities such that the majority of EBV-latent epitope-specific CD8 T cells express the IL-7Ra while EBV-lytic specificities show significantly lower levels of IL-7Ra expression.

Conclusions: These results indicate that responses to immediate early and early proteins are dominant over late responses and factors such as target cell recognition and functional avidity are not related to the selection of these particular responses into memory. Additionally, the selective expression of the IL-7Ra on antiviral human T cells could suggest differential maintenance of these antigen-specific populations whereas the relevance of IL-15Ra expression patterns remains to be determined.

BZLF1 inhibits C/EBP alpha and C/EBP beta mediated activation of the TNF alpha receptor promoter

Morrison T, Bristol J, Kenney SC

Contact:
Jillian Bristol
University of North Carolina at Chapel Hill
Lineberger Comprehensive Cancer Center
Medicine, Microbiology and Immunology (Chapel Hill, USA)
Email: jbristo@email.unc.edu

Abstract:
Objective: The immediate-early protein BZLF1 (Z) inhibits expression of the TNF-alpha receptor, TNFR1, and thus prevents TNF-alpha signaling in the host cell. The mechanism for this effect, however, is unknown. As Z directly interacts with C/EBP alpha (Wu et al., 2003), and the TNFR1 promoter contains a potential C/EBP binding motif, we hypothesized that Z inhibits the ability of one or more C/EBP family members to activate this promoter.

Methods: Various regions of the TNFR1 promoter were linked to the CAT reporter gene. The TNFR1 promoter constructs were co-transfected into HeLa and 293 cells with C/EBP alpha or C/EBP beta, in the presence or absence of Z, and CAT activity was quantitated. Electrophoretic mobility shift assays (EMSAs) were performed, using extracts from transfected cells, or in vitro translated proteins, to determine if C/EBP alpha or C/EBP beta bind to the TNFR1 promoter. GST pull-down assays were performed with a GST-Z fusion protein and 35S-labeled in vitro translated C/EBP alpha and C/EBP beta proteins to determine if these proteins interact.

Results: Both C/EBP alpha and C/EBP beta activated the TNFR1 promoter. Sequences in the TNFR1 promoter between -154/+35 were sufficient for C/EBP alpha and C/EBP beta activation. Interestingly, Z prevented the turn on of TNFR1 by both C/EBP alpha and C/EBP beta. EMSAs showed that in vitro translated C/EBP alpha and C/EBP beta both bind to the TNFR1 promoter region from -94 to -75, which contains the C/EBP-like motif, TGTTGCAAC. Mutation of this sequence in the TNFR1 154/+35-CAT construct inhibited C/EBP alpha and C/EBP beta activation. GST pull-down experiments demonstrated that C/EBP alpha and C/EBP beta both interact with GST-Z. Preliminary results indicate that Z expression in cells inhibits the binding of endogenous C/EBP alpha and C/EBP beta to the TNFR1 promoter.

Conclusions: C/EBP alpha, and C/EBP beta both activate the TNFR1 promoter and this effect requires a C/EBP binding site. Although Z has been shown to enhance C/EBP alpha-mediated activation of certain promoters (Wu et al., 2003), our results indicate that Z inhibits C/EBP alpha and C/EBP beta activation of the TNFR1 promoter. Given that C/EBP beta plays an important role in immune activation, the ability of Z to inhibit certain C/EBP beta responsive target genes may help EBV to evade the host immune response.

Potent T Cell Response to a Class-I-binding Epitope from BZLF1 of Extraordinary Length

Burrows SR, Miles JJ, Green KJ, van Zuylen WJM, Borg N, Elhassen D, McCluskey J, Rossjohn J

Contact:
Dr. Scott Burrows
Queensland Institute of Medical Research
Cellular Immunology Laboratory (Brisbane, Australia)
Email: scottB@qimr.edu.au

Abstract:
Objective: This investigation has characterised a cytotoxic T lymphocyte (CTL) epitope from the BZLF1 or ZEBRA protein from EBV (the HLA B35-binding epitope EPLPQGQLTAY), revealing several unique features that redefine the selection requirements for viral CTL epitopes.

Methods: Healthy EBV-seropositive laboratory blood donors who express HLA B35 were the subjects for this investigation.

Results: An analysis of the CTL response to the EPLPQGQLTAY epitope in PBMCs from nine HLA B35+, EBV+ individuals revealed that 5 donors responded very strongly while 4 donors showed no detectible response. HLA subtyping of the B35 antigen in each donor revealed that a single HLA amino acid difference prevents responses to this epitope. While all B*3501+ donors showed strong responses, EBV-exposed donors expressing HLA B*3503 showed no significant CTL response to this epitope because the single amino acid difference between B*3501 and B*3503 within the F pocket inhibited HLA binding by the peptide. Although B*3508+ individuals failed to respond to the EPLPQGQLTAY 11-mer peptide, strong responses were shown to the extraordinarily long overlapping 13-mer peptide, LPEPLPQGQLTAY. HLA B*3508 differs from B*3501 at a single position within the D pocket (B*3501, 156Leucine; B*3508, 156Arginine) and this minor difference was shown to enhance binding of the 13-mer peptide, presumably through a stabilizing interaction between the negatively charged glutamate at position 3 of the peptide and the positively charged arginine at HLA position 156.

Conclusions: The 13-mer epitope defined in this study represents the longest class-I-binding viral epitope identified to date as a minimal determinant. Furthermore, the potency of the response indicates that peptides of this length do not present a major structural barrier to CTL recognition, and their role in the CTL control of infecting pathogens can be significant.

A Bulging BZLF1 Epitope Shapes T Cell Receptor Patterning

Miles JM, Borg N, Silins SL, Elhassen D, Rossjohn J, McCluskey J, Burrows SR

Contact:
John Miles
Queensland Institute of Medical Research
Division of Infectious Diseases and Immunology (Brisbane, Australia)
Email: johnMi@qimr.edu.au

Abstract:
Objective: To understand why an EBV epitope raises T cells with identical T cell receptors (TCR) in unrelated people; a probability of 100 million to 1.

Methods: Solving the crystal structure of HLA B3501 in complex with EPLPQGQLTAY (EPLP), a highly immunogenic 11mer from the BZLF1 gene of EBV. Using Tetramer, TCR Ab, PCR and amino acid replacement technology the TCR response was studied.

Results: The crystal structure HLA B3501-EPLP complex shows that the terminal bonds in EPLP are conserved causing the middle to bulge high above the MHC cleft. This bulging causes EPLP to be highly mobile with only the terminal anchors firmly grasping the MHC. In addition, the 'E' at position 1 (P1) manipulates residues on HLA B3501, changing the conformation of the MHC. The effect of this 11mer epitope on the TCR repertoire is potent. From a potential inventory of one hundred million unique TCRs, only two patterns emerge. The first is the TRBV10/TRAV1 TCR and the second is the TRAV28/TRAV3 TCR. These two TCR were found in all four donors and were either identical (Public) or comprised negligible changes. These TCR were found at high frequency in all donors sometimes reaching 11% of total population of CD8+ T cells. The DNA analysis of the public TCR revealed they had undergone very limited recombination in the thymus and consisted almost completely of germline DNA. The peptide replacement assay revealed that 'E' at P1 that was essential for any TCR/pMHC recognition.

Conclusions: The structure of the HLA B3501-EPLP complex reveals a bulging and very mobile epitope. Combing both the problems of bulge size and high mobility would likely turn many potential TCR away from this awkward MHC composite surface, thus retaining only a strict selection which we are seeing in HLA B3501 people. It is apparent that these public TCR are docking in a unique conformation to the modified pMHC. EBV is an ancient virus and HLA B35 is a very common HLA allele across all ethnic populations. It would be intriguing to speculate that these largely germline encoded public TCR have been selected through evolution to preempt an almost inevitable EBV infection in B35 people.

Enhanced In Vitro Expansion of EBNA1-specific CD8 T Cells Irrespective of Donor HLA Type Using a Glycine/Alanine Repeat Deletion Mutant of EBV

Fogg M, Quink C, Brander C, Wang F

Contact:
Dr. Fred Wang
Harvard Medical School
Department of Medicine, Brigham & Women's Hospital (Boston, USA)
Email: fwang@rics.bwh.harvard.edu

Abstract:
EBNA1 is the predominant viral protein expressed during viral persistence and in EBV-associated malignancies, but until recently, cytotoxic T cell (CTL) responses against EBNA1 have been described as ineffective due to inhibitory effects of the Glycine/Alanine repeat domain (GAr) on antigen presentation. A virus with an incompletely deleted GAr has been described as viable, but a virus with the entire GAr removed has not been reported.

We rescued an EBV B95-8 genome in E. coli after homologous recombination of F plasmid sequences into the major internal repeat and utilized recombinant BAC technology to generate a recombinant EBV where the entire GAr coding region had been deleted from the EBNA1 gene. The GAr deleted virus (dGAr) was able to immortalize B cells and the viral genome was maintained episomally in similar copy numbers as a wild type EBV BAC derived virus suggesting that none of the GAr is required per se for virus persistence in B cells. Steady state levels of EBNA1 protein were comparable in dGAr and wild type immortalized B cells, but upon inhibition of protein synthesis, the half-life of the EBNA1 protein was reduced in the dGAr virus immortalized B cells.
To assess the role of the GAr on presentation of EBNA1 peptides in the context of a virus-infected B cell, we assessed the ability of dGAr or wild type EBV immortalized B cells to stimulate EBNA1 specific, autologous CD8+ T cells in vitro using IFN-γ ELISPOT analysis. Wild type EBV immortalized B cells were able to stimulate small numbers of EBNA1 specific IFN-γ responsive T cells, whereas the dGAr immortalized autologous B cells stimulated a near 10-fold higher number of EBNA1 specific T cells.
We have demonstrated that the EBV major internal repeat can be used as an innocuous site for inserting a large amount of recombinant sequences and that none of the EBNA1 GAr is required for B cell immortalization or episomal persistence in tissue culture. We find evidence that in the context of a virus infected B cell, the GAr can inhibit stimulation of EBNA1 specific responses consistent with an inhibitory effect on antigen presentation. The ability to use the dGAr virus and greatly increase the stimulation of EBNA1 specific CD8+ T cells irrespective of an individual's HLA type may have potential therapeutic value for adoptive immunotherapy and control of EBNA1 expressing malignancies.

CD4+ T cell responses to EBNA2-derived epitopes presented in the context of different HLA class II alleles

Long HM, Taylor GS, Haigh TA, Tsang CW, Gudgeon NH, Rickinson AB

Contact:
Dr. Heather Long
University of Birmingham
CRUK Institute for Cancer Studies (Birmingham, UK)
Email: HeatherLong@cancer.bham.ac.uk

Abstract:
CD4+ T cell responses to EBV latent cycle antigens are of potential therapeutic importance but still relatively little is known about immunodominant epitopes, their restriction elements and their level of presentation on the surface of EBV-transformed lymphoblastoid cell lines (LCLs). Previous work using peptide panels in interferon-gamma Elispot asays showed that EBNA1 and EBNA3C are much more frequent targets of CD4+ T cell responses in healthy virus carriers than are LMP1 and LMP2. We have now extended this work to more donors and added a further two EBV latent antigens, EBNA2 and EBNA3A. Seven of 23 healthy laboratory donors screened against a panel of EBNA3A peptides responded to one or more of the 3 novel epitopes identified. In similar assays, 16 of 24 donors responded to one or more peptides within EBNA2, identifying 6 epitopes of which 5 are novel. This establishes the hierarchy of immunodominance for CD4+ T-cell responses as EBNA1 > EBNA3C > EBNA2 > EBNA3A, LMP1, LMP2 in terms of numbers of identified epitopes, and as EBNA1, EBNA2, EBNA3C > EBNA3A >> LMP1, LMP2 in terms of frequency of donors with responses to those epitopes.

Focusing on EBNA2-induced responses, we established CD4+ T-cell clones specific for three of the above EBNA2 epitopes and in each case determined their HLA class II restriction, cytotoxic capacity, and functional avidity as measured in peptide titration assays. The two less frequently recognised epitopes were restricted through HLA-DR4 and HLA-DR17 respectively, whereas a much more frequently detected response against the EBNA2 276-295 epitope was restricted through different alleles in different donors, namely DR7 and three subtypes of DR52 (52a, b, c). Interestingly, all the epitope-specific CD4+ T cell clones restricted through DR52 subtypes showed 10-fold higher avidity in peptide titrations than the DR7-restricted clones or clones to the other EBNA2 epitopes. However the relative efficiency with which these clones recognised unmanipulated LCL targets was not directly related to avidity, being highest for DR52b-, intermediate for DR7-, and much lower for DR52a- and 52c-restricted effectors. This shows that the level of representation of a target epitope on the LCL surface is highly dependent not just upon the identity of the epitope peptide but also the identity of the restricting allele.

The XLP gene SAP is a target of p53

Nagy N, Takahara M, Klein E

Contact:
Noemi Nagy
Karolinska Institutet
Microbiology and Tumor Biology Center (Stockholm, Sweden)
Email: Noemi.Nagy@mtc.ki.se

Abstract:
Objective: SAP is deleted or mutated in the X linked lymphoproliferative disease (XLP), a primary immunodeficiency characterized by extreme sensitivity to EBV. XLP manifests by fatal infectious mononucleosis (50% of the cases), malignant lymphomas (30%) and dysgammaglobulemia (30%). XLP patients respond with uncontrolled T and B cell proliferation to EBV infection. This and the high incidence of lymphomas (200 times higher than in the general population) could, at least in part, be the consequence of a defect in cell cycle control and/ or in apoptosis. Therefore we studied whether SAP expression is linked to the p53 pathway, known to coordinate DNA repair, cell cycle progression and apoptosis.

Methods: The SAP negative BL41 line that carries mutant p53, has been transfected with a temperature sensitive p53 (ts p53). At 32°C, the mutant ts p53 acquires the wild type (wt) conformation, leading to apoptosis. In addition to this system, we investigated the effect of endogenous p53 on SAP expression following DNA damage. SAP protein and mRNA levels were monitored by Western blot, Northern blot and RT-PCR. Binding of p53 to the SAP gene in the context of organized chromatin was detected by chromatin immunoprecipitation (ChIP) assay.

Results: BL41 ts p53 became SAP positive when wt p53 was induced (at 32°C) and this was not a secondary consequence of apoptosis or cell cycle arrest. Furthermore, experiments with BLs and LCLs showed that wt but not mutant p53 carrying cells expressed SAP upon DNA damage. Regulation of SAP occurred at the mRNA level and it was independent of protein synthesis indicating that SAP is a direct target of p53. In addition, we have shown by ChIP assay the binding of wt p53 to the responsive element located in the promoter region of the SAP gene in DNA damaged BL lines. Induction of SAP was abolished by the p53 inhibitor pifithrin-α. Importantly, SAP levels were upregulated upon irradiation in T cells as well. SAP was also upregulated in late phases of PHA induced T cell activation in parallel with p53, when the proliferation of cells was ceased.

Conclusions: The p53 mediated induction of SAP in DNA damaged lymphocytes indicates a role of SAP in apoptosis, growth arrest or DNA repair, that could explain the uncontrolled lymphocyte proliferation and high incidence of lymphomas in XLP patients.

Changes in CD27+ memory B cells in HIV-Infected patients before and under ART. Correlation with EBV viral load.

Richard Y, Amiel C, Jeantils V, Mestivier D, Portier A, Dhello G, Feuillard J, Nicolas JC, Raphael M

Contact:
Dr. Yolande Richard
INSERM U131
Institut Paris-sud sur les cytokines (Clamart, France)
Email: yolande.richard@inserm.ipsc.u-psud.fr

Abstract:
Objectives: Patients infected with HIV-1 are at high risk of developing Epstein-Barr virus (EBV)-associated lymphoma but frequently exhibit a loss in memory B cells, the major reservoir of EBV in the periphery. This study re-evaluated the phenotypic status of the B cell compartment and EBV viral loads in HIV-infected patients before and under antiretroviral therapy (ART)

Methods: Phenotyping of B cells was performed by three-colour flow cytometry using whole blood from 23 HIV-infected patients and 30 healthy donors. HIV viral load and sCD27 were measured in plasma whereas EBV viral load was measured, by quantitative real-time PCR, in peripheral blood leukocytes. HIV-Infected patients were studied before and 9 months of ART

Results: Based on the percentage of circulating memory CD27+ B cells at baseline, we delineated three groups of patients: group A with normal percentages of CD27 (22±8%, n=11), group B with high percentages of CD27 (45±3%, n=4) and group C with low percentages of CD27 (6±3%, n=8). These three groups differed by their activation profile and EBV viral load. The EBV viral load was elevated in all groups as compared to controls, but higher in group B's patients. Our findings also pointed out a decrease in SIgD+CD23+ naive B cells, in all groups. While ART induced an increase in CD4 cell count, a strong decline of HIV plasma viremia and B cell activation, the deficit in SIgD+CD23+ naive B cells persisted in all groups. The frequency of memory B cells further decreased in patients of groups A and B whereas it increased by two fold in patients of group C. The dynamics of EBV content during ART was also different among the three groups: a strong decline in HIV-infected patients of group A and B but not in those of group C.

Conclusions: Our data establish a loss a naive SIgD+ CD23+ and switched memory B cells (CD27+SIgG+) in all groups that ART is unable to stop. The correlation between activation marker expression, frequency of CD27+B cells and EBV viral load suggests that, in groups B and C, B cell activation proceeds through different process and must be compared to the patterns of EBV gene expression in the context of immundeficiency.

Initiation of latency in infected B-lymphocytes is the third phase of EBV's life cycle
Altmann M, Hammerschmidt W

Contact:
Dr. Markus Altmann
GSF
Gene Vectors (Muenchen, Germany)
Email: m.altmann@gsf.de

Abstract:
Epstein-Barr-Virus immortalises primary B-lymphocytes efficiently in vitro establishing a latent infection. In these growth-transformed cells only a few viral genes are expressed. After induction of lytic genes the virus gives rise to progeny. To prevent apoptosis of virally infected cells several viruses encode apoptosis-inhibiting gene products related to the bcl-2 family or induce anti-apoptotic cellular genes.
In a genetic approach, the two bcl-2 homologues of EBV, BALF1 and BHRF1, are dispensable for efficient B-cell immortalisation when deleted individually, but a double knockout EBV mutant fails to immortalize B-lymphocytes. Although categorized as lytic genes, BHRF1 and BALF1 were found expressed very shortly after infection of primary B-Lymphocytes with wild-type EBV. BZLF1, the prototype immediately early gene of EBV, and members of the viral polymerase-complex were detected early in infection but no virus is produced.
Our data indicate that a third phase of EBVs life cycle has been overlooked. In the course of initiating EBV latency and B cell growth transformation a number of lytic genes is found expressed. In this phase, expression of BHRF1 and BALF1 act in an anti-apoptotic fashion to prevent cellular death presumably repressing EBVs lytic mode. This step seems to be critical since two bcl-2 homologues of EBV act redundantly in newly infected cells to establish a latent infection and stable virus-host relationship for a lifetime.

Epstein-Barr virus can establish a persistent infection in the absence of a classical memory B cell population

Conacher M, Callard R, McAulay K, Chapel H, Webster D, Kumararatne D, Spickett G, Crawford DH

Contact:
Prof. Dorothy Crawford
University of Edinburgh
School of Biomedical & Clinical Laboratory Sciences (Edinburgh, UK)
Email: D.Crawford@ed.ac.uk

Abstract:
Objective: Epstein-Barr virus (EBV) is a ubiquitous human herpes virus that persists in the body for life after primary infection. The primary site of EBV persistence is the memory B lymphocyte, but whether the virus initially infects naïve or memory B cells is still disputed. We have analysed EBV infection in 9 cases of X-linked hyper IgM syndrome who, due to a mutation in CD40 ligand gene, do not have a classical, class switched memory B cell population (IgD- CD27+).

Methods: Using a sensitive PCR technique to detect EBV DNA in blood and throat wash samples we found evidence of EBV infection in 67% of cases, which is similar to the infection rate found in the general UK population (60-70% for the relevant age range).

Results: We detected EBV DNA in peripheral blood B cells and showed in one case that the infection was restricted to the small population of non-classical, germinal centre-independent memory B cells (IgD+ CD27+) where the viral load was very high (approximately 1 EBV genome per 6 cells). Detection of EB small RNAs, latent membrane protein 2 and EB nuclear antigen (EBNA) 3C expression in peripheral blood indicated full viral gene expression in this population. Analysis of EBV DNA in serial samples showed variability over time suggesting cycles of infection and loss, however in one case where 2 throat wash samples taken 6 months apart were available, we detected identical EBNA 3C sequences in both samples.

Conclusions: Our results demonstrate that EBV persistence can occur in the absence of a germinal centre reaction and a classical memory B cell population. We have identified a unique form of EBV infection which could be explained by periodic re-infection of the B cell pool from an external source.

EBNA2 and LMP1 heteroduplex analysis of EBV strains in infectious mononucleosis patients: ex vivo material versus in vitro-rescued virus isolates

Tierney R, Croom-Carter D, Roy S, Edwards R, Sitki-Green D, Raab-Traub N, Rickinson AB

Contact:
Dr. Rose Tierney
University of Birmingham
Cancer Research UK Institute for Cancer Studies (Birmingham, UK)
Email: R.J.Tierney@bham.ac.uk

Abstract:
Objectives: Heteroduplex analysis of polymorphic LMP1 sequences PCR amplified from ex vivo material suggests that many individuals carry more than one EBV strain, whereas earlier work rescuing resident viruses by B cell transformation in vitro implied the presence of only one dominant strain. Here we attempt to reconcile these differences by heteroduplex analysis of paired ex vivo and in vitro-isolated samples from infectious mononucleosis (IM) patients undergoing primary EBV infection.

Methods: Peripheral blood mononuclear cells (PBMCs) and throat washing (TW) samples from 14 IM patients were analysed ex vivo alongside multiple PBMC-derived and TW-derived lymphoblastoid cell line (LCL) isolates from each patient. EBV type-specific assays across EBNA2 and EBNA3C loci, strain specific-assays across LMP1 30bp deletion and 33bp repeat regions were combined with an established LMP1 heteroduplex tracking assay distinguishing 7 different LMP1 variants and a new EBNA2 heteroduplex assay distinguishing 5 different type 1 EBNA2 variants and type 2 EBNA2.

Results: Of 14 IM patients analysed, only one carried a single virus strain (type 1) in ex vivo samples. Eleven patients carried either two or three different type 1 strains, one carried two type 1 plus one type 2 strain, and one carried two different type 2 strains. In most cases, all co-resident strains were detectable both in PBMCs and TWs and the EBNA2 and LMP1 assays were generally concordant. By contrast, in 12/14 patients only one of the identified strains was isolated in vitro, despite analysis of multiple PBMC- and TW-derived LCLs; furthermore this was not always the strain giving the strongest PCR signals on ex vivo material. In the other 2/14 cases, in vitro isolation identified the two co-resident type 1 virus strains and the co-resident type 1 and type 2 strains respectively.

Conclusion: This study using two sensitive heteroduplex tracking assays, now including one at the type-specific EBNA2 locus, confirms that most IM patients are infected with more than one EBV strain and supports the view that in Caucasian populations these strains are predominantly of type 1. By contrast, virus isolation in vitro often gives an incomplete record of resident strains and the factors influencing the selective rescue of some strains and not others need to be determined. Most importantly, these results indicate that the presence of co-resident strains in healthy virus carriers does not necessarily reflect serial acquisition but may be explained by simultaneous acquisition at the time of primary infection.

Epstein-Barr virus structural genomics

Burmeister WP, Buisson M, Tarbouriech N, Geoui T, Morand P, Cusack S

Contact:
Dr. Wim Burmeister
EMBL / Universite Joseph Fourier / Institut Universitaire de France
Virologie Moleculaire et Structurale (Grenoble, France)
Email: wpb@embl-grenoble.fr

Abstract:
Objective: A project on the structural genomics of EBV started in autumn 2002 as part of the European SPINE project. The aim is the determination of a number of 3-dimensional structures of EBV proteins aided by high-throughput platforms. This structural information will serve to find new targets for antiviral therapy as well as to understand better viral functions.

Methods: We expressed the proteins fused to a hexahistidine-tag via a TEV (tobacco etch virus) protease cleavage site in E. coli. We used an efficient 2 step purification protocol based the different binding of the expressed protein to nickel columns before and after protease cleavage. The purified protein is submitted to robotic crystallization trials in order to obtain crystals suitable for structure determination by X-ray crystallography. Targets with an enzymatic activity (about 13) were treated with priority; furthermore proteins with relatively well characterized functions or a predicted high amount of secondary structure. Membrane proteins have been left out from the target list.

Results: Since the start of the project 41 constructs from 21 open reading frames out of the about 75 open reading frames of EBV have been cloned. The first experiences gained showed that the major bottleneck is the production of soluble protein as only 8 out of the 41 constructs yielded soluble protein. We tried to use a variety of E. coli strains for better protein solubility as well as purification of denatured proteins followed by different renaturation protocols, but this problem cannot be easily overcome. The ongoing development of high-throughput platforms in the context of the PSB project in Grenoble will allow us to multiply the number of constructs tested for soluble expression in the future. Nevertheless, we obtained crystals of 5 of the proteins and very recently, the 3-dimensional structure of the EBV dUTPase.

Conclusions: We have to identify the reasons for the low yield of soluble protein. Expression tests of some of the insoluble proteins in the baculovirus system are envisaged. The available structures of the EBV protease and dUTPase will be used for the structure-based design of inhibitors of these enzymes. Our 3-dimensional structure of the protease opened new perspectives for the design of inhibitors.

High Epstein-Barr-Virus DNA-Load, Multiple Infection and Long-Term Persistence of Infectious Virions in Saliva after Infectious Mononucleosis: A Longitudinal Prospective Study

Fafi-Kremer S, Morand P, Brion JP, Pavese P, Baccard M, Germi R, Genoulaz O, Nicod S, Ruigrok R, Stahl JP, Seigneurin JM

Contact:
Dr. Samira Fafi-Kremer
Centre Hospitalo-Universitaire Michallon
Laboratoire de Virologie (Grenoble, France)
Email: sfafi@chu-grenoble.fr

Abstract:
Objective: The aim of the study was to investigate viral load, infectivity and variability of Epstein-Barr virus (EBV) during infectious mononucleosis (IM).

Methods: A prospective study was undertaken among 20 patients that were followed for 6 months after the onset of IM. Peripheral blood mononuclear cells (PBMCs), plasma and saliva samples were analysed at days 0, 3, 7, 15, 30, 60, 90 and 180 for EBV load by real-time quantitative PCR. Infectivity of the saliva was assessed by a cord blood cell-transformation assay at D0 and D180. EBV genotypes were assessed in PBMCs and saliva at D0 and D180 by cloning and sequencing the EBV gene that codes for the latent membrane protein 1 (LMP-1).

Results: All patients displayed high levels of EBV DNA in the saliva (median: 6.53 and 5.83 log copies/ml at D0 and D180 respectively), with a persistent infectivity of saliva after 6 months. In contrast, the detection of EBV DNA in plasma was positive in 95% of plasma samples at D0, but was always negative after D15. The viral load in PBMCs decreased significantly from D0 to D180 (median: 3.17 log and 1.92 log copies/µg DNA at D0 and D180, respectively; $p<0.0001$) in spite of a transient viral rebound between D30 and D90 in 90% of the patients. Multiple LMP-1 genotypes were found in 40 % of the patients, in most cases with the same predominant strain in the blood and the saliva.

Conclusions: These data show that IM patients harbour different viral load patterns and multiple EBV genotypes in saliva and blood. The persistent high level of viral load in saliva suggests that the patients remain infectious during IM convalescence.

Present EBV genome in blood and absent anti-EBV antibody is a novel one-point early diagnostic criterion for EBV primary infection

Dohno S, Maeda A, Ishiura Y, Imai S, Wakiguchi H

Contact:
Dr. med. Sumitaka Dohno
Kochi Medical School, National University Corporation Kochi University
Department of Molecular Microbiology and Infections Program of Biosignaling and Infection Control (Nankoku, Japan)
Email: sdohno@med.kochi-u.ac.jp

Abstract:
Objective: The diagnosis for EBV primary infection, infectious mononucleosis (IM), requires repeated anti-EBV antibody tests including anti-viral capsid antigen (VCA)-IgG, anti-VCA-IgM, anti-Early antigen (EA)-IgG and anti-EBV nuclear antigen (EBNA) antibodies in combination. Our preliminary study suggests that infants with IM often show negative results for above serologic tests at the early phase of the course. We examined the clinical significance of positive EBV-genome and negative antibodies in the patients manifested by IM-like symptoms.

Methods: We reviewed EBV serostatus of the IM children who were diagnosed by Sumaya's criteria, and analyzed the relationship between their age and the antibody titers against EBV. Antibody titers were measured by the commercial-based immunofluorescent assay. In addition, we report three patients with IM-like symptoms who showed present EBV genome in peripheral blood mononuclear cells (PBMC) and absent anti-EBV antibodies soon after disease onset. EBV genome was detected and measured by semi-quantitative PCR using limiting dilution or quantitative real time PCR.

Results: Thirty-five percents of the infants with IM revealed negative serologic tests against EBV before two weeks after disease onset. We experienced three infants (6 months, 8 months, and 12 months of age) with prolonged fever, hepatosplenomegaly, lymphadenopathy, skin rash, upper eye lid swelling, elevated liver enzyme value, and atypical lymphocytosis. Their antibody titers against EBV resulted in solely negative, and EBV genome was detectable by PCR in the acute phase (on the 28th, the 11th, and the 9th day after onset, respectively). The serostatus was followed and the seroconversion of anti-VCA-IgG was confirmed on the 42th, 36th, and 15th day, respectively, leading to the definitive diagnosis for EBV primary infection.

Conclusions: The serologic test alone in the early phase is not very useful for diagnosis of IM in the case of infants, but EBV-seronegative individuals carrying EBV genome in PBMC are strongly suspected as EBV primary infection. We propose that EBV genome positive and seronegative result is a novel diagnostic criterion for IM, which is particularly useful for infantile cases.

EBV infection of oropharyngeal mucosal epithelium

Tugizov S, Herrera R, Xiao J, Berline J, Veluppillai P, Greenspan D, Palefsky JM

Contact:
Dr. Sharof Tugizov
Department of Medicine
University of California, San Francisco (San Francisco, USA)
Email: tugizov@itsa.ucsf.edu

Abstract:
It has been suggested that the primary infection with EBV may occur at the oropharyngeal mucosal surface. To study the mechanisms of EBV infection in oropharyngeal epithelium we developed an ex vivo short term floating raft culture model using biopsy materials from tongue and buccal tissues. The floating raft tissue was infected with EBV B95-8. At 24 and 72 hours postinfection the raft tissue was fixed and cross-sectioned. To detect EBV proteins the sections were subjected to immunofluorescence assays using high-titer anti-EBV human serum. Confocal microscopy analysis of EBV-infected buccal and tongue raft cultures at 24 hrs postinfection showed EBV-infected cells, mostly within the submucosa. EBV-positive cells expressed CD45, CD86 and CD68 markers suggesting that these cells could be B cells, macrophages or dendritic cells. Most of the clusters of EBV-infected cells were localized in close proximity to the basement membranes but some were detected in the basal layer of mucosal epithelium. At 72 hrs postinfection a small number of EBV-positive and CD45-, CD68-, CD86- and CD1a-positive cells were detected within stratum basale, stratum spinosum and the lower part of stratum granulosum layers of the mucosal epithelium. There were no EBV-positive cells in the upper part of the stratum granulosum or the stratum corneum of tongue and buccal mucosal epithelium. These findings suggest that cell-free EBV entry does not occur from the intact surface of mucosal epithelium consistent with our earlier findings that showed the absence of EBV infection from the apical surface of polarized tongue and pharyngeal epithelial cells (Tugizov et al., Nature Medicine 9:307-314, 2003). Instead, EBV-infected submucosal B cells, macrophages or dendritic cells may migrate into the epithelial layer from the submucosa and initiate dissemination of EBV within the mucosal epithelium.

Kinetics of EBV-specific antibody responses in symptomatic and asymptomatic primary Epstein-Barr virus infection in Chinese children

Chiang AKS, Lam SY, Chan KH

Contact:
Dr. Alan Kwok Shing Chiang
The University of Hong Kong
Paediatrics and Adolescent Medicine (Hong Kong, HK)
Email: chiangak@hkucc.hku.hk

Abstract:
Objective: To study and compare the kinetics of EBV-specific antibody responses in symptomatic and asymptomatic primary Epstein-Barr virus infection

Methods: Fifteen Chinese children with symptomatic or asymptomatic primary EBV infection confirmed by serologic profile were followed longitudinally from the time of diagnosis to one year at defined intervals. The serum samples at each timepoint were tested simultaneously for VCA IgM, VCA IgG, EBNA and VCA IgG avidity for each patient.

Results: Among the symptomatic children (infectious mononucleosis [IM] group), VCA IgG titres were high (1: 2560 or above) at diagnosis and remained high at 6 months to one year. A four fold rise in VCA IgG titres could be documented in some patients within one month of diagnosis. The VCA IgG avidity converted from low to high at a range of 3-7 months while the EBNA antibodies became detectable at 3 months and continued to rise in titres towards one year. The VCA IgM were positive at diagnosis and became negative by 3-6 months. Among the asymptomatic children (non-IM group), the VCA IgG were also maintained at high titres (1: 1280 or above) from diagnosis to 1 year. However, they had high avidity VCA IgG at diagnosis and had relatively late emergence of EBNA antibodies when compared to the IM group.

Conclusions: While the VCA IgG response is equally robust between symptomatic and asymptomatic EBV infection in Chinese children, a difference in the time of emergence of the EBNA antibody may be observed.

Loss of Latent EBV Genome and Induction of Hydroxyurea Resistance in Hydroxyurea-treated Burkitt's Lymphoma Cell Line Raji

Jiang R, Zhang JL, Satoh Y, Sairenji T

Contact:
Prof. Dr. Takeshi Sairenji
Tottori University
Biomedical Sciences (Yonago, Japan)
Email: sairen@grape.med.tottori-u.ac.jp

Abstract:
Objective: Eliminating EBV genomes from infected cells is an intriguing theoretical strategy for EBV-associated malignant diseases. We have sought to characterize the mechanism by which hydroxyurea (HU), an inhibitor of ribonucleotide reductase (RR), leads to both elimination of latent EBV genome in the Raji Burkitt's lymphoma cell line and induction of HU resistance.

Methods: Raji cells were sub-cultured 20 times in medium containing 50 μM HU, over more than two months. Such HU-treated Raji cells were cloned by the limiting dilution method. These clones were analyzed for EBV genomes by Southern blot and fluorescence in situ hybridization, for EBV antigen expression by immunofluorescence and Western blotting, and for HU sensitivity by the cell growth MTT assay.

Results: EBV DNA was eliminated in about 40% of the cells in the HU-treated Raji cultures. Of 102 examined clones from such cultures, only EBV-positive and no EBV-negative clones could be isolated. No differences in EBV latent state, EBV-gene expression, and cell growth were observed between HU-untreated Raji cells and the clones. Relative to parental Raji cells, the Raji clones were almost 8-times more resistant to HU, and the expression of the R2 component of RR increased more than 3-5 times. The HU-resistant clones were sensitive to gemcitabine, another inhibitor of RR.

Conclusions: HU both efficiently eliminates EBV genome from Raji cells, and induces HU resistance. HU resistance is caused by over-expression of the R2 component of RR.

EBV modulates CXCR4 expression and migration in tonsil B lymphocytes.
Ehlin-Henriksson B, Mowafi F, Chiodi F, Klein G, Nilsson A

Contact:
Barbro Ehlin-Henriksson
Karolinska Institutet
Microbiology and Tumor Biology Center (Stockholm, Sweden)
Email: barbro.ehlin-henriksson@mtc.ki.se

Abstract:
EBV has previously been shown to induce changes in the cell surface expression of several chemokine receptors and the transfection of EBNA 2 or LMP 1 into the B cell lymphoma derived cell line, BJAB, decreased the expression of CXCR4.
In the present study we have infected primary tonsil B cells and then 1) followed and analysed the expression of CXCR4 by flow cytometry and 2) analysed the chemotactic response to the CXCR4 ligand SDF-1 in a Transwell migration assay.
We show that primary EBV infection in vitro reduces the expression of CXCR4 on primary tonsil B cells already 43 hrs after infection. Furthermore, EBV infection affects the chemotactic response with a reduction of the spontaneous and SDF-1-induced migration. To clarify whether this reduced migration is EBV specific or a consequence of cell activation, tonsillar B cells were either EBV infected, activated with anti-CD40 and IL-4 or kept in medium. Activation by anti-CD40 and IL-4 decreased the CXCR4 expression but the CD40+IL-4 stimulated cells showed no reduction of chemotactic efficacy.
SDF-1 is produced in the tonsil epithelium and it may be speculated that changing the SDF-1 response of the EBV infected B cells may serve the viral strategy by permitting the infected cells to move into the extrafollicular areas, rather than to be retained in the lymphoepithelium.

An improved tandem affinity purification method for the identification and characterization of proteins associated with the EBV-encoded nuclear antigen 5 (EBNA5)

Stromberg A, Ruetschi U, Rymo L

Contact:
Alma Strömberg
Laboratory Medicine
Clinical Chemistry and Transfusion Medicine (Gothenburg, Sweden)
Email: alma.stromberg@clinchem.gu.se

Abstract:
Objective: EBNA5 is one of the first viral proteins detected after primary EBV infection and has been shown to be required for efficient transformation of B lymphocytes. The primary known function of EBNA5 is cooperation with EBNA2 in the activation of the LMP1 promoter. Recently, we have shown that EBNA5 has the potential to inhibit pre-mRNA cleavage and polyadenylation. EBNA5 is also known as a multifunctional protein that interacts with various components of the cellular machinery. Our goal is to identify proteins that associate directly and indirectly with EBNA5.

Method: To further extend our search for cellular partners of EBNA5, we developed an improved tandem affinity purification method for isolation of protein complexes in mammalian cells. This method is a modification of the Tandem Affinity Purification (TAP) method by Puig et al. The TAP method was developed for isolation of protein complexes in yeast. When employed to mammalian cells, this method produced high background due to unspecific binding. The improved method allows rapid purification of protein complexes under native conditions, even when expressed at biologically relevant levels. In addition, this system provides an indication of the approximate stoichiometry of the proteins present in a given complex. This modified tandem affinity purification tag consists of a Protein A domain and the StrepTag II separated by two Tobacco Etch Virus (TEV) protease cleavage sites, fused to the C-terminus of EBNA5. The ProtA binds to an IgG matrix and the protein complex is eluted by cleavage with the TEV protease. The eluate from the IgG affinity resin is then incubated with the StrepTactin matrix. The bound material is released from the StrepTactin beads by the specific competition of D-desthiobiotin.

Results: With this improved tandem affinity purification, several specific interacting proteins with EBNA5 were identified. The resulting protein mixture after the purification procedure was analysed by SDS-PAGE and the proteins were visualised by silver staining. Identification of some of the putative cellular partners of EBNA5 has been performed by mass spectrometry.

Conclusion: We have developed a method for isolation of protein complexes in mammalian cells. By use of this method we have identified several interacting partners with EBNA5.

Structure and catalytic activity of the EBV protease

Buisson M, Hernandez JF, Forest E, Arlaud G, Morand P, Seigneurin JM, Burmeister WP

Contact:
Dr. Marlyse Buisson
EMBL / CHU Grenoble/Universite Joseph Fourier (Grenoble, France)
Email: buisson@embl-grenoble.fr

Abstract:
Objectives: The herpesvirus proteases are found in all herpesviruses and are essential for viral replication. To design new antiviral agents we characterized the crystal structure and the catalytic activity of the Epstein-Barr virus protease.

Methods: The protease domain of the assembling-protease of EBV has been expressed in E. coli and purified. As for the other herpesviruses, it forms a monomer-dimer equilibrium in solution. After inhibition of the protease with diisopropyl-fluorophosphate (DFP) we could crystallize the protease domain. A colorimetric assay was developed to study the catalytic activity of the protease. The chromogenic peptide substrate turnover was monitored by the colorimetric detection of free para-nitroaniline at 410nm.

Results: The structure was solved by X-ray crystallography to 2.3Å resolution. The overall structure confirms the conservation the homodimer and its structure throughout the α, β, and γ-herpesvirinae. We noticed that the specific rearrangement of the processed C-terminus of the protease domain is only possible after its release from the assemblin precursor protein implicating a conformational change after precursor cleavage. We furthermore could show that this precursor has a much reduced catalytic activity. The Km value for the peptide cleavage (M-site mimic) by the protease domain was 0.8 mM and the catalytic efficiency of 10 min-1 mM-1. These kinetic parameters are similar to the ones previously obtained for fluorescent and chromogenic substrates on other herpesvirus proteases.

Conclusions: Our work showed the importance of precursor cleavage for structure and activity of the EBV protease. Kinetic parameters and Ki's of a number of inhibitors have been determined that will allow us in the future to search for protease inhibitors with a potential antiviral activity.

Epstein-Barr virus infection in a human signet ring cell gastric carcinoma cell line, HSC-39

Luo B, Murakami M, Fukuda M, Fujioka A, Yanagihara K, Sairenji T

Contact:
Prof. Dr. Takeshi Sairenji
Tottori University
Biomedical Sciences (Yonago, Japan)
Email: sairen@grape.med.tottori-u.ac.jp

Abstract:
Objective:To study the mechanism of EBV infection in gastric epithelial cells, we characterized the EBV infection in signet ring cell line HSC-39, derived from a human gastric carcinoma.

Methods: HSC-39 cells were infected with Akata and P3HR-1 EBV. The infected cell clones were isolated and analyzed for the change of cellular phenotype with EBV infection.

Results: EBNA and EBER were detected in 50-70% of Akata or P3HR-1 virus-infected cells on days 1 and 2 post-infection and then decreased on serial passage. The Akata and P3HR 1 EBV-infected clones differed from each other in morphology and growth patterns. Akata EBV-infected clones had lower growth rates than did P3HR-1 EBV-infected clones in both liquid and soft agar media. Both the infected HSC-39 cells and the clones expressed EBNA1 and EBER, but did not express EBNA2, latent membrane protein (LMP) 1 and LMP2A. The Q promoter (Qp), but not the Cp/Wp for EBNA transcription, was active in the infected HSC-39 cells and all clones. No lytic infection was observed in either infected parental cells or any clones. Uninfected HSC-39 cells did not express a principal EBV receptor CD21; however, Akata but not P3HR-1 EBV-infected clones expressed CD21 mRNA.

Conclusions: HSC-39 cells were highly susceptible to cell-free EBV infection by Akata and P3HR-1 EBV strains; however, EBV genome in the infected cells is not stably maintained. The cellular phenotypes of HSC-39 cells are altered by EBV infection in strain-specific manner. We propose the HSC-39 cell line as a model target for the study of the mechanism and significance of EBV infection in gastric carcinoma.

Epstein-Barr virus (EBV)-associated gastric carcinomas in Germany: first retrospective clinico-pathological study

zur Hausen A, Brucken U, Weber A, Monig SP, Schneider PM, Holscher AH, Odenthal MO, Dienes HP, Baldus SE

Contact:
Dr. Axel zur Hausen
University Hospital Cologne
Pathology (Cologne, Germany)
Email: zhausen@aol.com

Abstract:
Objective: The association of EBV with gastric carcinomas (GC) is well established. With respect to the worldwide incidence GC comprise one of the most frequent EBV-associated human malignancy. EBV-associated GC are characterized by distinct chromosomal aberrations and a unique viral latency type. In the present study we analyzed GC for the presence of EBV to determine its clinico-pathological relevance in a large German GC patient cohort.

Methods: DNA of paraffin-embedded tumor material (n=176) was submitted to nested PCR testing. EBER-RNA in situ hybridization (RISH) was carried out on the EBV DNA-positive cases. The EBV-status was correlated with clinico-pathological features, including immunohistochemistry for p53 and p21.

Results: EBV was detected in 6.25% (n=11) of GC. EBV-positive GC were significantly more frequent in males (p=0.01) and the mean age of patients was higher (EBV+: 65.4, EBV-: 60.1). EBV was significantly more frequent in the unclassified type of GC (Laurén-classification) (p=0.02). No correlation was observed with pTNM-stage and the overall survival. However, 50% of the EBV-negative group were dead after 1.9 years, in contrast to 5.4 years in the EBV-positive group. This effect was abrogated after 6.7 years when 75% of both patients were dead. No difference was found between these two groups concerning the expression of the cell cycle regulators p53 and p21.

Conclusions: Our data of the first retrospective German clinico-pathological study on the relevance of EBV in GC strongly indicate that EBV-associated GC patients benefit of the presence of EBV with respect to the first 5 years after surgery, which is in line with our recent data of the Dutch D1D2 gastric cancer trial (566 GC; 41 (7.2%) EBV-positive). EBV-associated GC constitute a distinct clinico-pathological entity. The incidence of EBV-associated GC (6.25%) in Germany is comparable to other European countries.

EBP2 is Important for the Attachment of EBNA1 to Human Mitotic Chromosomes and is Regulated by Ipl1/Aurora Kinases

Kapoor P, Lavoie BD, Frappier L

Contact:
Priya Kapoor
University of Toronto
Department of Medical Genetics and Microbiology (Toronto, Canada)
Email: priya.kapoor@utoronto.ca

Abstract:
The EBNA1 latency protein mediates the segregation of EBV episomes in human cells by tethering them to the host mitotic chromosomes. This requires the interaction of the EBNA1 DNA-binding (amino acids 452-607) and segregation (amino acids 325-376) domains with the EBV segregation element FR and mitotic chromosomes, respectively. Using an EBV-based plasmid segregation system in S. cerevisiae, we have previously shown that EBNA1-mediated segregation of a FR-containing yeast plasmid requires EBP2, a human mitotic chromosomal protein that interacts with EBNA1 through amino acids 325-376. Functional studies of EBP2 using this system have indicated that EBP2 binds yeast mitotic chromosomes in a cell cycle dependent manner and is required for the attachment of EBNA1 to mitotic chromosomes. While our results indicate that EBP2 can enable EBNA1 to partition plasmids through chromosome attachment, they do not address the relative importance of EBP2 as an EBNA1 attachment site in human mitotic chromosomes. We have now assessed the importance of EBP2 for EBNA1-dependent segregation in humans using RNAi experiments to silence EBP2 in several human cell lines. We find that the silencing of EBP2 has pronounced effects on the ability of EBNA1 to bind mitotic chromosomes but does not affect EBNA1 localization during interphase. We have also investigated the mechanism of EBP2 attachment to mitotic chromosomes by screening a variety of yeast ts mutants for proteins that affect EBP2 association with yeast chromosomes. The interaction of EBP2 with mitotic chromosomes was not dependent on any of the yeast condensin or cohesion subunits, but was found to require the Ipl1 mitotic kinase. In keeping with these results, RNAi experiments in human cells demonstrated that the localization of EBP2 is partially dependent on the Aurora B kinase, the human homolog of Ipl1. These results indicate that EBP2 plays an important role in EBNA1-mediated segregation in human cells lines and that this process is regulated by the Ipl1/Aurora family of kinases.

Interactions of EBV capsid and capsid associated proteins with gB and the p38/BFLF2 complex

Ahuja M, Lake C, Hutt-Fletcher L

Contact:
Prof. Dr. Lindsey Hutt-Fletcher
Louisiana State University Health Sciences Center
Microbiology and Immunology and the Feist Weiller Cancer Center (Shreveport, USA)
Email: lhuttf@lsuhsc.edu

Abstract:
EBV membrane proteins are involved not only in entry of virus into its target cell, but also in assembly and egress of new virus from the cell in which it is replicating. Of the twelve membrane proteins currently known to be expressed only in the lytic cycle, five, gp350/220, gH, gL, gp42 and gp150, are dispensable for assembly or egress. Of the remaining seven, four have, to date, been implicated either in assembly or envelopment and egress of virus. Glycoproteins gN and gM are required for secondary envelopment and we have recently shown that the BFRF1 protein, p38, interacts with the BFLF2 protein to colocalize both proteins to the nuclear membrane. This behavior is identical to that of its homologs in alpha and betaherpesviruses where the complex is critical to export from the nucleus. An unexpected finding made by others several years ago was that EBV gB is also required for assembly of virus; in its absence there is incomplete assembly of capsids. In order to examine the interactions between virus proteins that may be important to assembly and egress of virus we made a cDNA library from induced Akata cells and cloned it into a yeast two hybrid vector. To date we have screened the library with p38, BFLF2 and the cytoplasmic tail of gB. The gB screen identified three virus proteins, BcLF1, BTRF1 and BDRF1. BcLF1 is the major capsid protein, BTRF1 is a homolog of the alphaherpesvirus UL21 gene, which encodes capsid associated protein involved in capsid maturation, and BDRF1 is a homolog of UL15 which transiently associates with capsids and may be involved in packaging. The interactions were confirmed biochemically using a GST-gB fusion protein. The BcLF1 binding site is between residues 821 and 841 of the 857 residue protein, whereas BDRF1 and BTRF1 interact with residues 754 to 802. The BFLF2 screen also identified BcLF1. Data to this point suggest a model in which gB is involved in capsid assembly, possibly nucleating interactions of BcLF1, BTRF1 and BDRF1, and the BFRF1/p38 complex interacts with the mature capsid to facilitate its exit into the perinuclear space.

Proteins Associated with Purified Epstein Barr Virus

Johannsen E, Luftig M, Chase M, Weicksel S, Illanes D, Cahir-McFarland E, Sarracino D, Kieff E

Contact:
Dr. Eric Johannsen
Channing Laboratory, Harvard Medical School, and Brigham and Women's Hospital
Department of Medicine and Microbiology and Molecular Genetics (Boston, USA)
Email: ejohannsen@partners.org

Abstract:
We have determined the protein composition of highly purified enveloped extracellular EBV using LC/MS/MS and database searching algorithms that included the possibility of polypeptide synthesis from any EBV open reading frame. The purity of our preparations was validated by electron microscopy. The sensitivity of our approach was evident by the detection of the portal protein homolog (HSV UL6), which is in 12 copies per capsid, but was not previously detected in enveloped virus. We identified 32 viral-encoded proteins, many of which were known or predicted to be virion components based on homology to known structural proteins of other herpesviruses. Novel virion proteins included three potential gammaherpes virus unique tegument proteins. An unanticipated observation was the identification of a processing event in the EBV glycoprotein B homologue, which has been demonstrated for other herpesvirus gBs. In addition to viral-encoded proteins, over 50 cell proteins were detected in these preparations. Many were highly abundant cytoplasmic proteins that could be non-specifically incorporated during virion egress. However, that is not the case for some of these proteins. For example, the third most heavily stained protein in EBV preparations is almost entirely actin. Using Isotope Coded Affinity Tags (ICAT) to label detergent treated and untreated virions, we determined the relative amounts of proteins in enveloped and de-enveloped virus. Based on this, we classified EBV virion proteins as envelope (decreased by NP40), tegument (stable to NP40), or capsid associated (stable to NP40 + DOC). Surprisingly, some host proteins, including actin and tubulin were tightly capsid associated. Our results define the protein composition of EBV virions and suggest important avenues for future studies of herpesvirus virus morphogenesis.

Viral transforming protein LMP-1 plays a critical role in virus production

Ahsan MN, Kanda T, Takada K

Contact:
Mohammed Nazmul Ahsan
Genetic Medicine, Hokkaido university
Tumor Virology (Sapporo, Japan)
Email: shishir74@hotmail.com

Abstract:
Objective: LMP1 is essential for EBV-mediated B cell transformation. It is strongly upregulated upon induction of viral lytic cycle. However, the biological significance of this strong LMP1 induction has been unclear. Our objective is to clarify whether LMP1 plays additional roles during the EBV life cycle.

Methods: BAC system was used to mutate LMP1 gene of Akata EBV. A nonsense linker (encoding 3 stop codons) was introduced after codon 9 of the LMP1 gene of AK-BAC-GFP(BAC having Akata EBV genome with an inserted GFP transgene) via homologous recombination in E.coli. Akata cells were transfected with modified BAC clone to obtain cell clones harboring only LMP1 mutated EBV episomes. The cell clones were treated with anti-IgG to induce virus production, and were examined for LMP1 and viral late gene products expression, and for the level of viral DNA amplification. Culture supernatants were examined for infectivity to B cells and for the amount of virions. Nucleocapsid formation was checked by EM.

Results: The mutation completely abolished the expression of LMP1. However, the LMP1 mutated Akata EBV entered the lytic cycle as efficiently as the wild-type counterpart (AK-BAC-GFP). The degree of viral DNA amplification and the expression levels of viral late gene products (gp110 and gp350) after virus induction were found to be intact. By contrast, the culture supernatant of LMP1 mutated virus exhibited poor infectivity to B cells. The amount of virions in the culture supernatants of LMP1 mutated virus was far less than that obtained from cells harboring the wild-type counterpart. This reduction of virions in the culture supernatant was restored by supplying the truncated form of LMP1 protein to the virus-producing cells in trans. EM observation revealed that nucleocapsid formation inside nuclei was found even in the absence of LMP1, which suggests that LMP1 mutated EBV can produce mature nucleocapsids inside nuclei but the egression of nucleocapsid is impaired due to LMP1 loss, resulting in few infectious viruses in culture supernatant.

Conclusions: Results strongly suggest that, although LMP1 is dispensable for viral DNA amplification and nucleocapsid formation, it is critical for augmenting the egression of mature nucleocapsids. Our data provides the first direct evidence that a viral transforming gene product also plays a critical role for virion egression.

Gene regulation in Epstein-Barr virus lytic cycle
Amon W, Binne UK, Farrell PJ

Contact:
Wolfgang Amon
Ludwig Institute for Cancer Research
Imperial College Faculty of Medicine (London, UK)
Email: wolfgang.amon@imperial.ac.uk

Abstract:
Objective: The EBV lytic cycle is divided into three phases, which involve immediate early, early and late gene expression. The first step in EBV reactivation is induction of the viral transcription factor BZLF1 via its promoter Zp. Our objective is to identify molecular mechanisms that regulate various stages of EBV lytic cycle gene expression.

Methods: Reactivation from latency can be studied using a cell line in which the latent EBV is reactivated by cross linking the B-cell receptor (BCR) on the cell surface using antibody to mimic the binding of antigen to the BCR. This gives a rapid and efficient induction of the virus lytic cycle and may mimic a physiological reactivation in vivo. Episomal vectors containing the EBV oriP stably transfected into EBV positive Akata cells have been used to study viral gene regulation using luciferase as a reporter. This accurately mimics the normal regulation of gene promoters in the background of the complete virus genome.

Results: The Zp promoter of EBV but not the Rp promoter can be activated in the absence of protein synthesis in these oriP plasmids, casting doubt on the immediate early status of Rp. The relative timing of immediate early, early and late promoter activity was reconstituted in this system. Late lytic promoters were only active if the EBV origin of lytic replication (ori lyt) was present within the plasmid. A large ori lyt fragment conveyed late lytic cycle activity on late promoters that was sensitive to inhibitors of lytic DNA replication. A minimal ori lyt which barely undergoes any replication was still able to confer late lytic cycle activity specifically on late promoters, but this was not sensitive to inhibitors of lytic DNA replication. Deletion analysis of EBV late promoter sequences upstream of the transcription start site confirmed that sequences between -49 and +30 are sufficient for late gene expression.

Conclusions: The results cast doubt on the immediate early status of Rp and suggest a mechanism of late gene regulation more similar to that of other herpes viruses than proposed recently for EBV.

Epstein-Barr Virus Rta Response Elements (RREs) Vary Markedly in their Capacity to Bind and to be Activated by Rta

Chen LW, Chang PJ, Miller G

Contact:
Dr. George Miller
Yale University School of Medicine
Pediatrics, Epidemiology and Molecular Biophysics & Biochemistry (New Haven, USA)
Email: George.Miller@yale.edu

Abstract:
The EBV R transactivator (Rta) protein activates lytic cycle viral genes by several distinct mechanisms: direct binding to viral promoters, synergy with ZEBRA, and activation of cellular signaling pathways. In the direct and synergistic mechanisms of action, Rta is believed to bind to specific DNA sequences which are present in the promoters of responsive genes. These RREs fit the consensus GNCCN9GGNG. However, it has been difficult to study the capacity of Rta expressed in mammalian cells to bind DNA in vitro. We discovered that a C-terminal domain of Rta inhibits its ability to bind DNA. C-terminal truncated versions of Rta bind RREs efficiently and thus facilitated the comparison of the relative affinity of RREs from the promoters of five Rta responsive genes: BMLF1, BHLF1, BMRF1, BaRF1, and BLRF2. It is not yet known whether all these genes are direct targets of Rta. All the RREs found in the promoters of the five genes conform to the proposed consensus sequence; nonetheless, the RREs varied markedly in their ability to bind Rta in EMSA. Not all RREs that fit the consensus were functional. There was a strong correlation between binding affinity of the RREs and the capacity to be activated by Rta in reporter assays carried out in EBV-negative B cells. By creation of chimeras, inversions and point mutations, differences in binding affinity and transcriptional activation were found to be due to N9 sequence variation. The length of N9 was also critical for a maximal response. The optimal RRE appears to be: GTCCC/AT/CC/GNA/GNCA/GT/AGGGCG. The kinetics and abundance of the mRNAs representing the five responsive lytic cycle genes also differed dramatically in Raji cells that were transfected with Rta. Therefore, RRE affinity for Rta is likely to play an important role in temporal regulation and the level of lytic cycle EBV gene expression.

Signaling Pathways Affect Expression of Epstein-Barr Virus Latent-Lytic Switch BZLF1 Gene Promoter in Part via ZEB

Kraus R, Yu X, Perrigoue E, Mertz J

Contact:
Prof. Dr. Janet Mertz
University of Wisconsin-Madison
McArdle Laboratory for Cancer Research (Madison, USA)
Email: mertz@oncology.wisc.edu

Abstract:
Objective: Epstein-Barr virus (EBV) is a human gamma herpesvirus capable of establishing a latent state in B-lymphocytes. The product of the immediate-early BZLF1 gene, Zta, is a transcriptional trans-activator essential for viral DNA amplification and virion production. The promoter of the BZLF1 gene, Zp, is repressed during latency. However, multiple kinase pathways signal activation of Zp, with the target transactivators and their cognate binding sites within Zp being fairly well established. We previously identified a negative cis-acting element within Zp, termed ZV, and its trans-acting factor, ZEB; we also showed that TPA treatment leads to superactivation of ZV mutants of Zp relative to wild-type (Kraus et al., J. Virol. 75: 867, 2001; Kraus et al., J. Virol. 77: 199, 2003). Here, we began to examine molecular mechanisms by which inducers of EBV act in part by altering ZEB's activities.

Results: We found that ZEB is a phosphoprotein that is a target of the TPA signaling pathway, with the phosphorylation status dictating its DNA-binding affinity. Treatment of the B-lymphocytic cells lines DG75 and Raji with TPA enhanced the DNA-binding affinity of ZEB, but showed no effect on ZEB in the epithelial cell lines HEK293 and HeLa. Correspondingly, TPA treatment lead to low-level activation or, even, modest repression of wild-type Zp yet high-level stimulation of a ZV mutant of Zp in the former cells; however, it had no effect on either promoter in the latter ones. To begin to study the role of ZEB binding to the ZV site of Zp, we introduced base pair substitution mutations in the ZV site of Zp into the maxiEBV plasmid p2089 of Hammerschmidt and colleagues. Our preliminary results indicate that this mutant EBV could still establish a latent infection in 293 and EBV-negative Akata cells with a very low rate of spontaneous reactivation. We are beginning to analyze the phenotype of this latency and the efficiency and kinetics of reactivation by inducers of this mutant relative to wild-type EBV.

Conclusions: We conclude that ZEB binding to the ZV site of Zp contributes significantly to the regulation of Zp, with ZEB's activities being affected in a cell type-specific manner via signaling pathways that lead to alterations in ZEB's phosphorylation status.

Phosphorylation of Epstein-Barr Virus ZEBRA Protein at its Casein Kinase 2 Sites Mediates its Ability to Repress Rta Activation of a Viral Lytic Cycle Late Gene by Rta

El-Guindy AS, Miller G

Contact:
Dr. Ayman El-Guindy
Yale University
Molecular Biophysics and Biochemistry (New Haven, USA)
Email: ayman.el-guindy@yale.edu

Abstract:
ZEBRA, a member of the bZIP family, serves as a master switch between latent and lytic cycle EBV gene expression. ZEBRA influences the activity of another viral transactivator, Rta, in a gene specific manner. Some early lytic cycle genes, such as BMRF1, are activated in synergy by ZEBRA and Rta. However, ZEBRA suppresses Rta's ability to activate a late gene, BLRF2. Here we show that this repressive activity is dependent on the phosphorylation state of ZEBRA. We find that two residues of ZEBRA, S167 and S173, that are phosphorylated by casein kinase 2 (CK2) in vitro, are also phosphorylated in vivo. Inhibition of ZEBRA phosphorylation at the CK2 substrate motif, either by serine-to-alanine substitutions or by use of a specific inhibitor of CK2, abolished ZEBRA's capacity to repress Rta activation of the BLRF2 gene, but did not alter its ability to initiate the lytic cycle or to synergize with Rta in activation of the BMRF1 early lytic cycle gene. These studies illustrate how the phosphorylation state of a transcriptional activator can modulate its behavior as an activator or repressor of gene expression. Phosphorylation of ZEBRA at its CK2 sites is likely to play an essential role in proper temporal control of the EBV lytic life cycle.

Implications for Pathogenesis: Post-translational Control of Epstein-Barr Virus Lytic Cycle Gene BZLF1 is impaired in a Strain Isolated from a Case of Chronic Active Infection.

Jager M, Konig U, Niller HH, Larcher C, Huemer H, Mitterer M, Wolf H, Schwarzmann F

Contact:
Dr. Fritz Schwarzmann
Universität Regensburg
Institut für Medizinische Mikrobiologie und Hygiene
Forschungszentrum (Regensburg, Germany)
Email: fritz.schwarzmann@gmx.de

Abstract:
So far, pathogenesis of chronic active infection (CAEBV) is unclear. Immunological disorders and non-transforming viral strains are discussed. Previously, we described CAEBV in a case of persistent polyclonal B-cell lymphocytosis (PPBL). Serology and transcriptional analysis pointed to enhanced systemic replication of EBV. Transcription of the viral key regulator of replication, BZLF1, was detected in peripheral B-lymphocytes of the patient confirming lytic viral replication. The impaired capacity of the strain to immortalise B-lymphocytes correlated with reduced transcription of latency-associated genes and simultaneously significantly increased activity of BZLF1.
We report characterisation of this immortalisation-impaired EBV strain SM in order to identify the molecular basis for its bias to lytic replication. The genomic structure of strain SM, analysed by Southern blotting, precluded major deletions or rearrangements. Selected viral genes with key regulatory functions for latency and replication like EBNA2, BZLF1, and the EBERs were isolated and sequencing revealed characteristic nucleotide variations. Band shift and co-immunoprecipitation assays confirmed binding of EBNA2 to CBF1 and reporter assays showed trans-activation of a target promoter simmilar to strain B95-8. One of the nucleotide variations was located in the dimerisation domain of BZLF1. Trans-activation by BZLF1 and interaction with post-translational regulators p53 and NF-kappa-B were comparable to the B958-strain. However, co-immunoprecipitation demonstrated a failure to bind to the retinoic acid receptor alpha (RAR-alpha), which is a known negative regulator of BZLF1. As expected, RAR-alpha was impaired to down-regulate autokrine stimulation of BZLF1.
In conclusion, we identified a nucleotide variation in an EBV-isolate of CAEBV, which affects posttranslational control of BZLF1. This sequence variation could be a reasonable cause for the non-immortalising phenotype of this isolate.

Interaction of the Epstein-Barr virus (EBV) mRNA export factor EB2 with a novel human Spen protein, OTT3, suggests a link between Spen proteins and mRNA splicing and export

Hiriart E, Keppler S, Mikaelian I, Buisson M, Meresse P, Mercher T, Bernard O, Sergeant A, Manet E

Contact:
Dr. Evelyne Manet
INSERM U412, Ecole Normale Supérieure de Lyon
Unité de Virologie Humaine (Lyon, France)
Email: emanet@ens-lyon.fr

Abstract:
Objective: The Epstein-Barr virus (EBV) protein EB2 (also called Mta, SM or BMLF1) is essential for the production of infectious EBV virions and has properties in common with mRNA export factors. EB2 binds RNA and shuttles between the cytoplasm and the nucleus. EB2 induces the cytoplasmic accumulation of mRNAs generated from intronless genes and unspliced mRNAs if they contain introns normally excised by the use of cryptic splice sites. We have previously shown that EB2 is found in complexes containing the REF and TAP cellular export factors together with mRNA. In an effort to understand the function of EB2 better, our objectives have been to characterize potential cellular partners of the EB2 protein.

Methods: We have performed a yeast two-hybrid screen using an EBV-transformed human peripheral lymphocytes cDNA library cloned in the two hybrid vector pACT with EB2 as the bait. Interactions were further analysed by co-immunoprecipitation assays, GST pull-down and colocalisation of the proteins within the cells using confocal microscopy.

Results: The yeast two-hybrid screen using EB2 as the bait led us to the isolation of a novel human gene, huOTT3, a new member of the split end (spen) family of proteins (huSHARP, huOTT1, DoSpen, MuMINT). Spen proteins are characterized by several N-terminal RNA Recognition Motifs (RRM) and a highly conserved C-terminal SPOC domain (Spen Paralog and Ortholog C-terminal domain). huOTT3 is a 890 amino-acid protein that can be immunoprecipitated from human B and epithelial cells by a monoclonal antibody (1D2), as a 110 kDa protein. When expressed from a tranfected plasmid, huOTT3 is a non-shuttling nuclear protein that colocalizes with co-expressed EB2. We have characterized the interaction domains in both proteins. Recent cristallographic studies have shown that the SPOC domain of Spen proteins is highly conserved and could have an essential repression function by directly interacting with SMRT/NCoR corepressors. We find that the SPOC domain of huOTT3 recruits both SMRT and EB2.

Conclusions: Our results suggest a new function for the huSpen proteins related to mRNA export and splicing. Interestingly, huSHARP and huOTT1 were recently identified as components of the spliceosome.

Characterization of the minimal replicator of Kaposi's sarcoma-associated herpesvirus
Hu JH, Renne R

Contact:
Dr. Rolf Renne
Case Western Reserve University
Division of Hematology/Oncology and Dept. of Microbiology (Cleveland, USA)
Email: rfr3@po.cwru.edu

Abstract:
The latency-associated nuclear antigen (LANA) of Kaposi's sarcoma-associated herpesvirus (KSHV) is required for long-term maintenance of terminal repeat (TR)-containing plasmids. LANA has also been shown to support DNA replication of TR-containing plasmids in dividing cells. We have previously reported that a single copy of TR replicates DNA with the same efficiency as two copies of TR. These data showed that all necessary cis-regulatory elements for ori function are located within TR. Deletion analysis revealed two sequence elements that are required for LANA-dependent ori function: two LANA binding sites (LBS1/2) and a 62-bp GC-rich sequence upstream of LBS1/2.
In the present study, we performed a detailed mapping analysis to define cis-regulatory elements and the minimal replicator within the KSHV TR. Our data show that a 70 bp long fragment replicates with the same efficiency than full-length TR in the presence ofLANA. Next, to LBS1/2 we mapped a 29 bp long GC-rich element, which we term replication element (RE). The minimal replicator of KSHV is encompassed by two Sp1 binding sites, however, Sp1 binding does not contribute to DNA replication of small plasmids.
We have previously shown that TR sequences have enhancer activity. Using DN/Sp1 in reporter assays in combination with EMSAs we demonstrate that Sp1 binds to TR and augments transcription. Sp1 binding sites have been described in many cellular and viral origins of replication as well as in the terminal repeats of the Epstein-Barr virus. In summary, we have identified three sequence elements within TR: LBS1/2, RE element and Sp1 binding sites. Although Sp1 sites are not required for replication of plasmids we cannot rule out a supportive role for DNA replication in the context of large viral episomes. In addition, structural similarities and differences between the latent origin of KSHV and oriP of EBV will be discussed.

Dynamics of Epstein-Barr virus EBNA1 protein binding to viral genome and subnuclear redistribution from latent to lytic infection

Daikoku T, Kudoh A, Tsurumi T

Contact:
Dr. Tohru Daikoku
Aichi Cancer Center Research Institute
Division of Virology (Nagoya, Japan)
Email: tdaikoku@aichi-cc.jp

Abstract:
Epstein-Barr virus, a lymphotropic human herpesvirus, possesses two life styles; latent and lytic infections. During the latent infection, EBV genomes are maintained as double-stranded DNA episomes, and replicatd once per cell cycle during the S phase, following the rules of chromosome replication. The viral latent gene products, EBNA1, binds directly to latent replication origin, oriP, as homodimer but lacks any activity predicted to be required for replication initiation. Lytic replication differs from the latent amplification state in that multiple rounds of replication are initiated within lytic replication origin, oriLyt, and the replication process has a greater dependence on seven EBV-encoded lytic replication proteins. A low level of EBNA1 transcripts is still produced during the viral productive cycle. Understanding protein-DNA interactions in vivo at origins of DNA replication throughout the cell cycle and lytic replication may shed further insight on EBNA1 functions on replication control.
Here, we report on EBNA1 binding to the EBV genome-wide mapping through latent and lytic replication by ChIP assay. It was turned out that EBNA1 binds to the oriP region of the EBV genome throughout the cell cycle. Even after induction of lytic replication EBNA1 still continued to bind to oriP. From the confocal microscopy analyses, lytic DNA replication occurred at discrete sites in nuclei, called replication compartments, where viral replication proteins were clustered. During the lytic replication, no chromosomal DNA replication occurred. In latent infection EBNA1 was distributed broadly in nuclei as fine punctate dots with weak, diffuse stainig. After induction of lytic replication, EBNA1 was redistributed and clustered to replication compartments with bright granular spots. However, the spots of EBNA1 did not completely coincide with BrdU stainig or viral replication proteins, but rather located side by side with the viral replication proteins and viral progeny DNA.

New insights into the molecular functions of the regulatory elements of the latent origin of DNA replication of EBV

Ott E, Pich D, Thomae A, Schepers A

Contact:
Dr. Aloys Schepers
GSF-National Research Centre for Environment and Health
Deptartment of Gene Vectors (München, Germany)
Email: schepers@gsf.de

Abstract:
In EBV latently infected lymphocytes, the viral genome persists as episomes that are replicated once per cell cycle using the host's replication initiation and elongation machinery. Only two viral elements are required for stable replication and maintenance: The latent origin of DNA replication, oriP, in cis and the EBNA1 protein in trans. OriP consists of two elements: the dyad symmetry element (DS) and the family of repeats (FR) both containing clusters of binding sites for EBNA1. It is generally accepted that the DS-element functions as viral replicator by recruiting the cellular origin recognition complex to oriP, whereas the FR tether oriP plasmids to condensed chromosomes during mitosis.

We used the mini-EBV technology to dissect the molecular functions of the oriP-elements individually in their native context and at ectopic positions, addressing the following points: (i) What is the necessity for virus production and the immortalization process? (ii) In contrast to small oriP plasmids, where replication is dependent on the DS-element, it was found that this element is dispensable on the EBV-genome. We addressed these apparently conflicting observations and asked, whether the viral replicator is important for integrating viral replication into the cell cycle of the host. (iii) Although it is known that the FR are essential for stable maintenance of small plasmids, it is not known whether they are also redundant in context of the viral genome. We will present data on these projects that shed new light on the molecular functions of the individual oriP-elements.

A super-tight EBNA1- and oriP-based doxycycline-regulatable episomal 'one-plasmid' vector

Bornkamm GW, Berens C, Kuklik-Roos C, Bechet JM, Laux G, Bachl J, Korndoerfer M, Schlee M, Holzel M, Chapman R, Nimmerjahn F, Mautner J, Hillen W, Bujard H, Feuillard J

Contact:
Prof. Dr. med. Georg W. Bornkamm
GSF-Research Center for Environment and Health
Institute of Clinical Molecular Biology and Tumor Genetics (München, Germany)
Email: bornkamm@gsf.de

Abstract:
Objective: Conditional expression systems are of pivotal importance for the dissection of complex biological phenomena. Most conditional systems suffer, however, from leakiness. Here we describe a novel EBNA1- and oriP-based episomal doxycycline-regulatable vector (pRTST-1) with a unique combination of features. The vector is characterized by (i) its extremely low background activity in the absence, (ii) its high inducibility in the presence of doxycycline, and (iii) its graded response to increasing doxycycline or tetracycline concentrations.

Methods, Results, Conlusions: The novel vector pRTST-1 carries all the elements for conditional gene expression including the gene of interest on one EBV-derived episomally replicating plasmid. The chicken beta actin promoter and an element of the murine immunoglobulin heavy chain intron enhancer drive constitutive expression of a bicistronic expression cassette that encodes the optimized, highly doxycycline-sensitive reverse transactivator-VP16 fusion protein (rtTA2s-M2) (referred to as the reverse tet-transactivator) and a tetracycline repressor-KRAB fusion protein (referred to as tet-silencer) placed behind an internal ribosomal entry site. The gene of interest is expressed from a bidirectional doxycycline or tetracycline regulatable promoter that allows simultaneous expression of a second gene, e. g. eGFP or truncated NGF receptor that may be used as surrogate markers for the expression of the gene of interest. Tight regulation is achieved by binding of the repressor to the doxycycline-regulated bidirectional promoter in the absence of doxycycline, and combined relief of repression and binding of the reverse transactivator in the presence of doxycycline. In human B lymphoma lines stably transfected with pRTST-1, basal levels of eGFP and luciferase expression were invariably very low in the absence of doxycycline, whereas inducibility varied among different clones and reached levels of 10,000 to 140,000 fold in individual single cell clones. An EBV-immortalized cell line stably transfected with Influenza virus matrix protein 1 (M1) cloned into pRTST-1 elicited an antigen-specific, HLA class II-restricted T cell response in the presence, but not in the absence of doxycycline. Stable transfection of pRTST-1 into rodent fibroblasts revealed also excellent regulation and maintenance of multiple episomal copies.

Inhibition of Epstein-Barr virus latent replication by tankyrase and PARP1 poly-ADP ribosylation of EBNA1

Deng Z, Atanasiu C, Sbodio JI, Chen CJ, Chi NW, Lieberman PM

Contact:
Dr. Zhong Deng
The Wistar Institute
The Molecular Genetics (Philadelphia, Germany)
Email: dengz@wistar.upenn.edu

Abstract:
Objective: The family of poly-ADP ribose polymerases (PARPs) affects numerous cellular processes by transiently modifying proteins with heterogeneous polymers of poly-ADP ribose (PAR). TNKS1 and PARP1 were previously found to be highly enriched in OriP-specific DNA affinity purified protein preparations, but their molecular associations and potential functions at OriP have not been explored in detail. In this study, we intend to understand how PARPs modulate the plasmid maintenance and DNA replication activity of latent EBV.

Results: Our data indicate that PARP1 and tankyrase (TNKS), a telomere-associated PARP, inhibit Epstein-Barr virus (EBV) OriP replication function through a physical interaction and enzymatic-modification of EBNA1. Previous work had identified PARP1 and TNKS1 as OriP-associated proteins. We now show that pharmacological inhibitors of PARP enzymes and shRNA targeted knock-down of PARP1 and TNKS1 enhance OriP-dependent plasmid maintenance and DNA replication. OriP-dependent DNA replication was also inhibited by overexpression of TNKS1 or TNKS2, but not by a PARP-inactive mutant of TNKS2 (M1054V). Coimmunoprecipitation and indirect-immunofluorescence indicated that EBNA1 and TNKS proteins interact in living cells. Two RXXPDG-like TNKS-interacting motifs in the EBNA1 amino-terminal domain mediated binding with the ankyrin repeat domain of TNKS. Mutations of both motifs at EBNA1 G81 and G425 abrogated TNKS binding and enhanced EBNA1-dependent replication of OriP. We show that EBNA1 was subject to PAR-modification in vivo, and that recombinant PARP1 and TNKS1 can modify purified recombinant EBNA1 in vitro. These findings demonstrate that PARP and TNKS inhibit EBNA1 function at OriP in PAR-dependent manner, and suggest that PARP enzymes regulate viral episome maintenance by direct modification of EBNA1.

Conclusions: Our results suggest that cellular PARPs modify DS-associated proteins that modulate the plasmid maintenance and DNA replication activity of latent EBV. PAR modifications may play an important role in the rapid remodeling of protein complexes at OriP. TNKS may interact with EBNA1 in a transient and dynamic fashion in vivo. PAR-modification of EBNA1 negatively regulates OriP replication and plasmid maintenance. The antiviral function of TNKS and PARP1 may provide EBV with a copy number control mechanism.

Tagging gammaherpesvirus episomes for live cell studies

White RE, Allday MJ

Contact:
Dr. Rob E. White
St Mary's Campus, Imperial College
Department of Virology (London, UK)
Email: robert.e.white@imperial.ac.uk

Abstract:
Objective: Gammaherpesvirus latency, and the transition into the lytic cycle is well understood at a transcriptional level, but the nature and timing of interactions with nuclear structures during latency are much more poorly understood. In order to investigate any such interactions, particularly those with ND10 domains, we aimed to directly label herpesvirus episomes with a fluorescent protein, to allow the tracking of individual episomes.

Methods: Strategies for directly labelling DNA have previously been used to tag genomic loci and HSV amplicons. This entails cloning an array of binding sites for a DNA-binding protein (either lac repressor or tet repressor). We have cloned an array of 168 tetO sites (binding sites for the tet repressor) and cloned it into plasmids carrying EBV oriP and an expression cassette for a tetR-GFP fusion protein. Transfection of this into 293 cells expressing EBNA-1 allowed selection of cells containing tagged episomes. We have also subcloned the tetO array into a BAC carrying the herpesvirus saimiri genome.

Results: Confocal microscopy of cells carrying tagged episomes shows fluorescent foci representing the individual EBV episomes. The HVS-BAC carrying the tetO array is capable of producing infectious virions in permissive cells, with tetO distribution suggestive of replication compartments. Real-time studies, and associations with nuclear structures are ongoing for both these systems.

Conclusions: This data suggests that this strategy for tagging -herpesvirus episomes and genomes offers promise for elucidating the real-time interactions between individual episomes and nuclear substructures.

Epstein-Barr virus (EBV) infection in epithelial cells in vivo: rare detection of EBV replication in tongue mucosa but not in salivary glands

Frangou P, Buttner M, Niedobitek G

Contact:
Dr. med. Maike Büttner
Pathologisches Institut
Friedrich-Alexander-Universität (Erlangen, Germany)
Email: Maike.Buettner@patho.imed.uni-erlangen.de

Abstract:
Objective: The Epstein-Barr virus (EBV) establishes a persistent infection of B-lymphocytes. Infectious virus is shed into the saliva but the cellular source of this virus has remained controversial. Putative reservoirs are tongue epithelial cells, where the virus can replicate in HIV-infected individuals, and salivary glands.

Methods: 232 samples from the margins of the tongue from 217 patients, 241 salivary gland specimens, and 63 squamous cell carcinomas of the tongue were investigated. Expression of the EBV-encoded immediate early (BZLF1), early (EA), viral capsid (VCA) and membrane (MA) antigens was detected by immunohistochemistry. Viral DNA and the EBV-encoded RNAs (EBERs) were identified by in situ hybridization.

Results: Full viral replication was seen in 3 of 232 (1.3%) mucosal samples from the tongue. No latent EBV infection was found at this site. No evidence of latent or lytic EBV infection was detected in salivary glands, and all carcinomas of the tongue were EBV-negative.

Conclusions: EBV replication occurs only infrequently in normal tongue epithelial cells. Salivary glands are unlikely to be a site of lytic or latent EBV infection. Finally, EBV is not involved in the pathogenesis of carcinomas of the tongue.

Epstein-Barr virus lytic replication evokes ATM checkpoint signal transduction while preventing p53-downstream signaling

Kudoh A, Tsurumi T

Contact:
Ayumi Kudoh
Aichi Cancer Center Research Institute
Division of Virology (Nagoya, Japan)
Email: akudoh@aichi-cc.jp

Abstract:
When exposed to genotoxic stress, eukaryotic cells activate a rapid and specific DNA damage response to delay or arrest cell-cycle progression, providing time for DNA repair. Induction of lytic program in B lymphocytes latently infected with Epstein-Barr virus (EBV) results in an explosive viral genome synthesis and arrest of cell cycle progression. Here we show that EBV lytic program elicits a cellular DNA damage response, with an activation of ATM signal transduction pathway, leading to phosphorylation of Chk2, Chk1, histon H2AX, and p53. The lytic program, however, blocks the p53-downstream signaling through physical interaction of p53 with the BZLF1 viral protein and its degradation by proteasome, resulting in maintaining constant levels of p53-target genes. Despite the activation of ATM checkpoint signaling, S-phase CDK activity was increased and hyperphosphorylated forms of Rb protein and E2F-1 were accumulated with the progression of lytic infection. Thus, EBV lytic replication skillfully evades host checkpoint security system and actively promotes an S-phase like environment that is advantageous for viral lytic replication.

Activation of EBV lytic cycle protects Raji cells from apoptosis and concomitantly increases nuclear matrix protein levels

Matusali G, Compagnoni S, Di Renzo L, D'Erme M, Mattia E

Contact:
Dr. Elena Mattia
University "La Sapienza"
Dept. of Public Health Sciences (Roma, Italy)
Email: elena.mattia@uniroma1.it

Abstract:
Expression of EBV lytic cycle genes is induced in Burkitts lymphoma-derived cells by treatment with phorbol esters, n-butirate, TGFβ, and anti-immunoglobulin. It has been reported that although all these agents induce apoptosis, lytic gene expression protects from cell death. Because treatment with inhibitors of viral DNA synthesis prior to induction of the lytic cycle, blocked the protective effect, it was hypothesized the latter being due to the expression of a late gene.
We have treated Raji cells with phorbol 12-13 dibutyrate P(BU)2, TGFβ2 and n-butyrate to quantitatively induce EBV lytic cycle gene expression and report here that they are protected from apoptosis. Because EBV does not express late antigens in Raji cells, an immediate early or an early EBV gene is likely to be responsible for preventing cell death. At 8 hours after induction of EBV lytic cycle, our results show a 5 fold increment of the viral oncoprotein LMP1 paralleled by a 10 fold and 2 fold increment of the cellular antiapoptotic proteins Mcl1 and Bcl2, respectively and a 70% decrement of p53 protein levels.
To investigate the effects of EBV lytic cycle induction on nuclear matrix proteins, we have evaluated the concentration of SAF-A, nuclear lamins B2 and D/E, poly-ADP ribose-polymerase (PARP) and nuclear actin, following the addition of P(BU)2, TGFb2 and n-butyrate to Raji cells and to Ramos, EBV-negative cells.
We detect a substantial increment in Raji cells of all the nuclear matrix components analyzed. In contrast, the levels of these proteins appear to diminish during the treatment of Ramos cells with the inducers, with the production of the apoptotic form of SAF-A and PARP. RT-PCR experiments indicate a post-transcriptional control of nuclear matrix proteins, following induction of EBV lytic cycle.

Towards Structural Characterization of the ZEBRA / DNA Complex

Morand P, Petosa C, Moulin M, Perissin M, Baudin F, Tarbouriech N, Seigneurin JM, Mueller C

Contact:
Dr. Patrice Morand
CHU Grenoble
Virology (Grenoble, France)
Email: pmorand@chu-grenoble.fr

Abstract:
Objective:The viral transcription factor ZEBRA (also known as Zta, Z, EB1) belongs to the basic-leucine zipper transcription factor-family (bZip). The Zebra's DNA binding domain is homologous to the basic domain of the Fos/Jun proto-oncogen family and binds specifically to AP1-like DNA sequences termed ZREs (ZEBRA Response Element) as a homodimer through the folding of a coiled-coil interface within the dimerization regions adjacent to the DNA binding sites. Our aim is to solve the crystal structure of the DNA binding and dimerization domains of ZEBRA bound to DNA.

Methods: Guided by sequence analysis and partial proteolysis data, we have generated in E. coli a set of different forms of non-tagged N-terminally truncated ZEBRAs (8Kd) containing the DNA and dimerization domains. In some constructs, additional site directed mutagenesis has been performed in order to remove the cystein in the DNA binding site and/or to shorten the C-terminal part of the protein to improve the crystallization of the protein/DNA complex. The ZEBRA Proteins were purified by cation exchange chromatography and gel filtration and used for: (i) Bandshift assays with synthetic oligonucleotides (11 to 21 mer) derived from the ZREs of the EBV genes BSLF2 and BMLF1, (ii) Circular dichroism analysis, (iii) Crystallization trials by the hanging drop vapour diffusion method.

Results and Conclusion: (i) Bandshift assays showed that the all the ZEBRA fragments specifically bind to ZREs. (ii) Circular dichroism confirmed the alpha helical structure of the constructs but, in contrast with other bZip, the DNA binding did not enhance the percentage of alpha helix of the protein. (iii) Crystals of ZEBRA bound to DNA have been obtained and diffract at 3.4 Å and are currently analyzed. Crystallization trials with the dimerization domain alone, are also ongoing.

The EBV Immediate-Early Protein BZLF1 Binds to Mitotic Chromosomes.
Adamson A

Contact:
Dr. Amy Adamson
Biology
University of North Carolina at Greensboro (Greensboro, USA)
Email: aladamso@uncg.edu

Abstract:
Objective: We have found that the EBV immediate-early protein BZLF1 binds to mitotic chromosomes. To decipher the mechanism by which this occurs, we have determined the domains of BZLF1 that are necessary for chromosome binding to occur. Since BZLF1 binds to several cellular proteins, we have also examined whether known BZLF1-interacting proteins are brought to mitotic chromosomes in the presence of BZLF1. These studies have shed light upon this otherwise-unknown phenomenon of BZLF1 binding to chromosomes.

Methods: The method used for all of these studies was immunocytochemistry.

Results: We have found that BZLF1 binds to mitotic chromosomes in a variety of cell types, including HeLa cells, NIH3T3 cells, and EBV-positive D98/HE-R1 cells. BZLF1 protein was found to bind to mitotic chromosomes throughout mitosis, including the prophase, metaphase, and anaphase stages. This chromosome binding requires an intact DNA binding domain within BZLF1. In addition, we have found that BZLF1 is able to transport at least a subset of it's cellular binding partners such that they also bind to chromosomes during mitosis.

Conclusions: The binding of BZLF1 protein to chromosomes had not been previously demonstrated, yet presents an interesting area of study. During mitosis, most proteins such as transcription factors are excluded from chromatin in order to aid DNA compaction. The binding of BZLF1, as well as BZLF1 binding partners, to the chromatin likely has a negative effect upon DNA compaction, and may result in abnormal mitotic events. As for it's role in EBV replication, we speculate that BZLF1 binding to chromosomes during mitosis may help to equally partition the replicating EBV genomes or the BZLF1 protein to the two daughter cells.

TAP-dependant peptide transport is impaired in EBV lytic cycle

Ressing ME, Keating S, van Leeuwen D, Koppers-Lalic D, Pappworth IY, Wiertz EJHJ, Rowe M

Contact:
Prof. Dr. Martin Rowe
University of Wales College of Medicine
Section of Infection & Immunity (Cardiff, UK)
Email: RoweM@cf.ac.uk

Abstract:
Objective: Previously, we reported that expression of HLA class I and class II was reduced at the cell surface of B cells supporting EBV lytic cycle (J. Virol. 2002, 76: 8179). To investigate the functional significance of and the biochemical mechanism(s) for these observations, we developed a novel protocol to isolate cells in lytic cycle.

Methods: A reporter plasmid was made to permit expression of a marker gene product (extracellular and transmembrane domains of rat CD2 fused to GFP) to be regulated by the early lytic cycle promoter of BMRF1. This plasmid was stably transfected into EBV+ Akata Burkitt's lymphoma cells, which show a latency I type of infection and, therefore, did not express the CD2/EGFP protein. Upon treatment with anti-IgG, lytic cycle was induced in a subpopulation of cells with concomitant CD2/EGFP expression. Productively infected cells were isolated by immunomagnetic bead separation of CD2+ cells. TAP-dependent transport was measured based on acquisition of N-linked glycans by iodinated peptides upon transport into the ER of streptolysin O-permeabilised cells. Total protein levels were determined by immunoblotting. De novo protein synthesis was measured by metabolic labeling with 35S-methionine.

Results: Purity of productively infected Akata cells upon EBV reactivation varies between 10-40%, but exceeds 90% following immunomagnetic sorting for CD2+ cells. This permitted investigation of TAP-dependent peptide transport function in these cells. Peptide transport during lytic cycle was reduced to about 40% of that in latently infected cells. Steady-state levels of TAP proteins displayed little difference for lytic or latent infection. Unexpectedly, de novo synthesis of cellular proteins was shut down, whereas synthesis of EBV lytic cycle gene products was detectable.

Conclusions: We have isolated almost pure populations of B cells in lytic EBV cycle. In these cells, reduced TAP peptide transport provides further evidence for impaired antigen presenting capacity. EBV lytic cycle induction caused a shutdown in cellular protein synthesis. Host protein synthesis shutdown is well-studied for alpha-herpesviruses, but does not occur in beta-herpesviruses and, to our knowledge, has not previously been reported for EBV. These data suggest that EBV, like other herpesviruses, employs multiple strategies to evade T cell recognition.

Induction of Latent Membrane Protein 1 during Epstein-Barr Virus Reactivation

Chang Y, Lee HH, Chang SS, Tsai CH

Contact:
Dr. Yao Chang
Graduate Institute of Microbiology, College of Medicine
National Taiwan University (Taipei, Taiwan)
Email: yao1224@sina.com.tw

Abstract:
Latent membrane protein 1 (LMP1) of Epstein-Barr virus (EBV) acts as a viral oncoprotein that triggers multiple signaling pathways and dysregulates cellular gene expression. Considering its roles in the EBV-associated oncogenesis, how LMP1 expression is regulated in the EBV life cycle becomes an important issue. Although LMP1 can be expressed in some states of EBV latency, significant induction of full-length LMP1 is also observed frequently during virus reactivation into the lytic cycle. It is still unclear how LMP1 expression is induced during the lytic stage. In this study we identify two independent mechanisms for the induction. First, treatment with phorbol ester and n-butyrate can upregulate LMP1 expression even when the expression cascade of lytic genes is inhibited by Zta-targeted RNA interference. Therefore the LMP1 expression induced by the EBV reactivation-inducing agents is independent of the lytic cycle per se. On the other hand, we notice that LMP1 expression is not only associated with but also a downstream event of the spontaneous virus reactivation in EBV-infected 293 cell clones. It raises the possibility that LMP1 can be upregulated by at least one EBV lytic gene product. Indeed we find that LMP1 expression can be induced by ectopic expression of Rta, an immediate-early lytic protein. The Rta-mediated LMP1 induction is independent of another immediate-early protein Zta. Rta induces LMP1 at the RNA level and can activate both the proximal and distal promoters of the LMP1 gene. Further experiments demonstrate that Rta is an effective transcriptional activator of the LMP1 gene in both epithelial and B lymphoid cells. In addition, both the amino- and carboxyl-termini of the Rta protein are required for the induction of LMP1. This study expands the knowledge of LMP1 regulation in the EBV life cycle.

Cloning, expression and biophysical characterization of EBV uracil DNA glycosylase
Geoui T, Buisson M, Tarbouriech N, Burmeister WP

Contact:
Thibault Geoui
EMBL / Universite Joseph Fourier
Virologie Moleculaire et Structurale (Grenoble, France)
Email: geoui@embl-grenoble.fr

Abstract:
Objective: EBV is a double stranded DNA virus, which infects quiescent as well as actively dividing cells. In order to maintain the integrity of its genetic material in quiescent cells, EBV codes for a DNA repair enzyme, uracil DNA-glycosylase (UNG), which specifically hydrolyzes uracil misincorporated in DNA or resulting from chemical or enzymatic deamination of cytosin. The goal of our work is to produce, to characterize UNG and to solve its 3-dimensional structure.

Methods: The UNG gene has been amplified by PCR and cloned in a pPROEX HTb vector. The protein is produced in E.coli (BL21 DE3 PlysS strain) fused to a hexahistidine-tag via a TEV (tobacco etch virus) protease cleavage site. We used an efficient 2 step purification protocol based on the different binding of the expressed protein to nickel columns before and after TEV protease cleavage. Different constructs have been designed and produced based on alignments and limited proteolysis. The purified protein passes a series of quality control tests and an activity assay prior to the screening of crystallization conditions. These use either the protein alone or in complex with either DNA or the uracil DNA-glycosylase inhibitor protein (UGI).

Results: Since the start of the project the wild type protein, a trypsin digested form and 4 shorter (N terminal deletions) constructs have been tested with up to 4000 crystallization conditions without yielding reproducible crystals. The UNGΔ24 construct (UNG wild type truncated for the first 24 amino acids) is very promising since its very soluble, highly stable, active and yields very homogenous samples. It is now possible to obtain up to 15 mg of pure (crystallization suitable) protein per liter of bacteria culture.

Conclusions: Although the protein is very soluble (it can be concentrated up to 150mg/mL) and extremely homogenous (monodisperse) the protein alone has never yielded any crystals suggesting that UNG is intrinsically flexible.We are now trying to lock the protein in one conformation using different types of enzyme-inhibitor complexes.

Characterization of the promoter region of the Epstein-Barr virus BFRF1 gene
Granato M, Farina A, Gonnella R, Santarelli R, Faggioni A, Angeloni A

Contact:
Prof. Dr. med. Alberto Faggioni
Università di Roma
Experimental Medicine and Pathology (Roma, Italy)
Email: alberto.faggioni@uniroma1.it

Abstract:
Objective: The BFRF1 gene has been shown to be transcribed during the early phase of the EBV lytic cycle. The switch from latency to replication of the EBV is driven by the expression of ZEBRA and RTA. Lytic genes have been shown to be responsive to either ZEBRA, RTA or a co-operation of these two viral transactivators. Here we characterized the BFRF1 promoter to study the mechanisms that regulate the expression of this viral gene and to provide a new element in the cascade of events that lead to EBV replication.

Methods: A computer analysis of the BFRF1 promoter region has revealed two potential ZEBRA responsive elements (ZREs) and one potential RTA responsive element (RRE). The responsiveness to either ZEBRA, RTA, ZEBRA+RTA in the EBV negative transfected cell line DG75 was analyzed by CAT assay. The capacity of a ZEBRA mutant Z(S186A) that has a substitution in the DNA binding region was also tested. The activities of the same transactivators were also assayed in Raji cells by both immunofluorescence and Western blot analysis. Finally, the 5'-end of the BFRF1 mRNA was determined by the RACE method.

Results: In Raji cells, TPA treatment induces BFRF1 expression. However, in DG75 cells no BFRF1 CAT construct was activated by TPA. ZEBRA transfection is sufficient to stimulate BFRF1 expression in Raji cells unlike RTA. However, in DG75 cells, both ZEBRA and RTA are able to induce the CAT activity from the constructs that retain the ZRE (-373 from the ATG) as well as the RRE (-518). The Z(S186A) mutant lost the capacity to transactivate BFRF1 but it can be rescued by co-transfection of RTA. Moreover, the analysis of the mRNA of the BFRF1 region has shown that the 5' end is located at -306 bps from the ATG.

Conclusions: In EBV positive cell lines BFRF1 expression is obtained after induction of the lytic cycle by TPA and butyrate. However, BFRF1 activation seems to be mediated by the viral transactivator ZEBRA. The other major EBV transactivator RTA co-operates with ZEBRA, but is not sufficient to induce BFRF1. Still, RTA can restore the capacity of the ZEBRA mutant Z(S186A) to induce BFRF1 expression. Taken altogether these results seem to suggest that the canonical ZIIIA element located at -373 is required for ZEBRA induced transactivation of the BFRF1 gene and that the RRDR-1 located at position -518 may be involved, although to a lesser extent.

Expression and localization of Epstein-Barr virus encoded protein kinase, a novel determinant of infectious virus production

Gershburg E, Hong K, Delecluse HJ, Pagano JS

Contact:
Dr. Edward Gershburg
University of North Carolina at Chapel Hill
Lineberger Comprehensive Cancer Center (Chapel Hill, USA)
Email: ste30@med.unc.edu

Abstract:
Objective: The only protein kinase (PK) encoded by the Epstein-Barr Virus (EBV), the BGLF4 gene product, belongs to the HSV UL13 group of conserved herpesvirus protein kinases, whose biological role and importance for viral infection are yet to be established. Here we have studied inhibition of the expression of EBV PK and its effects on production of infectious virus. We also analyzed the kinetics of expression and subcellular localization of EBV PK during reactivation of the viral lytic cycle.

Methods: To inhibit the expression of EBV PK we used RNAi in 293 cells infected with recombinant EBV tagged with the gene for green fluorescent protein. Lytic cycle was reactivated, and infection of Raji cells with the resultant virus served as a measure of infectious virus production. The expression pattern of EBV PK and its localization were analyzed in Akata cells with a polyclonal antiserum raised against a peptide corresponding to its N-terminus.

Results: Inhibition of EBV PK expression resulted in a ~90% reduction of ability of the virus to infect Raji cells. By biochemical fractionation, the protein could be detected mainly in the nuclear fraction at 4 hours after viral reactivation in Akata cells. Nuclear localization could also be visualized by indirect immunofluorescence in HeLa cells transiently expressing EBV BGLF4 in the absence of other viral products. Transient expression of 3'-terminal deletion mutants of EBV BGLF4 resulted in cytoplasmic localization, confirming the presence of a noncanonical nuclear localization site in the C-terminal region of the protein. In contrast to wild type EBV PK, all these mutants were unable to hyperphosphorylate EA-D.

Conclusions: The results demonstrate that: I) EBV PK expression is critical for the efficient production of infectious viral particles; II) EBV PK is an early-late protein, requiring viral DNA replication for maximum expression; and III) the nuclear localization as well as the kinase activity of BGFL4 are dependent on an intact C-terminal region.

Transcription and Translation Control of EBV BGLF4 Protein Kinase

Chen MR, Chen HH, Chen YL, Yan PW, Wang JT, Han CH

Contact:
Dr. Mei-Ru Chen
College of Medicine, National Taiwan University
Department and Graduate Institute of Microbiology (Taipei, Taiwan)
Email: mrc@ha.mc.ntu.edu.tw

Abstract:
BGLF4 of EBV encodes a serine/threonine protein kinase, which was demonstrated to phosphorylate viral DNA polymerase accessory factor, EA-D and a nucleoside analog ganciclovir. Since both BGLF4 protein kinase and thymidine kinase are considered as candidates for drug targeting of EBV replicating cells, the transcription and translation control of BGLF4 was studied in this report. We found the expression of BGLF4 is independent of viral DNA synthesis. The promoter of BGLF4 is mainly up-regulated by the immediate early transactivator Rta rather than Zta as demonstrated by luciferase reporter assay. The 5'-end of BGLF4 containing transcripts mapped by 5'-RACE protocol identified two populations of cDNA clones containing sequences from -201 or -255 upstream to the first in frame ATG of BGLF4. Since both transcriptional initiation sites are relatively far away from the first in frame ATG, the translation initiation site was confirmed by mutagenesis. The ability of the 5'-end nontranslation region functioning as ribosome entry site (IRES) was examined in a bicistronic construct. Expression of BGLF4 transcripts was also detected by RT-PCR in 5/8 nasopharyngeal carcinoma tissues. Results of this study thus provide the basic knowledge of transcription and translation control of BGLF4 and the possibility for future design of kinase dependent treatment of EBV associated malignant diseases.

High resolution 3D structure of the EBV dUTPase

Tarbouriech N, Buisson M, Geoui T, Morand P, Seigneurin JM, Cusack S, Burmeister WP

Contact:
Dr. Nicolas Tarbouriech
EMBL-Grenoble (Grenoble, France)
Email: tarbour@embl-grenoble.fr

Abstract:
Objective: The 3-dimensional structure of the EBV dUTPase has been solved by X-ray crystallography as part of the European SPINE project for structural biology. This high resolution structure leads to new insights on the enzyme activity at a molecular level and open new opportunities for drug design.

Methods: The protein was expressed in recombinant bacteria and purified by affinity chromatography. It was then crystallized and data were collected at the ESRF on native crystals as well as crystals grown in presence of dUTP. The structure was solved by the single anomalous dispersion method using an Europium soaked crystal. The first model obtained was then used together with the higher resolution complexed data to get the 3D structure of this enzyme in complex with the product dUMP.

Results: The 3D structure of the EBV dUTPase has been solved at 1.5Å resolution in complex with dUMP. The comparison of this new structure with the other dUTPases already known from other organisms is being investigated.

Conclusions: This structure is the first structure of a monomeric dUTPase, a specificity of the Herpes virus family. The comparison with the structures of dUTPases from other viruses as well as other organisms showed some features that may be used in the development of new specific inhibitors against this enzyme.

Molecular Biology of Hodgkin's Lymphoma

Kuppers R

Contact:
Prof. Dr. Ralf Küppers
Institute for Cell Biology (Tumor Research)
University of Essen, Medical School (Essen, Germany)
Email: ralf.kuppers@uni-essen.de

Abstract:
Hodgkin and Reed/Sternberg (HRS) cells represent the malignant cells in classical Hodgkins lymphoma (HL). Their immunophenotype cannot be attributed to any normal cell of the hematopoietic lineage, but molecular studies established their derivation from germinal center (GC) B cells. Gene expression profiles generated by SAGE and DNA chip microarrays from HL cell lines were compared with those of normal B cell subsets, focussing here on the expression of B lineage markers. This analysis revealed decreased mRNA levels for nearly all established B lineage-specific genes. In particular, multiple components of signaling pathways active in B cells, including B cell receptor (BCR) signaling, were severely affected. We propose that the lost B lineage identity in HRS cells may explain their survival without BCR expression and reflect a fundamental defect in maintaining the B cell differentiation state in HRS cells. Notably, the downregulation of several molecules that are important for signaling by the EBV-encoded LMP2a gene questions whether this gene is indeed capable to replace BCR signaling in the established HRS cell clone of EBV-positive HL cases, as was previously discussed.

In rare cases, a HL and a B cell Non-Hodgkin lymphoma (B-NHL) occur in the same patient. We analysed several of such composite lymphomas for the clonal relationship of the two tumors. In most cases, the two lymphomas were found to derive from a common precursor. Since these lymphomas represent interesting models to study the multistep transformation process in lymphomagenesis, the HRS and B-NHL cells with a common origin were analysed for shared as well as distinct transforming events. It was found that in two combinations of HL and follicular lymphoma, the same bcl-2/IgH translocation was present in the two types of tumor cells. Hence, these translocations occurred early during lymphomagenesis in a shared precursor of the two lymphomas. In a combination of HL and diffuse large cell lymphoma, somatic p53 gene mutations were present only in the B-NHL, but not in the HRS cells, showing that in this instance, the p53 gene mutations happened late in the development of the diffuse large cell lymphoma. In one composite lymphoma, EBV was found only in a subclone of the HRS cells, defined by a distinct V gene mutation pattern. This strongly suggests that EBV infection was a late event in HRS cell development, likely happening in a GC B cell lymphoma precursor.

The viral etiology of Hodgkin's lymphoma: Epidemiologic evidence for a role of EBV in both EBV(+) and EBV(-) HD

Mueller NE,Chang E, Levin L, Ambinder R, Lennette E, Zhang T

Contact:
Dr. Nancy Mueller
Harvard School of Pubic Health
Epidemiology (Boston, MA, USA)
Email: nmueller@hsph.harvard.edu

Abstract:
Objective: To determine whether EBV infection plays a role in both EBV(+) and EBV(-)HD

Methods: We conducted a multi-project research program including a population-based case-control study involving 565 cases and 679 population controls, and a cohort study in the Department of Defense Serum Repository with pre-diagnosis samples obtained from 126 cases and matched controls. In the case-control study we obtained risk factor information by interview and post-treatment blood samples from cases. In the cohort study we obtained the earliest specimen preceding malignancy. The pathology was verified by study pathologists and tested for EBV(+)or EBV(-) status.

Results: In the case-control study we found that the major risk factors for HD included lack of day care/nursery school - the adjusted odds ratio (OR) for HD associated with greater than or equal to l year was 0.64 (95% confidence interval, 0.45-0.92) for cases aged 15-54 years; this finding is consistent with previous evidence that protection from early exposure to infections such as the EBV is a risk factor. We found that lack of regular aspirin use was a risk factor for HD at all ages; the OR for greater than or equal to2 tablets per week was 0.60 (0.42-0.85). This new finding is consistent with the selective down-regulation of NFkB by aspirin. These two novel associations did not differ by EBV-status of tumors; neither did they differ by history of IM. However, both the post-diagnosis and the pre-diagnosis antibody profiles for the EBV(+) cases were significantly abnormal, while those for the EBV(-) cases were not. In the pre-diagnosis cohort samples, there was significantly elevated free EBV-DNA viral load in the EBV(+) HD cases. EBV(+) HD cases were more likely to be male, to have mixed cellularity histology, have less education, and be more likely to have smoked cigarettes than the EBV(-) cases. These findings suggest that EBV(+) HD cases are more likely to have impaired immunity than EBV(-) cases.

Conclusions: We conclude that there is no evidence that EBV infection plays a role in EBV(-) HD. Our observation that early infectious exposure via day care is also protective for EBV(-) HD underlines the likelihood that it is also related to a 'late' infection with an oncogenic virus. The candidate virus appears to share transmission patterns with EBV, and that its oncogenic potential is evident in more immunocompetent populations.

Induction of Autotaxin by the Epstein-Barr Virus is specific to Hodgkin's Lymphoma Cells and Promotes their Growth and Survival through the Generation of Lysophosphatidic Acid

Baumforth KR, Flavell JR, Reynolds GM, Davies GL, Pettit T, Wei W, Morgan SL, Nowakova M, Stankovic T, Pratt G, Aoki J, Wakelam MJO, Young LS, Murray PG

Contact:
Dr. Karl Baumforth
University of Birmingham
Cancer Research U.K. Institute for Cancer Studies (Birmingham, UK)
Email: karlb@cancer.bham.ac.uk

Abstract:
Objective: A proportion of Hodgkin's lymphomas (HL) carry EBV in their tumor cells. Although it is generally assumed that EBV contributes to the malignant phenotype of HL cells, direct evidence in support of this is lacking. We have previously shown that EBV infection results in the enhanced growth and survival of HL cells. This study sought to determine the mechanisms that underlie these effects.

Methods: Phenotypic consequences of EBV infection of HL cells were confirmed by proliferation & viability assays. Transcriptional profiling of EBV-positive & EBV-negative KM-H2 cells was performed using Affymetrix Genechips. mRNA expression was confirmed using gene specific RT-PCR & protein expression using western blotting & immunohistochemistry. Small interfering RNAs were used to knock down mRNA expression in HL cells.

Results: EBV-positive HL cells expressing a limited repertoire of virus genes displayed enhanced growth & survival compared with their EBV-negative counterparts. Transcriptional changes accompanying EBV infection included the upregulation of autotaxin (ATX), a secreted tumor motility-promoting factor with lysophospholipase D activity that promotes the proliferation & survival of a range of cancer cell types. ATX protein was also elevated in supernatants of EBV-infected cells & this was accompanied by increased production of lysophosphatidic acid (LPA) from lysophosphatidylcholine. ATX expression was low/undetectable in EBV-transformed lymphoblastoid cell lines and also in a range of cell lines derived from Burkitt's lymphomas or nasopharyngeal carcinomas and was unaffected by the presence of EBV in these cells. While upregulation of ATX was evident in EBV-positive primary HRS cells, most primary non-Hodgkin's lymphomas lacked detectable ATX protein. Importantly, specific downregulation of ATX expression through RNA interference resulted in significantly reduced growth & viability of EBV-infected HL cells.

Conclusions: The induction of ATX & the subsequent generation of LPA is an important mechanism through which EBV contributes to the growth & survival of HL cells & is the first demonstration that the tumour promoting lipid, LPA, is the target of a transforming virus. Furthermore, this effect appears to be restricted to HL cells. Targeting this pathway could provide novel opportunities for therapeutic intervention in Hodgkin's lymphoma.

LMP1 and LMP2 specific T-cell stimulation in EBV+ Hodgkin's Disease

Lauterslager TGM, Klarenbeek JB, Bloemena E, Middeldorp JM

Contact:
Dr. Tosca Lauterslager
Vrije Universiteit Medical Center
Department of Pathology (Amsterdam, The Netherlands)
Email: tgm.lauterslager@vumc.nl

Abstract:
EBNA1, LMP1 and LMP2 are putative targets for anti-tumour immune responses but tumour cells expressing these proteins can avoid elimination. EBNA1 can induce CD4 T-cell responses, but escapes CTL recognition. LMP1 and LMP2 are potential CTL targets but, although CD4-and CD8-epitopes are described, the T-cell precursor frequency is very low. LMP1 contains in its first transmembrane region an immunosuppressive domain and is secreted via exosomes. Dendritic cells (DC) may cross-present these proteins and evoke or boost LMP1 and LMP2 CTL responses. The aim of our study is to enhance anti-LMP1 and anti-LMP2 T-cell responses in patients with EBV+ Hodgkin's disease using DC loaded with recombinant LMP1 and -LMP2 and EBNA1 for bystander T-cell help.
Recombinant proteins were expressed using the baculovirus-expression system and purified via affinity chomatography. Immature monocyte-derived DC were subsequently loaded with purified protein and matured using TNFα, IL-1β, IL-6, PGE2, GM-CSF and IL-4. Uptake by and influence on the DC was assessed. IFNγ -ELISPOT and pentamer staining were used to monitor the induction of specific T-cells.
We have purified baculovirus-expressed full-length LMP1, LMP1 lacking the immunosuppressive region (DTM1-LMP1), full-length LMP2 and EBNA1 deleted of the Gly-Ala-repeat region. These proteins are indeed taken up by immature DC and thus can be used for antigen-presentation. The proteins do not influence maturation of the DC when proper maturation stimuli were added. Without additional maturation cytokines, however, full-length LMP1 but not DTM1-LMP1 kept the DC to a more immature phenotype suggesting that LMP1 may influence the migration of protein-loaded DC to the lymph nodes. Since the frequency of LMP1 and LMP2 specific CD8 T-cells is very low, monitoring assays must be sensitive enough to detect these low frequent T-cells. Using blood of healthy donors, we have established reliable and sensitive assays based on pentamer staining and IFNγ production which enables us to screen large groups of people.
At this moment, we are able to routinely purify several latent EBV proteins and present them to DC. Functional studies are in progress. Low frequencies of LMP1 and LMP2 reactive T-cells can be detected which may be boosted to higher levels by DC cross-presentation but full length LMP1 may disturb this process.

The generation and characterisation of EBV-negative and Latency I L591 cells identifies important phenotypic and cellular gene transcriptional consequences of EBV infection of Hodgkin/Reed-Sternberg cells

Flavell JR, Baumforth KR, Reynolds GM, Wei W, Klein E, Young LS, Murray PG

Contact:
Joanne Flavell
University of Birmingham
Cancer Research U.K. Institute for Cancer Studies (Birmingham, UK)
Email: joannef@cancer.bham.ac.uk

Abstract:
Objective: EBV is present in the malignant Hodgkin/Reed-Sternberg cells of approximately one half of all cases of Hodgkin's lymphoma (HL). However, the contribution of EBV to the pathogenesis of HL a has yet to be defined. In order to investigate the phenotypic and transcriptional consequences of EBV infection of HL cells, we generated variants of the Lat III L591 EBV-positive HL cell line, including EBV-negative L591 cells and L591 cells displaying a restricted (Lat I) pattern of virus latent gene expression.

Methods: Limiting dilution cloning was used to establish EBV-negative clones from parental EBV-positive L591 cells. Progressive loss of the EBV genome was monitored by immunohistochemistry for EBNA1 and Q-PCR for detection of the viral DNA polymerase gene. Serial passage of parental L591 cells was used to generate L591 cells with a virus gene expression program limited to the EBERs, EBNA1 and BARTs (Lat I).

Results: L591 cells lacking the EBV genome were dramatically less viable and proliferated less well than their EBV-positive counterparts. L591 cells exhibiting a restricted latency pattern of gene expression also showed reduced viability and proliferation but this was less marked than that observed for EBV-negative L591 cells. Gene expression analysis identified cellular genes whose expression was significantly altered following either the loss of the EBV genome from L591 cells or the acquisition of a limited virus gene expression profile. We demonstrate here that several of the genes upregulated either in Lat I or Lat III L591 cells compared with EBV-negative L591 cells are not only highly expressed in primary EBV-positive HRS cells, but also contribute to the growth and survival promoting effects of EBV we have observed in L591 cells.

Conclusions: Our results demonstrate important growth and survival promoting effects for EBV latent genes in the context of HL and identify several novel targets of EBV infection of HL cells. Work is underway to identify which of the latent genes is responsible for these phenotypic and transcriptional effects. The Latency I and EBV-negative L591 systems provide an ideal model to determine the contribution of the different latent genes to the pathogenesis of EBV-positive HL.

EBV latent membrane protein 1 induced alterations in gene transcription in Hodgkin lymphoma cell lines.

Vockerodt M, Baumforth KR, Davies GL, Kube D, Wei W, Young LS, Murray PG

Contact:
Dr. Martina Vockerodt
Georg-August-Universitaet Fachbereich Humanmedizin
Zentrum Kinderheilkunde und Jugendmedizin
Abteilung Paediatrie I, (Goettingen, Germany)
Email: mvocker@gwdg.de

Abstract:
Classical Hodgkin Lymphoma (cHL) is a distinct malignancy of the immune system. Despite the progress made in the understanding of the biology of cHL, the transforming events remain to be elucidated. The EBV latent membrane protein 1 (LMP1) is suggested to play a central role in the pathogenesis of EBV-positive cHL. To characterise cellular genes involved in LMP1-mediated transformation oligonucleotide-microarray experiments were performed. Expression profiles of L428 HL cells transfected with vector control or LMP1 were compared and 260 genes were found to be upregulated.
The LMP1-dependent gene profile comprises chemokines, cytokines, receptors, apoptotic regulators, intracellular signaling molecules, and transcription factors, the majority of which maintain a marker-like expression in Hodgkin Reed Sternberg (HRS) cells. Within this set of genes, a subset was modulated in agreement with previous reports demonstrating their regulation by LMP1 or NF-kappaB in other cell types and are expressed in HRS cells. Remarkably, we found 124 novel LMP1 target genes which expression is presently confirmed in additional cHL cell lines by real-time RT-PCR, Western Blot or ELISA. The gene profile underscores a central role of LMP1-mediated activation of NF-kappaB in the pathogenesis of cHL and potentially of other tumors with constitutive NF-kappaB activation. The newly identified LMP1-regulated HL-specific genes may represent potentially novel diagnostic markers, can be considered for new drug targeting.

Identification of the cellular binding partners of Epstein-Barr nuclear antigen-1 in Hodgkin's lymphoma cells

Davies GL, Shire K, Frappier L, Vockerodt M, Kube D, Martin A, Dawson C, Young LS, Murray PG

Contact:
Gillian Davies
The University of Birmingham
Cancer Studies (Birmingham, UK)
Email: gilliand@cancer.bham.ac.uk

Abstract:
Objective: The EBNA1 protein is essential for viral episome replication and maintenance and is therefore consistently expressed in all EBV-associated malignancies. However, it is not clear whether EBNA1 has any direct role in the development or progression of EBV-associated tumors. In this context the effects of EBNA1 on cellular processes leading to transformation are likely to be mediated through its interactions with specific host cellular proteins. However, few of these cellular protein interactions have been identified and none have been studied in the context of Hodgkin's lymphoma.

Methods: Cellular binding partners of EBNA1 were identified by mass spectrometry following binding to EBNA1 affinity columns (Holowaty M et al., J Biol Chem 2003; 278: 29987-94). Co-immunoprecipitation was used to confirm these interactions in EBNA1-expressing HL cell lines, including the naturally occurring L591 line and in EBNA1-positive HL cells generated by transfection.

Results: A number of cellular binding partners were shown to physically interact with EBNA1 in HL-derived cell lines, some of which have previously been described in association with EBNA1 in other cellular environments. Among these was the ubiquitin specific protease, USP7, previously identified to interact with EBNA1 in HeLa cells.

Conclusions: Although binding partners of EBNA1 have been previously identified in a variety of cellular backgrounds it has not been clear whether such interactions also occur in EBV-associated tumours such as Hodgkin's lymphoma. This study confirms that the previously observed interactions do indeed occur in this setting and are likely to be important either for the maintenance of the viral genome or more directly for transformation. USP7 has a role in stabilising p53 and it has been suggested that sequestration of USP7 by EBNA1 might represent an alternative route to abrogate p53 activity in EBV-infected tumor cells. This is particularly interesting in light of the fact that EBV-associated HL rarely harbors p53 mutations. The observations presented here indicate that the influence of EBNA1 expression in Hodgkin's lymphoma is likely to be far greater than simply the maintenance and replication of the EBV genome.

Relationship of chromosomal imbalances to EBV status in paediatric Hodgkin's Lymphoma

Morgan SL, Heys D, Arrand JR, Bose S, Dyer S, Baumforth KR, Grundy RG, Parkes SE, Jarrett R, Chui D, Mann JR, Young LS, Murray PG

Contact:
Dr. med. Susan Morgan
Birmingham University
Institute of Cancer Studies/Department of Pathology (Birmingham, UK)
Email: lara@doctors.org.uk

Abstract:
Objective: The underlying genetic changes responsible for the development of Hodgkin's lymphoma (HL) are poorly understood, primarily because of the technical problems involved in isolating the Hodgkin/Reed-Sternberg(HRS) cells from the surrounding tissue. To date there is no information on the cytogenetics of paediatric HL. Clinical and epidemiological studies on paediatric classical HL suggest that the EBV-positive and EBV-negative forms of this disease might be aetiologically distinct. Here we have investigated whether this is reflected in their differing patterns of chromosome imbalances.

Methods: Malignant HRS cells were isolated from CD30 stained frozen sections using a laser microdissection microscope. DNA was extracted and amplified using a degenerate oligonucleotide primed PCR reaction. Conventional Comparative Genomic Hybridisation was then performed.

Results: 4/14 tumours were EBV-negative. The EBV-negative cases had an average of 18.5 imbalances (range, 4-34), including 39 gains (mean, 9.75 per tumour) and 36 deletions (mean, 9.00 per tumour). Chromosomal losses most frequently (i. e. 50% or more of EBV-negative tumours) affected arms 1p, 2p, 5p, 6p, 6q (50%) and 13q (75%). Chromosomal gains most frequently affected 2p, 2q (50%), 3q (75%), 4p, 5p, 5q, 9p, 14q, 15q, 17q (50%), 19p (75%), 20p and 21q (25%). There were 10 EBV-positive cases and imbalances were detected in all but two of these. There was an average of 4.7 imbalances per EBV-positive tumour (range, 0-17). These included 24 gains (mean, 2.4 per tumour) and 23 deletions (mean, 2.3 per tumour).

Conclusions: The results of this preliminary analysis support the hypothesis that the EBV positive and EBV negative forms of paediatric Hodgkin's Lymphoma are aetiologically distinct. Our data suggest that virus infection might substitute for genetic events that are necessary for the development of EBV negative tumours. The potential role of individual genes within commonly affected chromosomal regions are discussed.

The prevalence and level of Epstein-Barr virus (EBV) antibodies by time to diagnosis in Hodgkin's Lymphoma (HL) with EBV genome status

Levin L, Chang E, Lennette E, Rubertone M, Ambinder R, Mueller N

Contact:
Dr. Lynn Levin
Walter Reed Army Institute of Research
Epidemiology (Silver Spring, USA)
Email: lynn.levin@na.amedd.army.mil

Abstract:
Objective: To determine if the EBV serologic pattern differs with time to diagnosis among EBV-positive [EBV (+)] HL cases, EBV-negative [EBV (-)] HL cases, and controls.

Methods: Using a nested case-control design, we examined 126 incident HL cases and 366 matched controls from active-duty military personnel with prediagnosis archived serum in the US Department of Defense Serum Repository. Tissue blocks from cases were tested for EBV genome status. Linear regression methods were used to test the linear change in antibody geometric mean titers (GMTs) (reciprocal of the dilution) over time for IgG to VCA, EBNA-1, and EBNA-2 in the two case groups and compared to controls. Logistic regression was used to test for changes in the prevalence of an elevated titer for EA and a low ratio of EBNA-1/EBNA-2 defined as EBNA-1/EBNA-2 lower than or equal to 1.0.

Results: At the index (earliest) blood draw, 32 (88.9%) of EBV (+) HL cases, 79 (87.8%) of EBV (-) HL cases, and 333 (92%) of controls were EBV seropositive. Of note, all four of the EBV (+) cases that were seronegative at the first serum collection subsequently seroconverted by later blood draws. The mean interval between the index serum specimen and date of diagnosis = 36.4 months; range, 6 months to 8 years. Controlling for age, gender, and race, there was a linear increase in GMTs for IgG to VCA comparing EBV (+) HL cases with EBV (-) HL cases ($p=0.01$), with time to diagnosis as a continuous variable. The GMT ratio for EBNA-1/EBNA-2 was diminished overall for EBV (+) HL cases versus the EBV (-) HL cases. The prevalence of a low ratio of EBNA-1/EBNA-2 also significantly increased with time comparing EBV (+) HL cases to controls ($p=0.009$). There was no other evidence of a significant temporal trend in time to diagnosis comparing the case groups to controls or to each other.

Conclusions: Although the comparisons are based on rather small numbers, these data show that the low ratio of EBNA-1/EBNA-2 was more prevalent in EBV (+) HL cases as time to diagnosis approached.

Familial aggregation of EBV antibody titres among relatives of patients with Hodgkin's Lymphoma

Besson C, Le-Pendeven C, Amiel C, Brice P, Ferme C, Carde P, Hermine O, Nicolas JC, Abel L

Contact:
Dr. Caroline Besson
Inserm, Faculté Necker
Laboratoire de Génétique Humaine des Maladies Infectieuses, U550 (Paris, France)
Email: besson@necker.fr

Abstract:
Objective: Epstein Barr virus (EBV) is found in 30% of cases of Hodgkin's lymphoma (HL) by in situ hybridization. Patients with HL have higher antibody titres against EBV than controls not only at diagnosis but also years before and after diagnosis. These elevated titres could reflect an immune « dysfunction » against EBV that may predispose to HL. Our main objective is to search for genetic factors controlling immune response against EBV.

Methods: HL cases, aged 15 to 35 years, are recruited in 3 haematology units in the Paris area since December 2002. They are proposed, as well as their parents and siblings, to undergo a medical interview and to be taken a blood sample. Anti-EBNA, VCA, EA IgG and anti-VCA IgM are measured by ELISA in plasma. EBV load is quantified by real-time quantitative PCR in peripheral blood mononuclear cells. Familial correlations are studied by analysis of variance and multiple regression using appropriate methods which allow estimating correlation coefficients, denoted as r, between different kinds of relatives: father-child, mother-child, sib-sib, and between spouses.

Results: So far, 240 subjects, including 80 cases, have been analyzed. The results are presented for anti-EBNA antibodies in the 227 seropositive subjects (95% of the studied population). Anti-EBNA titres decrease with age ($p=0.03$), and do not differ between cases and their family members. They are not correlated with EBV load. The 227 studied subjects belong to 64 families. Analysis of variance shows that anti-EBNA IgG displays a strong familial component ($p<10\text{-}4$). Interestingly, significant positive correlations for EBNA titres are found between fathers and children ($r=0.32$, $p=3.10\text{-}4$) and between children ($r=0.22$, $p=0.04$), with a trend in favour of a positive correlation between mothers and children ($r=0.15$, $p=0.09$). In contrast, there is no evidence for a correlation between spouses ($p=0.65$).

Conclusions: The existence of correlations for anti-EBNA titres between biologically related subjects, and not between spouses, supports the hypothesis of their genetic control. The collection of a larger sample of families is ongoing in order to map the gene(s) controlling EBV response by genome-wide linkage analysis.

Molecular Signatures and Classification of Nasopharyngeal Carcinoma's

Pegtel DM, Ramaswamy A, Tsai CH, Golub TR, Thorley-Lawson DA

Contact:
Dr. Dirk Michiel Pegtel
Tufts University School of Medicine
Graduate Program in Immunology, Sackler School of Biomedical Sciences (Boston, USA)
Email: Dirk.Pegtel@tufts.edu

Abstract:
Non-keratininzing NPC is a highly metastatic tumor that is always associated with Epstein-Barr virus. Though very common in south East Asia, NPC has not been yet been characterized at the molecular genetic level in a comprehensive way. We conducted 'consensus clustering' on Affymetrix data from a small set of NPC biopsies to look for genetically distinct subsets. The clustering identified two strongly distinct groups of non-keratinizing NPC's with a large set of genes differentially expressed and a high degree of statistical significance. This represents a previously unrecognized subdivision of NPC since the two clusters did not correlate with the traditional histo-pathological WHO criteria that distinguish two forms of non-keratinzing NPC differentiated (type II) and undifferentiated (type III). This suggests that the molecular signature we have detected is more dominant than a possible differentiation related signature. The top hundred markers included a group of cell-cycle, DNA damage repair and synthesis related genes suggesting different rates of cell proliferation and perhaps tumor progression between the two clusters. The clusters did not correlate with expression levels of EBV genes including LMP1 (the main viral oncogene), LMP2a and EBER.
A priori marker analysis to look for genetic differences between type II and type III NPC identified a gene 'signature' that included a large set of interferon Inducible Genes (ISG's) and monocyte/macrophage markers and related genes that are highly expressed in Type III NPC's. Gene Set Enrichment Analysis (GSEA) independently verified enrichment of ISG's with a strikingly high correlation with the subset of ISG's known to be highly expressed in PBMC's derived from SLE patients. The ISG's did not correlate with transcript expression of type I and II interferons, nor with EBV replication, a potential source of interferon activation. This suggests that the source of interferon lies outside of the tumor. This may relate to the well know observation that little EBV replication occurs in the tumors, yet relapse is highly correlated with anti-virion antibodies raising the possibility that EBV is replicating adjacent to the tumor and inducing interferon which is then impacting the characteristics of a histological subset of tumors. We have found a novel molecular basis for the classification of NPC tumors unrelated to current histological distinctions. The discovery of new molecular classes of NPC might be a valuable tool in tailoring treatment of this heterogeneous tumor.

Alteration of the Global Gene Expression in Nasopharyngeal Carcinoma by EBV Infection
Lin CT

Contact:
Prof. Dr. Chin-Tarng Lin
Department Of Pathology
National Taiwan University Hospital (Taipei, Taiwan)
Email: ctl@ha.mc.ntu.edu.tw

Abstract:
Objective: The purpose of this investigation was to observe the change of global gene expression in EBV-infected NPC cell lines.

Methods: cDNA microarray analysis of five NPC cell lines and three normal epithelial cultures, and of five EBV-infected and EBV-free NPC cell lines.

Results: All genes could be classified into four distinct categories, such as differentially, non-regulated, randomly and ambiguously expressed groups. From 7500 genes, only 4022 genes were identifiable significantly in the NPC-normal array gene set. Only those genes which showed altered expression in the majority of cell lines, at least 4 out of 5 lines were classified into differential groups. In comparison of the gene expression from NPC-normal set and EBV-free and EBV-infected array, only 88 genes were altered in both NPC-normal set and EBV-free and EBV-infected array sets, but 48 genes were altered in NPC-normal arrays and were not EBV target genes. On the contrary, 59 genes were nonregulated in NPC-normal set and became EBV target genes, while 69 genes were nonregulated in NPC-normal set and also not EBV target genes. The fold changes of gene expression were rather mild. When these data were verified by Q-RT-PCR, about 70-75 percent similarity was demonstrated in cDNA microarray data and Q-RT-PCR results. From those selected EBV target genes, we compared the alteration of nucleolin, EGFR, casein kinase-2 and mdm2 gene expressions in response to EBV infected and LMP-1 transfected NPC cells. Results showed that EBV could mildly to moderately up-regulate those 4 gene expressions, while LMP-1 could enhance slightly higher expression than that of EBV did. Cotransfection of pmdm2 promoter-luciferase with pSV-40-p53 and pCMV-LMP-1 in the p53 mutant cell line revealed marked elevation of pmdm2 promoter activity, while cotransfection of pmdm2-luc and pCMV-LMP-1 could not up-regulate mdm2 promoter activity.

Conclusions: It is concluded that EBV preferentially targets genes differentially expressed in NPC, and the alteration of EBV target gene expression is rather weaker than LMP-1 transfectants. Although, LMP-1 and/or EBV can not turn on the unexpressed host genes, but can enhance indirectly the expression of the expressed host genes, suggesting that EBV plays a role in enhancing NPC pathogenesis.

Characterisation of the transcriptional and phenotypic consequences of EBV infection of nasopharyngeal carcinoma cells identifies novel cellular targets of EBV

Waites ER, Stewart SE, Arrand JR, Wei W, Dawson C, Laverick L, Takada K, Young LS, Murray PG

Contact:
Elyse Waites
CRUK Institute for Cancer Studies
University of Birmingham (Birmingham, UK)
Email: elysew@cancer.bham.ac.uk

Abstract:
Introduction: Although EBV is consistently associated with undifferentiated nasopharyngeal carcinoma (NPC), the precise contribution of EBV to the malignant phenotype of NPC remains to be established. We took advantage of the finding that most cell lines derived from primary NPC eventually lose the viral episome and can be stably infected with recombinant EBVs in vitro.

Methods: We compared the phenotype and gene expression profiles of parental EBV-negative HONE-1 NPC cells with those of EBV-positive HONE-1 cells generated by infection with either wild type Akata-derived recombinant EBV or the same virus lacking the LMP2A gene. As described elsewhere (Stewart S., et al., abstract 08.08), HONE-1 cells carrying wild type Akata-derived virus do not express LMP1. However, in the absence of the LMP2A gene, LMP1 expression is induced in these cells. The phenotypic consequences of EBV infection were determined by measurement of proliferation and survival and by transwell and wound healing assays. The transcriptional changes accompanying infection were investigated by Affymetrix GeneChip analysis.

Results: HONE-1 cells infected with wild-type virus or virus lacking the LMP2A gene displayed marked phenotypic changes compared with EBV-negative HONE-1 cells, including increased motility and invasion. Analysis of cellular transcriptional changes following EBV infection identified a number of alterations in gene expression, which were consistent with the phenotypic effects we had observed. In particular, a number of genes associated with progression of the cell cycle and with cell motility were upregulated in EBV-infected cells compared to parent HONE-1 cells. Furthermore, EBV infection was associated with the downregulation of genes associated with differentiation, adhesion and the ubiquitin -proteasome system. Importantly, we demonstrate that several of the genes upregulated in EBV-positive HONE-1 cells are also highly expressed in primary NPC tumours.

Conclusions: EBV induces important phenotypic and transcriptional effects in nasopharyngeal carcinoma cells. The functional consequences of expression of several of the EBV-regulated genes we have identified are currently under investigation and will be presented at the meeting.

The role of ID1 expression in immortalization and transformation of nasopharyngeal epithelial cells

Tsao SW, Li HM, Man C, Yip YL, Young LS, Huang DP

Contact:
Prof. George Sai-Wah Tsao
University of Hong Kong
Anatomy (Hong Kong, Hong Kong)
Email: gswtsao@hkucc.hku.hk

Abstract:
Objective: To examine the mechanism of ID1 expression in LMP1 transformed nasopharyngeal epithelial cells

Methods: Expression of ID1 and LMP1 in nasopharyngeal epithelial cell systems

Results: ID1 overexpression is common in nasopharyngeal carcinoma cells. LMP1 expression in an immortalized nasopharyngeal epithelial cell (NP69) induced ID1 expression. Expression of ID1 by LMP1 could be suppressed by chemical and genetic inhibitors of NF-kB. LMP1 mutants deleted in NF-KB activation domains (CTAR1 and CTAR2) are defective in induction of ID1. The prevalent LMP1 variant in Hong Kong nasopharyngeal carcinoma is more potent in activation of NF-kB and induction of ID1 expression. ID1 expression was observed in nasopharyngeal epithelial cell immortalized by telomerase. Expression of ID1 facilitates immortalization of primary nasopharyngeal epithelial cells by telomerase. In addition, ID1 expression stimulates the growth of immortalized nasopharyngeal epithelial cells, induces centrosomal and chromosomal aberrations.

Conclusions: ID1 could be activated by the EBV encoded LMP1 through activation of NF-kB and may play a role in facilitation of immortalization of premalignant nasopharyngeal epithelial cells.

Expression of (active) caspase 3 in tumour cells relates to clinical outcome in patients with nasopharyngeal carcinoma (NPC)

Harijadi A, Hariwiyanto B, Haryana SM, Oudejans JJ, Middeldorp JM

Contact:
Prof. Dr. Jaap M Middeldorp
VU Medical Center
Pathology (Amsterdam, The Netherlands)
Email: j.middeldorp@vumc.nl

Abstract:
Although NPC is a radiosensitive tumor, a large proportion of tumors relapse with increasing resistance to radio and/or chemotherapy. In vitro data showed that the cell-death inducing effects to radio- and/or chemotherapy are mediated by activation of the apoptosis cascade. Tumors failing to respond to therapy may have defects in the apoptosis pathway.
Aim was to investigate the relationship between the expression of (active) caspase 3 and clinical outcome of NPC patients. To clarify effects of radiotherapy on caspase 9 pathway, we also examined the expression of p53, p21, bcl-2, pro-caspase 3 and X-linked inhibitory apoptosis protein (XIAP) in 41 Indonesian NPC biopsies from patients without evidence of distant metastasis who were treated with radiotherapy only. Standard immunohistochemistry staining with monoclonal antibodies against pro-caspase 3, active caspase 3, XIAP, p53, p21 and bcl-2. Levels of caspase 3 activation were determined by quantifying positive staining tumor cells. Expression of pro-caspase 3 was scored relative to expression levels in surrounding lymphocytes. Tumour-infiltrating activated cytotoxic T-lymphocytes were detected via Granzyme-B staining as described before (Oudejans et al., J.Pathol. (2002) 98; 468-475).

Low levels of caspase 3 activation were associated with high levels of granzyme-B expressing tumour-infiltrating cytotoxic lymphocytes ($p=0.001$). High levels of active caspase 3 showed a strongly associated with good clinical outcome (CR; $P<0.0001$). Positive active caspase-3 was associated with younger age ($p=0.04$), but not with gender. A positive correlation was found between detectable caspase 3 activation and expression levels of pro-caspase 3 ($p=0.02$). High levels of active caspase 3 were associated with absence of XIAP expression ($p=0.05$). Bcl-2 was detected more frequently in cases with active caspase-3 ($p=0.07$), but P53 and p21 were not significantly different among cases positive or negative for active caspase 3.
Expression of active caspase 3 in NPC cells predicts good clinical outcome in NPC patients. Presence of abundant activated T-cell infiltrate correlates with absent caspase-3 and supports the hypothesis on their role in selecting apoptosis resistant tumour cells. Our data support the notion that an intact apoptosis cascade in tumor cells is essential for effective tumour cell killing by radiotherapy.

Epstein-Barr virus RK-BARF0 protein inhibits i-MFA-mediated repression of WNT signaling pathway by disruption of I-MFA-TCF/LEF-1 complex formation

Kusano S, Raab-Traub N

Contact:
Dr. Shuichi Kusano
St Marianna University School of Medicine
Department of Microbiology (Kawasaki, Japan)
Email: skusano@marianna-u.ac.jp

Abstract:
Objective: RK-BARF0 is a potential 279 amino acids protein, encoded by one of the transcripts from BamHI A region of the EBV genome. To determine the function of RK-BARF0 during latent EBV-infection and/or EBV-mediated tumor development, potential cellular targets of RK-BARF0 were identified by yeast two-hybrid screening. One of the strongest interacting proteins was I-mfa, an inhibitor of MyoD family proteins. Recent studies by ourselves and others have also shown that I-mfa is a strong negative regulator of Wnt signaling pathway through the interaction with T-cell factor (TCF) and lymphocyte enhancer factor-1 (LEF-1). In this report, we analyzed the effects of RK-BARF0 on I-mfa function in epithelial cells.

Methods: Interactions between I-mfa and RK-BARF0, or LEF-1 were determined by co-immunoprecipitation analysis. Intracellular localization of RK-BARF0 and I-mfa was determined by Subcellular Proteome Extraction Kit (CALBIOCHEM). The effect of RK-BARF0 for I-mfa-mediated repression of Wnt signaling was determined by analysis of TOP-flash (synthetic TCF-specific promoter, Upstate) activities in the presence and absence of RK-BARF0. The effect of RK-BARF0 for I-mfa-mediated stimulation of NF-kappaB signaling was determined by analysis of pNF-kappaB-SEAP (synthetic NF-kappaB specific promoter, Clontech) activities in the presence and absence of RK-BARF0.

Results: RK-BARF0 and I-mfa also interacted in vivo as demonstrated by co-immunoprecipitation analysis. This interaction repressed the nuclear translocation of RK-BARF0, I-mfa-mediated downregulation of reporter gene expression regulated by TCF/LEF-1, and also decreased the complex formation between I-mfa and LEF-1 in epithelial cells in vitro and in vivo. However co-immunoprecipitation analysis demonstrated that RK-BARF0 and LEF-1 required the different regions of I-mfa domain for complex formation with I-mfa. Interestingly, it is identified that I-mfa stimulates NF-kappaB-dependent transcription by its I-mfa domain, but RK-BARF0 does not show any effects for this regulation of NF-kappaB signaling pathway.

Conclusions: These data reveal that RK-BARF0 specifically interacts with I-mfa in vivo and inhibits I-mfa-mediated negative regulation of the Wnt signaling pathway. This interaction may contribute to tumor development in vivo through effects on the Wnt signaling pathway in EBV-infected cells.

Expression of the Epstein-Barr virus (EBV)-Encoded Latent Membrane Protein 2A (LMP2A) in EBV-Associated Nasopharyngeal Carcinoma

Heussinger N, Buttner M, Ott G, Brachtel E, Pilch BZ, Kremmer E, Niedobitek G

Contact:
Dr. med. Maike Büttner
Pathologisches Institut
Universität Erlangen-Nürnberg (Erlangen, Germany)
Email: Maike.buettner@patho.imed.uni-erlangen.de

Abstract:
Objective: The Epstein-Barr virus (EBV) is associated with virtually all cases of undifferentiated nasopharyngeal carcinoma (NPC). In this context the virus provides a possible target for immunotherapy of NPC since a limited number of viral genes are expressed in the neoplastic cells. The EBV-encoded latent membrane protein 2A (LMP2A) is considered a promising target since it provides epitopes recognised by EBV-specific T-cells. The objective of this study was to investigate whether LMP-2A is expressed at protein level in NPCs.

Methods: EBER-in situ hybridisation was performed of paraffin sections of 35 undifferentiated NPCs, 5 squamous cell NPCs and 12 tonsillar carcinomas to dectect EBV infection. Using immunohistochemistry the expression of LMP2A-protein was investigated.

Results: EBV infection was detected in the neoplastic cells of all 35 undifferentiated NPC biopsies. In contrast all 5 squamous cell NPCs and all 12 tonsillar carcinomas were EBV-negative. Expression of LMP2A was detected in 17 of 35 (48.6%) EBV-positive undifferentiated NPCs. In EBV-negative squamous cell NPCs and tonsillar carcinomas no LMP2A-specific staining was observed.

Conclusions: This finding provides evidence suggesting that NPC tumour cells may be susceptible to lysis by cytotoxic T-cells directed against LMP2A and should encourage efforts to develop immunotherapeutic approaches for the treatment of NPC.

What do we learn from EB virus encoded LMP1 story? (II) The Role of Signaling Pathways Activated by EBV-Encoded LMP1 in the Carcinogenesis of NPC

Cao Y

Contact:
Dr. Ya Cao
Cancer Research Institute (Changsha, China)
Email: ycao98@public.cs.hn.cn

Abstract:
EBV-Encoded LMP1 consists of a cytoplasmic N-terminus trail, transmembrane domain, and a cytoplasmic c-terminus containing three essential activating domains, namely, CTAR1, CTAR2 and CTAR3 and is known to activate NF-kB, AP-1, JAK3/STAT (Janus kinase 3/signal transducers and activator of transcription) pathways respectively. Further studies suggest that there was a cross-talk between NF- kB and AP-1 signaling pathways, and p65,p50 and c-Jun were involved in the process while JIP could block the cross-talk.
Our studies have demonstrated that these three signaling pathways activated exquisitely by LMP1 were related to many biologic manners of carcinoma involving in cell proliferation, cell apoptosis, metastasis, differentiation, invasion and also have identified some target genes of host such as cyclinD1, p16, EGFR, VEGF, MMP9, IL-8 as the down-stream genes of these signaling pathway activated by LMP1 .
During studies on NF-kB, AP-1, JAK3/STAT signaling pathways, we have defined some multihit targeted genes to interfere. Our previous studies showed that LMP1 activated the NF-kB signaling pathway via phosphorylation and degradation of IkB a. So we could inhibit NF-kB signaling pathway through the inhibition of IKK, NF-kB. We also could take JNK, JIP (JNK interacting protein), Jak3 and Stat as the targeted genes to inhibit the activity of the AP 1 and JAK3/STAT signaling pathways.
We proved that nuclear factors on the signaling pathways activated by LMP1 such as NF-kB, AP-1, performed their functions via translocation to the nucleus, phorsphorylation. c-Jun and Jun-B belong to the AP-1 family which always form dimers to function. EGFR as a transmembrane protein interfered by LMP1 translocated to the nucleus in a ligand-independent manner.
Our studies already proved that LMP1 continuously activated NF-kB. Based on this progress, we developed gene therapy strategies in vivo and in vitro target on NF-kB for EBV positive cancers. We introduced pLTR-tk plasmid into LMP1-expressing cells and dealt with GCV, and these cells were highly apoptotic. At the same time, we used NF-kB inhibitor to inhibit p65 activity and found it can induce EBV lytic replication, at last resulting in specific cell killing effects on EBV positive carcinoma cells in vitro.

Dissecting the role of LMP1 signalling in epithelial cells in vivo

Charalambous C, Wilson JB

Contact:
Chrystalla Charalambous
University of Glasgow
Molecular Genetics, I.B.L.S. (Glasgow, UK)
Email: 0105611c@student.gla.ac.uk

Abstract:
In order to explore the consequences of expression of Latent Membrane Protein 1 (LMP1) in epithelial cells, we have developed lines of transgenic mice which express the protein in the epidermis (Wilson et al., Cell 1990, 61, 1315-1327). The mice develop epidermal hyperplasia as a result of excess proliferation and are sensitised to chemical carcinogenesis (Curran et al., Cancer Research 2001, 61, 6730-6738). Further lines of mice have now been developed which express the LMP1CAO variant in the epidermis, under the control of the viral ED-L2 promoter (Stevenson et al., manuscript in preparation). These mice show an epidermal hyperplasia and inflammation that progresses with age. When brought into the FVB background, the mice develop papillomas spontaneously.
Several signalling pathways have been examined in the affected tissues in order to explore which are activated by LMP1 in the epithelium in vivo. Initial experiments showed upregulation of the Tumour Growth Factor a (TGFa) in transgenic tissues. Total levels of Epidemal Growth Factor Receptor (EGFR) protein showed no upregulation. However, upregulation of smaller phosphorylated EGFR products was observed in the transgenic tissues, implying a faster turnover. One of the pathways activated by EGFR is the Ras/MAPK pathway. It was seen in transgenic tissues that LMP1 activated MEK1/2 and ERK1/2. Further experiments, revealed that LMP1 in the epithelium in vivo leads to activation of p38, caspase 3 and upregulation of p53, VEGF and MMP9. The exact role that LMP1 plays in tumourigenesis and what other factors tip the balance towards proliferation and survival remain to be elucidated. In order to explore the contribution of LMP1 induced TGFa upregulation further, LMP1 transgenic mice have been crossed into a TGFa null background. Data from these experiments will be presented.

Epstein-Barr Virus Encoded Latent Membrane Protein 1 Modulates Nuclear Translocation of Telomerase Reverse Transcriptase Protein through Activating NF-kB p65 in Human Nasopharyngeal Carcinoma Cells

Ding L, Yang J, Li L, Tao Y, Ye M, Shi Y, Gong J, Cao Y

Contact:
Dr. Lin Ding
Cancer Research Intitute
Central South University (Changsha, China)
Email: lindeyre@hotmail.com

Abstract:
Objective: Study the mechanism of LMP1-induced telomerase activity in NPC

Methods: Telomerase PCR-ELISA; Cytoplasmic and Nuclear Fractionation; Western Blotting; Immunoprecipitation; Immunofluorescence Microscopy

Results: 1. We performed Western blotting and Immunofluorescence Confocal Microscopy to detect localization of hTERT and p65 in NPC cell lines.Two independent experiments have shown that hTERT protein was present in both cytoplasmic and nuclear fraction of LMP1-negative NPC cell lines, and LMP1 promoted nuclear accumulation of NF-kB p65 and hTERT. 2. Co-IP results suggest that LMP1 can induce direct binding of hTERT with NF-kB. 3. LMP1 induced telomerase activity through modulating translocation of hTERT and NF-kB. Conversely, a specificNF-kB nuclear translocation inhibitor Z-LLF-CHO, and HNE2-LMP1-DNM IkBa, in which degradation of IkB a is blocked and NF-kB stays at the cytoplasm, can block LMP1-induced nuclear translocation of hTERT.

Conclusions: LMP1 modulates telomerase activity by inducing translocation of hTERT protein directly bound to NF-kB p65 from the cytoplasm to the nucleus of NPC cells.

Involvement of cross-talk between c-Jun and Ets-1 in EBV-LMP1 regulating expression of MMP9 in nasopharyngeal carcinoma cells

Zeng L, Tao Y, Song X, Liu YP, Yi W, Li W, Cao Y

Contact:
Dr. Liang Zeng
Cancer Research Institute
Xiangya School of Medicine, Central South University (Changsha, China)
Email: zengL2004@hotmail.com

Abstract:
Objective: Epstein-Barr virus (EBV) is closely related to nasopharyngeal carcinoma (NPC) and EBV encoded latent membrane protein 1 (LMP1) is an oncogenic protein, involving carcinogenesis, invasion and metastasis. One of the mechanisms is abnormality in transcriptional regulation of tumor associated genes mediated by transcription factors. In this study, the effect of the adjacent AP-1 and Ets binding sites in the MMP9 promoter on the activation of MMP9 transcription by EBV-LMP1, and if cross-talk between c-Jun/Ets-1 is involved in the LMP1-mediated expression of MMP9 were investigated.

Methods: A NPC cell line, pTet-on-LMP1 HNE2 was used and characterized by LMP1 expression regulated by Doxycycline (Dox). RNA level, protein expression, protein phosphorylation and DNA binding activity were assayed respectively by RT-PCR, Western blot, co-immunoprecipitation, and EMSA. Activity of MMP9 was assayed with Gelatin Zymography; Site-directed mutagenesis technique was used to establish a series ofmutants, MMP9-Ets(-541)mt-CAT, MMP9-AP-1(-533)mt-CAT, MMP9-AP-1(-533)/Ets(-541) mt-CAT; respectively containing a mutation in the Ets binding site (-540) alone, a mutation in the AP-1 binding site (-533) alone, and mutations in both the Ets and AP-1 binding sites.Mutants were confirmed by DNA sequencing. The mutants were transfected into NPC cells by transient transfection .

Results: Ets-1 Expression was Dox-dependent to some extent, and LMP1 could regulate the expression of Ets-1 in transcriptional and protein level, activate it by protein phosphorylation and enhance its DNA binding activity. c-Jun protein expression was also Dox-dependent to some extent. Compared with MMP9-CAT wt, the activity of the reporter gene in MMP9-CAT Ets mt, MMP9-CAT AP-1 mt, and MMP9-CAT Ets/AP-1 mt decreased significantly, especially the MMP9-CAT Ets/AP-1 mt. After c-Jun alone, and Ets-1 alone, both c-Jun and Ets-1 were blocked by antisense oligonucleotide, the activity of MMP9 was inhibited greatly, especially when both c-Jun and Ets-1 were blocked. Under induction of Dox, Ets-1 expression was inhibited significantly after the blocking of c-Jun; c-Jun expression decreased greatly after the blocking of Ets-1.

Conclusions: c-Jun and Ets-1 play a crucial role in activation of MMP9 transcription induced by LMP1, and cross-talk between c-Jun/Ets-1 is involved in the LMP1-mediated expression of MMP9.

Evidence of LMP1 interaction with galectin 9 in lipid rafts of malignant NPC cells

Busson P, Durieu C, Keryer C, Nishi N, Faigle W, Middeldorp JM, Loew D

Contact:
Dr. med. Pierre Busson
CNRS-Institut Gustave Roussy
UMR 8126 (Villejuif, France)
Email: pbusson@igr.fr

Abstract:
Objective: A substantial fraction of LMP1 is consistently associated with lipid rafts in both lymphoid and epithelial cells, including NPC cells. LMP1 selectively recruits TRAF3 in lipid rafts whereas both TRAF2 and TRAF3 translocate into the rafts under activation of the CD40 receptor (Ardila-Osorio et al., Int. J. Cancer, in press). In order to better understand raft contribution to LMP1 signaling and trafficking, we have undertaken to identify novel LMP1 partners within rafts of NPC cells.

Methods: Rafts were isolated from the C15 NPC tumor line which has permanent spontaneous LMP1 expression. Preparative immuno-precipitation was performed with the OT21C monoclonal antibody. The precipitate was analysed using one dimension PAGE electrophoresis and/or mass spectrometry analysis. To perform MS-MS analysis samples were processed through nano-LC, electrospray ionization and Quad-TOF analysis.

Results: Nine protein species were visualized on silver-stained gels with molecular weights ranging from 187 to 39 kDa. The smaller species was identified by MS-MS as galectin 9. This beta-galactoside binding lectin has been initially described in malignant Hodgkin cells. It is known to have immune suppressive activity. We have found that galectin 9 is extremely abundant in NPC tumor lines and biopsies as well as in LCLs whereas it is not detectable in Burkitt lymphoma cells. Bi-directional co-immunoprecipitation of LMP1 and galectin 9 has been confirmed by Western blotting in NPC and LCL cells but not in co-transfected Hela cells, suggesting that the interaction is indirect. Experiments are in progress to investigate interactions of both proteins in vitro. In addition, we have obtained preliminary evidence that both proteins are secreted by NPC cells.

Conclusions: Galectin 9 seems to be a tracer of a multimolecular complex carrying LMP1 within lipid rafts and possibly secreted vesicules. LMP1-carrying complexes might contain distinct assortments of proteins and lipids depending on the host cell. It is interesting to observe that galectin 9 is abundant in several types of EBV-transformed cells which have spontaneous LMP1 expression (Hodgkin, NPC and LCLs). Our present aim is to confirm secretion of galectin 9 and LMP1 and to determine whether they are carried by the same extra-cellular structures.

EBV is colinear with the immunoglobulin gene loci

Niller HH, Salamon D, Rahmann S, Koroknai A, Banati F, Ilg K, Schwarzmann F, Wolf H, Minarovits J

Contact:
Dr. Hans Helmut Niller
University Regensburg, Research Centre
Institute for Medical Microbiology and Hygiene (Regensburg, Germany)
Email: Hans-Helmut.Niller@klinik.uni-regensburg.de

Abstract:
A 19 base pair in vivo-binding site for the oncoprotein c-Myc has been discovered in the EBER1 promoter of the EBV genome that specifies the causal role of EBV in lymphomagenesis*. This binding site has only one identical twin sequence in the human genome, namely at the very 5'-end of all the variable (V) genes of the lambda immunoglobulin (Ig) locus (GenBank/EMBL D86993). Furthermore, oriP of EBV is a functional homologue of the Ig intronic enhancer Ei/MAR, while the W-repeats of EBV contain functional switch signals like the Ig loci. Similar to the Ig-V-RNAs, the EBER-RNAs exhibit uninterrupted full length open reading frames with strong homologies to Ig V-peptides and secondary structures that expose somatic hypermutation signature motifs. Therefore, the left part of EBV exhibits a strong colinearity with the Ig-gene loci. This finding indicates that the left part of EBV co-originated with the Ig-loci. This may help elucidating the mechanisms of EBV-caused tumorigenesis. Specifically, the accidental presence of an active Ig-like domain (EBV) in epithelial cells of the nasopharynx may contribute to the origin of NPC through the induction of hypermutation mechanisms.

*Niller HH, D Salamon, K Ilg, A Koroknai, F Banati, G Bauml, O Rucker, F Schwarzmann, H Wolf, and J Minarovits. 2003. The in vivo binding site for oncoprotein c-Myc in the promoter for Epstein-Barr virus (EBV) encoding RNA (EBER) 1 suggests a specific role for EBV in lymphomagenesis. Med. Sci. Monit. 9:HY1-HY9.

*Niller HH, D Salamon, K Ilg, A Koroknai, F Banati, F Schwarzmann, H Wolf, and J Minarovits. 2004. EBV-associated neoplasms: alternative pathogenetic pathways. Med. Hypotheses 62:387-391.

A Functional Single Nucleotide Polymorphism Site Detected in Nasopharyngeal Carcinoma-Associated Transforming Gene Tx

Ren W, Zheng H, Li M, Deng L, Li XL, Pan KF, Lu YY, Cao Y

Contact:
Dr. Wei Ren
Cancer Research Institute, Xiangya School of Medicine
Central South University (Changsha, China)
Email: ycao98@public.cs.hn.cn

Abstract:
Tx is a transforming gene cloned from a nasopharyngeal carcinoma (NPC) cell line CNE2. Sequence analysis revealed that Tx encoded an aberrant immunoglobulin kappa light chain (Ig-E), which is abnormally expressed in epithelial tumor cells and plays an important role in nasopharyngeal carcinogenesis, we have confirmed its protein level could be regulated by Epstein Barr Virus (EBV) latent membrane protein 1 (LMP1) in EBV positive NPC cell lines. Bioinformatical analysis confirmed the presence of a single nucleotide polymorphism (SNP) site in Tx gene as matched to the Cancer Genome Anatomy Project (CGAP) SNP clusters database, which predicted 8 candidate SNP sites. Distribution of the confirmed SNP site in the genomes of healthy individuals and NPC patients was analyzed by denaturing high performance liquid chromatography (DHPLC). Heteroduplex genotype (GC/CG) occurred in NPC patients with a frequency significantly higher (52.44%) than that detected in healthy individuals (33.75%). In contrast, homoduplex genotype (CC) was less frequent in NPC patients (31.70%) than in normal individuals (56.25%), suggesting that heteroduplex genotype of Tx gene might be a risk factor for NPC.

Epstein Barr Virus infection and nasopharyngeal carcinoma in Indonesia

Haryana SM, Astuti I, Harijadi A, Fachiroh J, Paramita DK, Hariwiyanto B, Dahlia HL, Kurnianda J, Purwanto I, Maesadjie S, Dhamiyati W, Rahardjo MD, Widayati K, Middeldorp JM, Tan IB

Contact:
Prof. Dr. Sofia M. Haryana
Faculty of Medicine Gadjah Mada University (Yogyakarta, Indonesia)
Email: rikaharyana@yahoo.com, sari_wuland@yahoo.com

Abstract:
Objective: To develop simple, cheap and appropriate test for early detection which can be applied in remote areas of Indonesia. This objective becomes our main target to reach further goal to increase the success of therapy.Another aim of this project is to develop better clinical management, which consists of clinical departments (ENT, Internal Medicine, Radiodiagnostic, Radiotherapy) and laboratory site (Pathology and Histology) under coordination of ENT Department and to improve the data management.

Methods: The early detection was done by ELISA test using synthetic peptides of VCA and EBNA1 and also native EA protein, while immunoblotting method to the EBV protein derived from HH514.c16 induced cell lines used as confirmatory. To perform better diagnostic for pathology we tried simpler and cheaper method using EBNA1-EBV instead of routine EBER. For non invasive diagnostic we use nasal swab to look at EBV-viral load analysis and malignant cells using NASBA and RT PCR methods.

Results: IgA ELISA using VCA+EBNA1 synthetic peptide developed in our laboratory to more than 200 NPC sera have been tested and also sera from healthy donor as control. The results are very promising both on specificity and sensitivity (>90%). ELISA test using another antigen (native EA extract) also gave a promising result in specificity and sensitivity (89.26% and 97.62%). The test using synthetic peptide will be used to develop in more large scale which is now on progress under collaboration with several European Institutions (VUmc and AvL). Better diagnostic also shown in immunohistochemistry with Ab to EBNA1 from paraffin block tissue. RT-PCR /NASBA from nasal swab also showed promising results. By centralization of data and clinical management, it has been shown that the number of drop out NPC patients is decreased significantly.

Conclusions: Several methods in this project can be used for diagnosis, such as IgA ELISA using VCA+EBNA1 synthetic peptides, immunohistochemistry to EBNA1 protein and also by looking at the DNA viral load.All these methods hopefully can be used for early detection which will be very useful for success of treatment. The improvement of clinical and data management can minimize the number of drop out patients.

Proteomic analysis of nasopharyngeal tumorigenesis tissues of rat in the different stages

Tang F, Tian D, Gong Z, Duan C, Xiao Z, Tang Y

Contact:
Prof. Dr. Faqing Tang
Clinic Research Center
Xiangya Hospital (Changsha, China)
Email: tangfaqing333@hotmail.com

Abstract:
Objective: To investigate protein expression in tumorigenesis stages in nasopharyngeal carcinoma.

Methods: The rat nasopharyngeal carcinomas were induced with N, N-Dinitrosopiperazine (DNP). The simple hyperplasia, atypical hyperplasia, and nasopharyngeal carcinoma tissue were got, from which proteins were isolated, separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and identified by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry followed by peptide mass fingerprinting

Results: To contrast the simple hyperplasia, atypical hyperplasia and nasopharyngeal carcinoma tissue, a total of 67 unique proteins were identified, including ILGF, IL-14, onco-protein WNT-2, MAPK-8 et al. Bioinformatic analysis predicted glycosylation to be the most common explanation for multiple forms of the same protein.

Conclusions: These imply that, proteomic analysis of nasopharyngeal carcinoma is a promising tool to study nasopharyngeal carcinogensis and to determine biomarkers of nasopharyngeal carcinoma. ILGF, IL-14, oncoprotein WNT-2 and MAPK-8 may playan important role in the development of nasopharyngeal carcinoma

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Functional characterization of EBV-infected nasopharyngeal epithelial cells

Lo AKF, Lo KW, Tsao SW, Takada K, Huang DP

Contact:
Dr. Angela KF Lo
Johns Hopkins University
School of Medicine (Baltimore, USA)
Email: kfloa@yahoo.com

Abstract:
Nasopharyngeal carcinoma (NPC) is closely associated with Epstein-Barr virus (EBV) latent infection. The EBV encoded LMP1 and BARF1 have been reported being oncogenic, EBNA1 and EBERs have been suggested to play roles in escaping from immune responses. The reported interactions of these EBV latent proteins in different signal transduction proteins suggest that EBV infection can manipulate cellular host program. However, the pathological role of EBV infection in NPC malignancy remains unclear. In this study, we stably established EBV infection in nasopharyngeal epithelial cell lines with a recombinant EBV. All of the EBV infected cell lines exhibited EBV latency II pattern by expressing Qp-EBNA1, EBER1, LMP1, LMP2a, BamHI A fragment and BARF1 genes but not EBNA2, LMP2b, Cp/Wp-EBNA1. Lack of BZLF1 and BRLF1 expressions were observed indicating the absence of lytic EBV infection. These EBV expression patterns are similar to that in NPC biopsies. EBV infection could alter the cell cycle and invasive ability of immortalized cells but failed to enhance tumorigenicity of NPC cells. EBV infection also activated STAT3 and NFkB pathways but suppressed the activities of stress-activated protein kinases. Reduced expression of keratin 14 and 19 as well as inflammatory IFNα, IFNβ and TNFα cytokines in EBV infected cells was also observed. The current data suggests the roles of EBV latent infection in nasopharyngeal epithelial cells for inhibiting cell differentiation, preventing cell apoptosis and suppressing inflammatory responses of the infected cells.

Identification of Genes Related in the Early Stage of Tumorigenesis in Nasopharyngeal Carcinoma (NPC)

Huang DY, Hwang YC, Lee YCG, Huang CYF, Wu HC, Lin CT

Contact:
Dah-Yeou Huang
National Taiwan University, School of Medicine
Pathology (Taipei, Taiwan)
Email: ctl@ha.mc.ntu.edu.tw

Abstract:
Objective: Nasopharyngeal carcinoma (NPC) is one of the common cancers in Taiwan, South China and Singapore. The etiological factors are not well defined yet. However, environmental factors, such as eating salted fish, chinese herbs, long-term exposure to sulfuric acid vapor, genetic factors and viral infection, especially Epstein-Barr Virus (EBV) have been proposed to be closely associated with NPC. Our previous investigation suggests that EBV can promote NPC progression. In the present experiments, we want to identify the genes that have been altered during the early stage of NPC tumorigenesis.

Methods: We used microarray assay to compare gene expression profiles of 5 NPC cell lines with expression profiles of the primary cultures of normal nasal mucosal cells, respectively. Differentially expressed genes selected by microarray analysis were further confirmed by quantitative RT-PCR and statistical analysis.

Results: From microarray assay, we picked out 46 genes that were differentially expressed in between all 5 NPC cell lines and normal nasal mucosal cells. We further verified the microarray data by real time RT-PCR. By Student T-test, Pearson's Correlation test and two-way ANOVA analysis, we found that, among all the selected genes, 9 were identified as down regulated in NPC cell lines. We also found that 3 of these 9 genes were also confirmed as down regulated in NPC biopsies, comparing to normal tissues. The promoter regions of these 9 genes were further analyzed. They were regulated by a common transcription factor, SOX5, which itself were down regulated in both NPC cell lines and NPC biopsy specimens. SOX5 has many isoforms. So far, we have cloned isoform A, B, and several previously unidentified new isoforms. When we made transient expression of SOX5A in NPC cell lines, the migratory activity of NPC cells was retarded.

Conclusions: We have identified 9 down regulated genes among 8K gene chip from all 5 NPC cell lines and biopsy specimens. Furthermore, we have proposed a transcription factor SOX5, which is down regulated in NPC, may regulate above mentioned 9 down regulated genes and play a pivotal role in NPC oncogenesis.

The Preliminary Study on Molecular Classification of Nasopharyngeal Poorly-Differentiated Squamous Cell Carcinoma

Wang S, Lu LC, Li X, Xie W, Deng X, Yao KT

Contact:
Shuang Wang
First Military Medical University
Institute of Cancer Research (Guangzhou, China)
Email: wang3660@fimmu.com

Abstract:
Objective: Nasopharyngeal carcinoma (NPC) is a malignant tumor with high incidence in Southern China and Southern China and Southeastern Asia. 98% NPC patients suffer from poorly-differentiated squamous cell carcinoma. However, patients with NPC have highly variable clinical course. The five-year survival rate for NPC patients is under 60%. This clinical heterogeneity indicates there are sub-classes in NPC. The purpose of this study was to classify nasopharyngeal poorly-differentiated squamous cell carcinomas based on variations in gene expression patterns derived from cDNA microarrays.

Methods: The 32P-dCTP labeled cDNA probes prepared from total RNA extracted respectively from 24 samples that were diagnosed as nasopharyngeal poorly-differentiated squamous cell carcinoma and contained >75% cancer cells by analysis of corresponding H&E-stained sections. Pooled RNA from 24 normal nasopharyngeal tissue and 4 nasopharyngeal poorly-differentiated squamous cell lines was respectively hybridized to cDNA microarrays containing about 4000 known genes. The signal intensity in each microarray was normalized and calculated.

Results: By t-test, 1625 genes were identified with statistically significant difference ($P<0.05$) between NPC and normal nasopharyngeal tissues. Based on the expression patterns of 4132 genes and 1625 genes, 24 NPC samples were classified into two subclasses using hierarchical clustering. Both results were consistent. 78 genes were identified with statistically significant difference ($P<0.05$) between the two groups. The clustering result on the basis of the expression pattern of 78 genes was similar to the above ones, which suggested the information included in 78 gene expression pattern might be enough for the molecular classification of nasopharyngeal poorly-differentiated squamous cell carcinoma. Comparing the two gene expression profiles of NPC tissues and cell lines, 50 genes expressed in NPC tissues but not in cell lines were identified and regarded as the genes expressed in tumor stroma cells. The 78 genes did not include the above 50 genes expressed in NPC stroma cells, so they were preliminarily regarded as the candidate markers for molecular classification of nasopharyngeal poorly-differentiated squamous cell carcinoma.

Conclusions: At the molecular level, at least there exist two subclasses in nasopharyngeal poorly-differentiated squamous cell carcinoma.

The establishment of a transgenic mouse model susceptible to Epstein-Barr virus

Lu LC, He Y, Shen XM, Xu SJ, Wang S, Yao KT

Contact:
Dr. Li-chun Lu
First Military Medical University
Institute of Cancer Research (Guangzhou, China)
Email: lichunlu21@163.com

Abstract:
Objective: The most frequent problem met in studying the relationship between EBV and NPC in vivo is the looking of appropriate EBV-related NPC animal model. In this paper, we try to introduce the gene of EBV-related receptors into mouse nasopharyngeal epithelial cells, hoping to establish a kind of mouse model which could be infected by EBV naturally.

Methods: After constructed epithelium-specific eukaryotic expression vectors pEDL2-hCR2/pEDL2-pIgR, they were transferred into human immortalized keratinocyte cell line HaCaT and transformed epithelial cell line TMNE derived from mouse nasopharynx respectively by lipofectAMINE. Stable CR2/pIgR-expressing transfected cells were cloned. And then these clones were treated with EBV during cell culturing in vitro. Based on these experimental data, the fragments containing only ED-L2 promoter and target gene were injected into the male pronuclei of the mouse fertilized eggs and two transgenic mouse models were established.

Results: 1,782bp ED-L2 promoter was obtained by PCR and then pEDL2-hCR2/pEDL2-pIgR vectors were constructed. 2, Stable CR2/pIgR-expressing cell clones of HaCaT and TMNE were selected out. RT-PCR and immunohistochemical test showed both target proteins could express correctly in host cells. 3, EBV could infect both groups of cells treated with either EBV or EBV+TPA. EBV BamHI W fragment could be detected by PCR and Southern Blot. In-situ hybridization showed 18.96%«32.82% cells were EBER-1 positive. The infection ratios of HaCaT was higher than that of TMNE ($P<0.05$). 4, Screening by PCR and Southern blot, 4 of CR2 founders and 6 of pIgR ones were obtained. All positive mice were used as founders to establish transgenic mouse line. Verified by immunohistochemical method, 1 mouse bearing pIgR were observed to express target protein mainly in squamous epithelial cells of tissues such as nasopharynx, esophagus and tongue. The rest 7 founders were treated with EBV for 1 to 3 months respectively. Yet ELISA test showed that EBV-IgG antibody in peripheral blood was negative. None of them was infected.

Conclusion: We have established two types of transgenic mice expressing EBV-related receptors especially at their nasopharyngeal epithelia for the first time. Though we did not observe EBV infection in any of these mice, it is still a beneficial try to construct an animal model susceptible to EBV.

Expression of EBV BGLF4 Protein Kinase in NPC Biopsies

Wang JT, Han CH, Sheen TS, Yan PW, Chen CL, Yu YC, Chen MR

Contact:
Jiin-Tarng Wang
Graduate Institute and Department of Microbiology, College of Medicine
National Taiwan University (Taipei, Taiwan)
Email: d90445007@ntu.edu.tw

Abstract:
Epstein-Barr virus (EBV) is highly associated with many human malignancies. Expression of limited viral lytic genes was observed in EBV-associated nasopharyngeal carcinoma (NPC) tissues previously and it raises the possibility that some vial lytic products may contribute to the pathogenesis of NPC. The EBV BGLF4 encodes a ser/thr protein kinase, which expresses early during virus replication. In order to examine the expression of BGLF4 protein in vivo, a panel of anti-BGLF4 monoclonal antibodies was generated. Antibodies against two different epitopes, one within amino acids 27-70, and the other within amino acids 326-429 of BGLF4, were further characterized. Expression of BGLF4 protein was detected in 20% NPC biopsies (6/30) by immunohistochemistry using monoclonal antibody 2224. The BGLF4 protein was observed in both cytoplasm and nucleus of the NPC cells. The presence of BGLF4 protein in NPC biopsies suggests the possibility that BGLF4 may function as its cellular homologue cdc2 in tumor cells. Our finding reveals the differential expression profile of EBV lytic genes in individual EBV positive NPC tumors and suggests the possibility to design virus-targeting protocol for cancer therapy.

Preliminary Study on Inhibition of EGFR Expression by RNAi in Nasopharyngeal Carcinoma Cells

Weng DS, Ding YQ, Feng TY

Contact:
Dr. Desheng Weng
First Military Medical University
Department of Pathology & Cancer Research Institute (Guangzhou, China)
Email: wds@fimmu.com

Abstract:
Objective: The development of EBV associated disease nasopharyngeal carcinoma (NPC) is a multifactor and multistage process. Deregulated and excessive expression of epidermal growth factor receptor (EGFR) was observed in NPC cases in some studies. In RNA interference (RNAi), duplexes of 21-nucleotide RNAs (small interfering RNA, siRNA) corresponding to mRNA sequences of particular genes are used to efficiently inhibit the expression of the target proteins in mammalian cells. Here we used RNAi to suppress the expression of EGFR in NPC cells, to explore the possible relations between EGFR and NPC.

Methods: After synthesized 3 EGFR specific siRNAs by in vitro transcription, we transiently transfected 10nM, 20nM, 40nM and 60nM siRNAs into CNE1 and 5-8F respectively by lipofectamine. Total RNA and protein were extracted after 24h, 48h, 96h and 144h. EGFR expression levels were detected by semi-quantitative RT-PCR and Western Blot. The cells were harvested and counted by trypan blue exclusion every day after transfection, and cell cycle distribution were analyzed using FACS.

Results: Only one of the 3 siRNAs can suppress EGFR expression effectively. 24h after siRNA transfection, CNE1 and 5-8F EGFR mRNA content was decreased by approximately 71.7% and 67.5% respectively. The results of RT-PCR and Western Blot showed that siRNA silencing effect ascended with the increasing of tranfection concentration to some extent. The effect got to top when siRNA transfection concentration was 40nM. We also found that the silencing effect can only last for about 48h. Transfection with siRNA targeted EGFR resulted in reduction of CNE1 and 5-8F cells proliferation. FACS results indicated cell cycle arrest at G1 phase but not apoptosis in association with EGFR silencing.

Conclusions: EGFR may play an important role in NPC evolvement. siRNA-mediated inhibition of EGFR may constitute a useful therapeutic approach in the treatment of NPC.

Molocular cloning of the V-val subtype of EBNA-1: sequence, expression and application
Chao M, Lee YM, Wang HN, Chang YS

Contact:
Prof. Dr. Mei Chao
Chang Gung University
Microbiology and Immunology (Tao-Yang, Taiwan)
Email: pa0728@mail.cgu.edu.tw

Abstract:
Epstein-Barr virus (EBV) establishes latent infection and is associated with various human cancers. Epstein-Barr nuclear antigen 1 (EBNA-1) is the only viral protein expressed in all EBV-associated malignant tissues. Sequence variations in EBNA-1 have been observed in different isolates. However, the functional differences, if any, among various EBNA-1 sequences remained unknown. We thereby PCR-amplified the full-length EBNA-1 gene from CG3 cell line, an EBV-carrying lymphoblastoid cell line derived from a myeloid leukemia Taiwanese patient. The sequencing data indicated that EBNA-1 in CG3 cells is V-val subtype. The sequences encoding the N- and C-terminal regions of CG3 EBNA-1 were subcloned and expressed as His-tagged fusion proteins in E. coli. The purified fusions were then used to raise rabbit antibodies specific for different domains of EBNA-1. Furthermore, the full-length V-val EBNA-1 was also obtained from a nasopharyngeal carcinoma (NPC) Taiwanese patient. The eukaryotic expression plasmids containing the full-length V-val EBNA-1 inserts have also been successfully expressed in the transfected cultured cells, which will provide an excellent experimental system to elucidate the role of V-val EBNA-1 in EBV-associated diseases.

Distribution and effects on transcriptional regulation of EBNA1-variants from Vietnamese NPCs

Nguyen Van D, Ernberg I, Almqvist J, Phan Thi PP, Tran Thi C, Zeng YX, Hu LF

Contact:
Dr. med. Do Nguyen Van
Karolinska Institute
Microbiology and Tumor Biology Center (Stockholm, Sweden)
Email: do.nguyen.van@mtc.ki.se

Abstract:
Objective: Sequence variation in the DNA binding domain of Epstein-Barr virus nuclear antigen-1 (EBNA-1) has been described and classified into five subtypes, namely P-ala, P-thr, V-pro, V-leu and V-val. We investigated the distribution of these EBNA 1 variants invietnamese cases of nasopharyngeal carcinoma (NPC) compared to chinese. The possible effect of the V-val-v1 variant on transcriptional regulation of the viral latent enhancer FR in ori P and the Q promotor (Qp) silencer was explored and compared to prototype EBNA 1

Methods: PCR based sequencing of EBNA1 from NPC-biopsies and blood from patients and healthy donors. Functional differences by mutations in the C-terminal part of EBNA 1 was assayed by replacing the P-ala containing EBNA-1 DNA-binding fragment from the prototype strain B95-8 for the corresponding fragment of the asian V-val-v1 subtype. A luciferase reporter vector containing FR upstream of a heterologous promoter was used for assaying FR-driven transcription

Results: The majority of NPC samples and matched samples from the same patients including fresh biopsies and peripheral blood and blood from healthy blood donors contained the V-val-v1 variant. Only one NPC matched sample contained the V-val-v2 variant. We detected two variants in V-val subtype from chinese (15 cases) and v ietnamese (9 cases) nasopharyngeal carcinoma (NPC) patients and healthy vietnamese controls (5 cases). The transcriptional activity on both the FR-enhancer in oriP and on the Qp-silencer of the major Asian EBNA-1 variant was reduced compared to the prototype P-ala ($p<0.05$). We are now also analyzing effects of variation in FR sequence from vietnamese NPC in the luciferase report system

Conclusions: EBNA 1 variants, which have been described before in Burkitts lymphoma and NPC are prevalent in asian, as examplified by vietnamese NPC. The aa-variation is localized in the DNA-binding region of EBNA 1, although not in the DNA contacting amino acids. One predominant variant was shown to exert reduced transcriptional activity through the FR enhancer, and also reduced repression of Qp. The effects are small but significant, and may play a role in host control of latency contributing to increasedtumor risk.

Epitope-specific CD4+ T cell responses to EBNA1 in Chinese individuals

Tsang CW, Lin XR, Taylor GS, Lee SP, Chan ATC, Rickinson AB

Contact:
Dr. Chi Wai Tsang
University of Birmingham
CRUK Institute for Cancer Studies (Birmingham, UK)
Email: Tsangcw@cancer.bham.ac.uk

Abstract:
Nasopharyngeal carcinoma (NPC), a common malignancy in Southern China, is a potential target for immunotherapy using T cells specific for viral antigens expressed in the tumour. One of the relevant antigens, EBNA1, is an immunodominant target for CD4+ T cell memory in 70% Caucasian virus carriers. Here we studied CD4+ T cell responses to EBNA1 in the Chinese population, where HLA class II allele distribution is quite different from that in Caucasians and where the prevalent EBV strains have a slightly different EBNA1 sequence.
CD8+ T cell-depleted peripheral blood mononuclear cells (PBMCs) were screened in interferon-Elispot assays against a panel of 81 EBNA1 peptides (20-mers overlapping by 5) from the Chinese EBNA1 sequence. Some 56/79 (71%) healthy Chinese donors tested responded to one or more EBNA1 peptides at levels (50-250 spot-forming cells per million) similar to those seen in Caucasians. These assays identified 9 epitopes, all in the C-terminal half of the protein, including 6 unique to Chinese donors. One epitope, EBNA1 564-583, was recognised by 40% of all individuals, often as the strongest detectable response. CD4+ T cell clones specific for this epitope were established from different donors and all clones were restricted through a HLA-DP allele, DP5, which turned out to be remarkably common (50% allele frequency) in Chinese populations. Further studies with CD4+ T cell clones, derived by peptide stimulation of Chinese donor PBMCs in vitro, are in progress to identify the various EBNA1 epitopes and their restriction elements unequivocally. In addition these EBNA1-specific clones are being assayed for their ability to recognise autologous LCL target cells endogenously expressing EBNA1 from the resident EBV genome and over-expressing EBNA1 from Modified Vaccinia Ankara-based vectors.
We conclude that EBNA1 is a significant target for CD4+ T cell immunity in Chinese donors. Vaccination strategies aiming to boost EBNA1-specific CD4+ T cell responses in NPC patients may have therapeutic benefit and the strength of such responses can be monitored using a selected panel of EBNA1 epitope peptides. CD4+ T cell responses in such patients are currently being analysed.

Effect of selective inhibition of EBV-encoded latent membrane protein-1 and bcl-2 gene by RNA interference on nasopharyngeal carcinoma cell lines

Yin Z, Yao KT

Contact:
Dr. Zhihua Yin
Cancer Research Institute
Central South University (Changsha, China)
Email: yzh2000@163.com

Abstract:
Objective: To observe the effect of selective silencing LMP1 and bcl-2 gene expression in NPC cell lines using RNAi technique.

Methods: Vectors that can express siLMP1s and sibcl-2s are constructed and transfected into CNE-LMP1 cell line or CNE1 cell line respectively. Stably expressing siLMP1 or sibcl-2 NPC cell lines are obtained by puromycin selection. Protein expression is detected by Western blotting. Cell proliferation ability and cell survival ratio are detected by MTT assay. Cell apoptosis is observed through fluorescent microscope and flow cytometry (FCM).

Results: Three CNE-LMP1 cell lines stably transfected with psLuc, psLMP1-1 and psLMP1-2 were established. MTT assay showed that cell growth of CNE-LMP1-psLMP1s was inhibited to the original cell proliferation level of CNE1. Flow cytometry analysis indicated that psLMP1s could arrest CNE-LMP1-psLMP1s at G1 phase and led to increase of the cell population in G1. Three CNE1 cell lines stably transfected psLuc, psbcl-2-1 and psbcl-2-2 were established. Western blotting showed CNE1-psbcl-2-1 and psbcl-2-2 expressed BCL-2 weakly. MTT assay and FCM showed that there was no distinct change among these cell lines. But psbcl-2-1 and psbcl-2-2 could increase chemosensitivity to cisplatin in CNE1 cells. The cell survival ratio of CNE1-psbcl-2s decreased distinctly when added 4mg/ml and 8mg/ml cisplatin. Compared with control, the difference was statistically significant. By fluorescent microscope we could observe more apoptotic cells in CNE1-psbcl-2-2 and CNE1-psbcl-2-1 than in CNE1 and CNE1-psLuc 48h after added 8 mg/ml cisplatin. FCM showed that the rates of cell apoptosis were 11.10%,15.86%, 22.12%, and 48.84% respectively when 8 mg/ml cisplatin was added to CNE1, CNE1-psLuc, CNE1-psbcl-2-1 and CNE1-psbcl-2-2 cell lines for 48h.

Conclusions: psLMP1s may influence cell growth of LMP1-positive NPC cell lines stably transfected psLMP1s; while psbcl-2s may not influence cell growth but they can increase chemosensitivity to cisplatin in NPC cell lines stably transfected psbcl-2s. This will help physicians to make some clinical trials of gene therapy on NPC by RNAi. Our study of stably silencing LMP1 on EBV-positive nasopharyngeal carcinoma cell line (5-8F) by RNAi is just going on. I think results will be obtained before July 25th.

LMP1 transgenic mice: A proteomic analysis
McLean A, Wilson JB

Contact:
Adele McLean
University of Glasgow
IBLS, Molecular Genetics (Glasgow, UK)
Email: 0205580m@student.gla.ac.uk

Abstract:
Objective: In an attempt to model the effects of latent membrane protein 1 (LMP1) expression in nasopharyngeal carcinoma (NPC), we have developed transgenic mouse lines expressing LMP1 of the Cao strain of Epstein-Barr virus (EBV). LMP1Cao expression is directed to the epidermis using the EBV lytic promoter ED-L2 (Stevenson, D. and Wilson, J. B., unpublished results). The most severe phenotype is seen in the ears of the mice and worsens through time. In early stages (<=30days), the ears become more vascularised with respect to transgene negative, age-matched controls. Over time, this progresses to epidermal and dermal hyperplasia, severe hyperplasia and finally with necrosis.

Methods: We plan to investigate the effects of LMP1 expression at the molecular level in this phenotypic tissue. To accomplish this a proteomic approach will be employed, namely the DiGE technology (Amersham biosciences), in which samples of interest and controls are labelled with different fluorescent dyes and run on the same 2D gel for direct comparison. This will allow identification of some of the proteins that are dis-regulated in response to LMP1Cao and which lead to the severe phenotype presented.
Additionally, the inhibition of LMP1 will be attempted using various carcinoma cell lines generated from our transgenic animals. This will be approached in two ways, firstly using the technique of RNA interference and secondly by using a dominant negative LMP1, known as LMP1AAAG (Brennan, P., Floettmann, E., Mehl, A., Jones, M. and Rowe, M. (2001), J. Biol. Chem., 276, 1195-1203), which possesses 4 point mutations in its crucial signalling regions, rendering wildtype LMP1 non-functional when it oligomerizes.

Stat3 Pathway is activated by EB virus encoded Latent Membrane Protein 1 in Nasopharyngeal Carcinomo cells

Tan Y, Tao Y, Li L, Liu S, Tang M, Cao Y

Contact:
Dr. Yunnian Tan
Cancer Research Institute
Xiangya School of Medicine (Changsha, China)
Email: tyncg@tom.com

Abstract:
It is controversial whether EB virus encoded latent membrane protein 1 (LMP1) activates JAK/STAT signal pathway in B cells. To determine whether this signal pathway exists and/or can be activated in nasopharyngeal carcinoma cells (NPC), we used one Tet-on-LMP1-HNE2 stable cell line in which LMP1 expression is tightly controlled by tetracycline derivative Dox as a cell model.

Western blotting and EMSA were used to detect that LMP1 can stimulate Stat3 tyrosine not serine phosphorylation and enhance Stat3 DNA binding ability in this tet-on cell line. Immunocytochemistry also show LMP1 can stimulate Stat3 705 tyrosine phosphorylation and nuclear translocation and accumulation.

After LMP1 C-terminus point mutation expression plasmids were co-transfected with pTKs3 luciferase reporter in HNE2 NPC cell, we found that LMP1 can drive this reporter and this function is dependent on the cooperation of it s c terminus activated regions 2 and 3.

Construction of a Recombinant Lentiviral Vector with LMP1 and Establishment of a Transgenic Mouse Model

Yang YF, Ding YQ, Feng TY, Su YF

Contact:
Dr. Yu-Fang Yang
First Military Medical University
Institute of Cancer Research (Guangzhou, China)
Email: yyf@fimmu.com

Abstract:
Objective:Epstein-Barr virus (EBV) is associated with a number of human malignancies, especially with nasopharyngeal carcinoma (NPC). However, due to lacking of evidence of animal models, the role of EB virus in NPC occurrence and development remains in theresearch hotspot. In view of this, we established EBV-related transgenic mouse model of NPC and using kerotinocyte-specific promoter ED-L2 which can lead to the efficient and specific expression of target genes in the nasopharynx.

Methods: We constructed the lentiviral vector carrying the enhanced green fluorescent protein (EGFP) gene driven by a kerotinocyte-specific promoter ED-L2. Based on this vector, we constructed ED-L2-N-LMP1-EGFP and ED-L2-B-LMP1-EGFP lentiviral vector. Microinjection was used to build transgenic mouse model. The founder transgenic mice were examined by PCR and Southern blot analysis.

Results: We firstly transferred N-LMP1 and B-LMP1 lentiviral vector into CNE1, 5-8F, 6-10B, 293T and NIH3T3 cells in vitro. The expression of enhanced green fluorescent protein could be observed under fluorescence microscope. The expression of LMP1 was detected positively by RT-PCR and Western blot in CNE1, 5-8F, 6-10B and 293T cells. But the target gene couldn't express in NIH3T3 cells. We obtained 3 founder mice expressing EGFP and N-LMP1. Results showed the target gene could express correctly in host cells and the founder mice were positive for gene integration.

Conclusions: Keratinocyte-specific promoter ED-L2 can effectively induce exogenous gene expression in epithelial cells. EBV-encoded latent membrane protein 1 (LMP1) gene had been integrated into the genome of epithelial cells by lentiviral mediated transfection and the target gene was expressed.

Selection Pressure Driven Evolution of the Epstein-Barr virus Encoded Oncogene, LMP1, in Virus Isolates from South-East Asia

Burrows JM, Bromham L, Woolfit M, Piganeau G, Tellam G, Connolly G, Webb N, Poulsen L, Cooper L, Burrows SR, Moss DM, Haryana SM, Ng M, Nicholls JM, Khanna R

Contact:
Jacqueline Burrows
Queensland Institute of Medical Research
Department of Immunology and Infectious Diseases (Brisbane, Australia)
Email: jacquiB@qimr.edu.au

Abstract:
Objective: The geographically constrained distribution of NPC in South-East Asian populations suggests that both viral and host genetics may influence disease risk. Although susceptibility loci have been mapped within the human genome, this investigation examines the role of viral genetics in the focal distribution of NPC.

Methods: A molecular phylogenetic analysis of the NPC-associated viral oncogene, LMP1 in a large panel of EBV isolates from NPC endemic (South-East Asia) and non-endemic (PNG, Africa and Australia) regions of the world.

Results: This analysis revealed that LMP1 sequences show a distinct geographic structure, indicating that the South-East Asian isolates have evolved as a lineage distinct from PNG, African and Australian isolates. Furthermore, a likelihood ratio test revealed that the LMP1 C-terminus sequences from the South East Asian lineage are under significant positive selection pressure, particularly at some sites within the C-terminal activator regions. We also present evidence which indicates that although the N-terminus and transmembrane regions of LMP1 have undergone recombination, the C-terminus region of the gene has evolved without any history of recombination.

Conclusions: Based on these observations, we speculate that selection pressure may be driving the LMP1 sequences in virus isolates from South-East Asia towards a more malignant phenotype thereby influencing the endemic distribution of NPC in this region.

The Epstein-Barr virus oncogene product, latent membrane protein 1, regulates Nm23-H1 at the transcriptional level

Tse KP, Chien CK, Wu CC, Yu JS, Chang YS

Contact:
Ka Po Tse
Institute of Microbiology and Immunology
National Yang Ming University (Taipei, Taiwan)
Email: bobotse.tw@yahoo.com.tw

Abstract:
Epstein-Barr Virus (EBV) is closely related with many human malignancies like Burkitt's lymphoma, Hodgkin's lymphoma and Nasopharyngeal carcinoma (NPC). Among them, NPC is prevalent in southern China and is notoriously metastatic. Latent membrane protein 1 (LMP1), one of the viral proteins expressed in NPC biopsies, has been reported to induce morphological changes and enhanced invasiveness of epithelial cells when over-expressed. However, little is known about the underlying mechanisms or target proteins regulated by LMP1. To get a global view of protein expression modulated by LMP1, proteomic strategies were used.

From cell lines that stably express LMP1 or the control vector, whole cell extracts were separated by two-dimensional electrophoresis, stained and analyzed. Protein differentially expressed in two cell lines were identified by matrix-associated laser desorption ionization mass spectrophotometry-time of flight (MALDI-TOF). Comparative analysis of the respective spot patterns on two-dimensional electrophoresis showed that Nm23-H1, which is a metastasis-suppressor, was down-regulated in the LMP1-expressing cell line. Reduction of Nm23-H1 protein and mRNA levels in LMP1-stable cell line were further confirmed by Western blot and quantitative real-time PCR, respectively. Data from Nm23-H1 promoter studies demonstrated that LMP1 represses NM23-H1 expression at the transcriptional level. Furthermore, the mechanism underlying the repression is studied. Since down-regulation of Nm23-H1 in NPC has been correlated with lymph node metastasis, our preliminary data further suggests that LMP1 may be the critical viral protein mediating Nm23-H1 down-regulation in NPC and which can explain the role of LMP1 in enhancing tumor metastatic ability.

The Inhibitory Effect of Chinese Herb Extract to the Infection Activity of Epstein-Barr virus in Population Highly Susceptible to Nasopharyngeal Carcinoma.

Tang F, Tian D, Wang C, Gong Z, Zhou H, Tang Y

Contact:
Prof. Dr. Faqing Tang
Clinic Research Center
Xiangya Hospital (Changsha, China)
Email: tangfaqing333@hotmail.com

Abstract:
Objective: Epstein-Barr virus (EBV) is etiologically associated with nasopharyngeal carcinoma (NPC), whose VCA-IgA is used as a biomarker in populations highly susceptible to NPC. Chinese Herb Extract, which was separated from Copti Chinese by gas chromatography-mass spectrometry, is used to treat NPC patients during or after radiotherapy and those with precancerous lesions in the nasopharynx, and satisfactory outcomes have been described in these clinical trials. In this paper, the inhibitory effect of Chinese Herb Extract was investigated on the infection activity of EBV in a population highly susceptible to NPC.

Methods: 111 cases of a population highly susceptible were randomly divided into two groups, given Chinese Herb Extract (treatment group, TG) and H2O (blank control group, BCG) respectively for 3 months. Serum samples of the cases were tested to evaluate the level changes of EBV-related antibodies IgA/VCA and IgA/EA.

Results: The results showed that, significantly effective rates and total effective rates for TG and BCG were 82.42%, 13.71%, and 67.55%, 5.53% respectively.The level ofEBV-related antibodies IgA/VCA and IgA/EA was decreased in TG after treatment with Chinese Herb Extract.

Conclusions: Chinese Herb Extract is significantly effective to inhibit the infection activity of EBV in cases highly susceptible to NPC, which could inhibit NPC tumorigenesis via inhibiting or eliminating EBV.

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Determination of circulating EBV DNA and anti-EBV IgA in serum of Nasopharyngeal Carcinoma (NPC) patients

Martins L, Banha L, Cunha M, Ornelas C

Contact:
Dr. Luis Martins
Instituto Português Oncologia Francisco Gentil, Centro Regional de Oncologia de Lisboa, SA
Laboratório Virologia (Lisbon, Portugal)
Email: lmartins@ipolisboa.min-saude.pt

Abstract:
Objective: Study of anti-EBV IgA markers detected by ELISA and circulating EBV DNA in serum of patients with NPC.

Methods: The serum of fourteen patients, 6 male and 8 females (mean age of 51years old, range from 24 to 74 years old), with histologically confirmed Nasopharyngeal Carcinoma (NPC), and fifty five healthy donors (included as control and reference group for EBV PCR and serology), were analyzed by Nested PCR (amplification of 54 bp from the DNA polimerase gene, with β-globin as internal control, and detection in agarose gel, 4%) and serological markers, by indirect ELISA: anti-EBV IgA (EA+VCA+EBNA), anti-EBV IgM (EA+VCA+EBNA), anti-EBV IgG (EA+VCA+EBNA), anti-EBV EA-D IgG and anti-EBV EBNA-1 IgG. The anti-EBV IgA (EA+VCA+EBNA), anti-EBV IgG (EA+VCA+EBNA) and anti-EBV IgM (EA+VCA+EBNA) have antigen control.

Results: All patients were EBV IgG and EBV IgA positive, 71,4% were positive for EBV EA-D IgG, 92.85%were positive for EBV EBNA-1 IgG and 71,42% had DNA detected in serum. In the control group, 87.3% were positive for EBV IgG, 41,8% for EBV IgA and 88.2% for EBV EBNA-1 IgG. All the donors were negative for EBV EA-D IgG and circulating DNA.

Concusions: The control group has 41.8% of anti-EBV IgA positive, with a mean optical density (OD) of 1.11, while in the patients group was present in all cases, with a mean OD of 2.16. EBV specific IgG was found in all the study cases and the antibody activity was significantly higher relative to healthy group (1502.5 UI/ml in NPC vs. 148 UI/ml in control group). These two markers (high levels of EBV IgG and high OD of EBV IgA) are good for determining the chronic persistence and/or reactivation of the Epstein-Barr virus. The detection of circulating EBV DNA, in serum, in association with anti-EBV IgA is a good tool for the diagnosis of undifferentiated Nasopharyngeal Carcinoma.

Brushing method for EBV and NPC detection

Dahlia HL, Hariwiyanto B, Haryana SM, Astuti I, Paramita DK, Fachiroh J, Tan IB, Middeldorp JM

Contact:
Dr. Healtho Lifeiantho Dahlia
Faculty of Medicine Gadjah Mada University
ENT (Yogyakarta, Indonesia)
Email: asialinknpc@med.ugm.ac.id, sari_wuland@yahoo.com

Abstract:
Objectives: 1) To know the validity to detect cell malignancy in NPC patients by brushing method. 2) To know the incidence of EBV in NPC by brushing method.

Methods: The specimens are taken using brushing method from all the patients then followed by biopsy method and each specimen is sent to the Pathology Anatomy and Histology department to detect EBV and malignancy.

Results: Brushing method has sensitivity of 86% (CI 95%=0.82-0.90), specificity of 76.5% (CI 95%=0.71-0.81), prediction value positive of 90.2% (CI 95%=0.86-0.94), prediction value negative of 68.4% (CI 95%=0.62-0.74) accuracy of 83.3% (CI 95%=0.78-0.88) likelihood ratio positive of 3.7 and likehood ratio negative of 0.2. EBV can be detected 100% in NPC patients and about 30% in non-NPC patients.

Conclusions: Brushing method is valid for the malignancy detection in NPC patients and EBV.

Native Early Antigen (EA) Extract of Epstein-Barr virus, a Promising Diagnostic Antigen for Nasopharyngeal Carcinoma

Paramita DK, Fachiroh J, Hariwiyanto B, Harijadi A, Artama WT, van Benthem E, Haryana SM, Middeldorp JM

Contact:
Dewi Kartikawati Paramita
Faculty of Medicine Gadjah Mada University
Tumour Diagnostic Laboratory Dept.Histology & Cell Biology (Yogyakarta, Indonesia)
Email: deardesay@yahoo.com

Abstract:
Objective: To develop the simple diagnostic test for NPC using native EA extract.

Methods: A simple procedure using low concentration of NaCl was developed to specifically extract native EA proteins from the nuclear fraction of EA-induced HH514.c16 cells removing cellular and EBNA1 proteins.IgG and IgA ELISA test was done using this extract as coating antigen.

Results: The EA-extract mainly consisted of DNA-binding proteins P47/54 (BMRF1) and p138 (BALF2) and was devoid of EBNA and VCA reactivity as revealed by immunoblot analysis using polyreactive human sera and a large panel of defined EBNA,EA-D and VCA reactive monoclonal antibodies. Serum IgA and IgG antibodies from NPC patients were measured using Enzyme-linked Immunosorbent Assay (ELISA) with EA extract as coating antigen. IgA reactivities in sera from NPC patients (n=121) were significantly higher than with blood donor sera from a regional blood bank (n=126), with a mean OD450 of 1.521 and 0.156 respectively ((p<0.001). IgG-EA responses also revealed significant differences, with mean OD450 of 1.688 and 0.253 respectively (p<0.001). Prior IgG-removal did not affect IgA-EA reactivity, suggesting different epitopes to be involved in IgG and IgA responses (see Fachiroh et al., abstract 13.10). Thus, IgA-EA-D ELISA using native antigen for coating seems promising as primary diagnostic or confirmation assay with a sensitivity and specificity (89.26% and 97.62%). 101/121 NPC sera tested by IgA-EA ELISA, were also tested by immunoblotting for reactivity to the individual baculovirus-expressed EA recombinant proteins such as (TK, DNase, EA-p47 and ZEBRA). High IgA-EA ELISA antibody reactivities correlated with positive response to TK (P=0.0107), EA-p47 (P<0.0001) and ZEBRA (0.0052). This correlation was also seen for IgG-EA ELISA and TK (P=0.0028), EA-p47 (P<0.0001) and ZEBRA (<0.0001).

Conclusions: The virtual absence of IgA antibodies to native EA-D protein epitopes in healthy EBV carriers in a population with high EBV infection rate and high NPC prevalence creates possibilities to further improve EBV-NPC serology.

Evaluation of Mikrogen EBV RecombLine assay for the diagnosis of Nasopharyngeal Carcinoma in Indonesia

Paramita DK, Fachiroh J, Haryana SM, Middeldorp JM

Contact:
Dewi Kartikawati Paramita
Faculty of Medicine Gadjah Mada University
Tumour Diagnostic Laboratory Dept.Histology & Cell Biology (Yogyakarta, Indonesia)
Email: deardesay@yahoo.com

Abstract:
Objective: Measuring IgM, IgG and IgA responses to individual EBV antigen complexes in patients with different EBV diseases, including infectious mononucleosis (IM) and NPC

Methods: The Mikrogen RecombLine assay employs the 5 immunodominant markers including p18 (BFRF3) and p23 (BLRF2) for VCA, p47/54 (BMRF1) and p138 (BALF2) for EAd and p72 (BKRF1) for EBNA, as purified recombinant protein sprayed on strips, allowing a simultaneous analysis of antibody responses to the different major EBV antigens. IgG, IgA antibodies of NPC sera was tested using this methods, compare with normal blood donor as control (IgG & IgA) and IM sera (IgG, IgA, and IgM).

Results: We evaluated the diagnostic value of the RecombLine assay for IgG and IgA reactivity in a panel of 108 histologically confirmed NPC patients and 60 regional blood donors from Yogyakarta, Indonesia and compared the responses to reference sera (n=16) and IM patients (n=10) from the Netherlands. EBV-specific ELISA tests and immunoblot were used for control. Monoclonal antibodies correctly identified the individual bands on the strips.
Virtually all Indonesian individuals had IgG antibodies to VCA (99,6%) and/or EBNA1 (93.8%). IgG to EA was borderline in 15-20% of the controls but significantly more reactive in NPC patients (98.2%), only 2 having a negative response ($p<0.001$). IgA-EA was detected in 87.4% of NPC patients, but had weaker intensity compared to IgG-EA. Only marginal IgA-EA responses were detected in 10 regional controls. In IM patients IgA antibodies to VCA, andEA-D were found in 90% of cases, with lower staining intensities as observed in NPC patients. Overall EA-IgG reactivity proved to be the best marker for discriminating NPC patients from regional controls, showing sensitivity and specificity of 92,6% and 96,8% respectively and yielding a PPV and NPV of 99% and 88%.

Conclusions: The Mikrogen RecombLine assays is a useful method for NPC diagnosis in a country with high NPC incidence.

Profile expression of IL-4, IFN γ, IgA anti - VCA and anti - EBNA of Infectious Mononucleosis and Nasopharyngeal Carcinoma patients

Astuti I, Budiani W, Haryadi A, Hariwiyanto B, Haryana SM

Contact:
Dr. med. Indwiani Astuti
Faculty of Medicine Gadjah Mada University
Medicine (Yogyakarta, Indonesia)
Email: indwiani@yahoo.com, sari_wuland@yahoo.com

Abstract:
Objective: To determine the levels of serum of IgA anti - VCA and anti - EBNA, serum of both IL-4 and IFNγ, and the expressions of IL - 4 and IFNγ m RNA in patients with EBV - associated WHO type III nasopharyngeal carcinoma (NPC) and I nfectious mononucleosis (IM).

Methods: Fortyseven samples consisting of 10 healthty subjects, 5 IM and 32 NPC were included. The levels of serum of IgA anti - VCA and anti - EBNA were determined by ELISA ent (Pan Bio). Serum of IL - 4 and IFN γ levels were assayed by Elisa and plastic blot respectively. The expression of IL - 4 and IFN γ mRNA of peripheral blood lymphocytes (PBL) was determined by card blot.

Results: The results showed that the levels of serum of IgA anti - EBNA were not significantly different among all groups. Higher levels of serum of IgA anti - VCA were seen in patients with NPC as compared to those with IM and healthy subjects. The levels of serum of IFN γ were predominantly observed in patients with NPC, those of patients with IM and healthy subjects were comparable. Decreased levels of serum IL - 4 of patients with IM and NPC as compared to healthy subjects were, however, determined. Interestingly, PBL - derived mRNA IL - 4 and IFN γ expression of all groups were not altered.

Conclusions: The results suggest, therefore that increased levels of serum IgA anti - VCA and IFN γ may be related with a lytic infection of EBV - associated NPC and may indicate relapse of the disease.

EBNA-1 immunohistochemistry staining to replace EBER 1,2 RISH in paraffin embedded tissue of nasopharyngeal carcinoma patients

Harijadi A, Murtono C, Hariwiyanto B, Haryana SM, Middeldorp JM

Contact:
Prof. Dr. Jaap M Middeldorp
VU Medical Center
Pathology (Amsterdam, The Netherlands)
Email: j.middeldorp@vumc.nl, sari_wuland@yahoo.com

Abstract:
Background: Epstein-Barr virus (EBV) encoded small non-coding RNAs EBER1 and EBER2 are abundantly expressed in all forms of EBV-latency in addition to the essential Epstein-Barr nuclear antigen-1 (EBNA1) protein, which is required for EBV genome maintenance in dividing cells. LMP1 is expressed in malignancies with EBV latency-II gene expression. EBER1,2 RISH is the gold standard method for defining the presence of EBV in EBV-related malignancies such as Hodgkin's lymphoma and nasopharyngeal carcinoma. However, several difficulties are associated with EBER1,2 RISH which make it less suitable for use in tropical environments, such as RNA instability. In addition the method is laborious and expensive. Immunohistochemical detection of EBV proteins EBNA1 and LMP1 using specific monoclonal antibodies may be more suited.

Aim: To study the diagnostic value of monoclonal antibody mediated immunohisto-chemistry staining for EBNA1 & LMP1 in comparison with EBER1,2 RISH in paraffin embedded tissue of NPC biopsies.

Methods: 87 NPC biopsies were stained immnunohistochemically using monoclonal antibodies anti-EBNA1 (OT1x) and LMP-1 (CS1-4, S12 and OT21C) combined with standard indirect biotinABC-HRP detection, and with the PNA-based EBER1,2 RISH kit from DAKO. Two cases of adenocarcinoma of mucous gland in the nasopharynx were used as negative control.

Results: Although the expression of EBNA1 revealed clustered and diffuse expression patterns in the nucleus of NPC tumour cells, a positive reaction for EBNA1 staining is correlated with positivity of EBER 1,2 RISH method ($p < 0.001$, rho 0.541). Two cases of adenocarcinoma were negative in both methods. LMP1 was detected in 50-70% of the EBNA1/EBER1,2 positive NPC cases, depending on the antibody used and revealed different staining patters, defined as coarse and diffuse.

Conclusion: Staining for EBNA1 by OT1x-immunohistochemistry can reliably replace EBER1,2 RISH method for detection of EBV infection in NPC tumour cells. Only part of NPC tumours express LMP1, which seems to have a variable expression pattern.

EBV immune response in first-degree relatives in families with a high risk of nasopharyngeal carcinoma

Friborg J, Jarrett R, Falk K, Koch A, Olsen OR, Melbye M

Contact:
Dr. Jeppe Friborg
Statens Serum Institut
Department of Epidemiology Research (Copenhagen, Denmark)
Email: jfr@ssi.dk

Abstract:
Objective: First-degree relatives of Inuit patients with undifferentiated nasopharyngeal carcinoma (NPC) have a 7-9 times increased risk of developing NPC and the similarly Epstein-Barr virus (EBV)-associated salivary gland carcinoma. Whether this increased risk is accompanied by changes in the EBV immune response is largely unknown, and we therefore performed a case-control study in Greenland measuring EBV viral load and serology in first-degree relatives in NPC/SGC high-risk families.

Methods: In the spring of 2003, 19 unaffected first-degree relatives of Greenlandic NPC/SGC families with at least two NPC or SGC cases among first-degree relatives, and 90 age- and sex-matched controls from families without NPC/SGC cases participated in the study. Peripheral blood mononuclear cells (PBMCs), plasma and saliva samples from those these 109 individuals were collected and EBV viral load was determined. In addition, anti-VCA IgG and IgA, anti-EBNA1 IgG, anti-EBNA2 IgG and anti-EA IgG titers were determined in plasma.

Results: Median EBV copy numbers in PBMCs did not differ between relatives (5.6/ul DNA) and controls (6.5/ul DNA), just as there was no difference between EBV viral load in plasma and saliva. All 109 individuals were anti-VCA IgG-positive, and EBV antibody titres were also equal except for significantly higher titres of anti-EBNA1 IgG in relatives. Seroprevalence of IgA antibodies to VCA was high, but equal among relatives (47.4%) and controls (53.4%). There was no significant difference in PBMC and saliva EBV load between individuals who were anti-VCA IgA seropositive with high titers, moderate titers and anti-VCA IgA seronegative.

Conclusions: The results indicate that the EBV-specific immune response in first-degree relatives in NPC-high risk families is similar to the response in individuals in non-NPC-affected families. Furthermore, that EBV viral load in lymphocytes and saliva is independent of anti-VCA IgA positivity.

Clonal analysis of Burkitt's Lymphoma cell lines carrying EBNA2-deleted and wild type Epstein-Barr virus genomes

Hutchings IA, Kelly GL, Bell A, Rickinson AB

Contact:
Dr. Isabel Hutchings
University of Birmingham
Cancer Research UK Institute for Cancer Studies (Birmingham, UK)
Email: Isabelh@cancer.bham.ac.uk

Abstract:
Objectives: We previously identified three cases of endemic Burkitts lymphoma (SAL-, OKU- and AVA-BL) with a novel form of virus latency in which EBNAs 1, 3A, 3B, 3C and a truncated EBNA-LP are expressed from the Wp promoter in the absence of EBNA2 and LMP1 transcription. These 'Wp-restricted' tumours all carried a wild-type transformation-competent EBV genome, which could be rescued into a lymphoblastoid cell line (LCL) in vitro, and an EBNA2-deleted genome. Here we used single cell cloning to analyse the EBV genome content of individual tumour cells in relation to gene expression.

Methods: Virus genome content was determined using two quantitative PCR assays specific for the EBNA2 gene and for a conserved lytic cycle gene respectively; virus strain identity was analysed by sequencing at polymorphic EBNA1 and LMP1 loci, and Wp/Cp methylation status by bisulphite sequencing at the Wp/Cp loci. Latency was characterised by quantitative RT-PCR for latent cycle transcripts and by Western blotting for latent antigens.

Results: For all three tumours, we established two types of single cell clone from early passage parental BL cell lines. Some clones carried both the wild-type and the EBNA2-deleted EBV genomes, in different proportions relative to one another, and other clones carried multiple copies of the EBNA2-deleted genome alone; we could never rescue the resident wild-type genome in BL cells in the absence of the EBNA2-deletant. Comparing sequences of the EBNA2-deleted genome isolated in BL clones and of the wild-type genome isolated in LCLs showed that they were derived from the same virus strain. All the clones resembled the original parent tumour in growth characteristics and germinal centre phenotype, and displayed the original 'Wp-restricted' pattern of viral transcription, in some cases with varying levels of Cp activity. At the protein level, all clones were EBNA1+, EBNA2-, LMP1- but varied in EBNA3A, 3B, 3C and truncated EBNA-LP status. Bisulphite sequence analysis on selected clones suggested that Wp/Cp activity is not always associated with demethylation at these promoters.

Conclusions: The data support the view that only the EBNA2-deleted genome is transcriptionally active in 'Wp-restricted' BL. The wild-type genome, from which the EBNA2-deletant has been derived, is only detectable in a subset of cells and appears to be transcriptionally silent.

EBV induces co-expression of CD40 and CD40L for host cell survival and transformation
Shirakata M, Imadome K

Contact:
Dr. Masaki Shirakata
Medical Research Institute
Tokyo Medical and Dental University (Tokyo, Japan)
Email: shirakata.creg@mri.tmd.ac.jp

Abstract:
CD40 is a TNFR-family of membrane receptor that plays important roles in antiapoptosis, growth and differentiation of B lymphocytes, natural host of latent infection of EBV. However, significance of CD40 in EBV infection is not fully recognized because it requires the specific ligand CD40L for activation, which is normally expressed only on activated T cells, and moreover, EBV expresses the latent membrane protein 1 (LMP1) that mimics a constitutively activated CD40. In this study, we show that EBV induces ectopic expression of CD40L in B cells and it has an essential role in antiapoptosis and transformation of infected cells. We detected CD40L expression by RT-PCR and FACS analyses in EBV-positive Burkitt's lymphoma lines, LCLs and the EBV-infected peripheral B cells. Expression of both CD40 and CD40L suggested that auto-activation via CD40 may occur in EBV-infected B cells. We blocked EBV-induced CD40L by addition of a CD40-Ig fusion protein in culture media and found that it resulted in loss of antiapoptotic ability of EBV-infected peripheral B cells and the reduced formation of LCL colonies. Importance of CD40L in EBV-infected B cells was further confirmed in the experiments using peripheral B cells from XHIM patients who do not express functional CD40L by genetic mutations. These results indicated that EBV-induced CD40L plays an important role in antiapoptosis and transformation. Interestingly EBV-positive CAEBV T cells lines and EBV-infected Jurkat T cells express CD40. EBV may also induce auto-activation of T cells. Our results also suggest that LMP1 is not sufficient for antiapoptosis and transformation of B cells because EBV-infected cells used in these experiments express LMP1. It has been known that both LMP1 and CD40 activate signal cascades leading to NF-kappa B, ERK, JNK, p38 MAPK and AKT through signal mediator TRAF, but there may be some differences in their down-stream signaling. It is important to identify CD40-specific signals for understanding collaboration between LMP1 and CD40 in viral infection.

Downregulation of a polyamine regulator, spermidine/spermine N1-acetyltransferase (SSAT), is associated with the EBV-induced tumor phenotype

Scott RS, Shi M, Davis T, Gan YJ, Su T, Sixbey JW

Contact:
Dr. Rona Scott
Louisiana State University Health Sciences Center-Shreveport
Microbiology and Immunology
Center for Molecular and Tumor Virology (Shreveport, USA)
Email: rscott1@lsuhsc.edu

Abstract:
In the Burkitt's lymphoma (BL) line, Akata, maintenance of the malignant phenotype is dependent on the presence of the viral genome despite the presence of the hallmark c-myc chromosomal translocation. To identify the contribution of EBV to this malignant phenotype, we examined changes in global cellular gene expression in paired EBV-positive and negative BL subclones cured of virus by treatment with hydroxyurea. In five independent experiments using Affymetrix DNA microarrays, spermidine/spermine N1-acetyltransferase (SSAT) was one of a limited number of genes differentially expressed, being 3-fold lower in EBV-positive BL than in the matched EBV-negative counterpart. Results were confirmed by real-time quantitative PCR demonstrating comparable SSAT mRNA down-regulation in multiple EBV-positive BL clones. SSAT is an inducible acetylating enzyme that is involved in the catabolism of polyamines, spermidine and spermine, and increased levels of these polyamines have been shown to facilitate cell growth and differentiation. Biochemical analyses confirmed decreased SSAT enzyme activity and increased polyamine levels in the EBV-positive BL clones compared to the EBV-negative BL counterparts. To determine if EBV infection directly affected polyamine catabolism during transformation of primary B-lymphocytes, we quantified SSAT mRNA in peripheral blood lymphocytes (PBLs) at multiple times after infection. Compared to the uninfected PBLs, there was a 30-fold decrease in SSAT mRNA in proliferating, EBV-transformed B cells. Re-infection of an EBV-negative BL clone with a neomycin recombinant Akata virus demonstrated down-regulation of SSAT after selection equivalent to that seen in the EBV-positive BL. These results suggest that EBV repression of SSAT and disruption of polyamine catabolism increases polyamine levels that may contribute to EBV-induced lymphoproliferation.

The EBERs: Small but Influential?

Repellin CE, Wilson JB

Contact:
Claire Repellin
University of Glasgow, IBLS
Molecular Genetics (Glasgow, UK)
Email: 0109261r@student.gla.ac.uk

Abstract:
Objective: The EBERs confer resistance to interferon (IFN)-a-induced apoptosis via binding of the IFN-inducible, double-stranded (ds) RNA-activated protein kinase PKR and inhibition of its activation by phosphorylation (Nanbo et al., Embo J, 2002, 21, 954-65). Recent evidence has suggested an oncogenic role of the EBERs in Burkitt's lymphoma (BL) cells, indicating their possible contribution to the disease process of EBV-associated tumours (Komano et al., J. Virol., 1999, 73, 9827-9831).
In order to investigate the role of the EBERs in the disease process of EBV, we have generated several lines of transgenic mice designed to express EBER1 in B-cells using three variant transgenes. The transgenes incorporate a novel combination of tissue-specific, RNA pol II and pol III elements.

Results: The efficacy of transgene expression has been confirmed in culture. To date, the first line (line 127) has been shown to express EBER1 specifically in lymphoid tissues. Further lines are currently under test. Mice of line EBER1.127 are viable, with the phenotypic consequences of EBER1 expression in vivo under examination. Preliminary FACS analysis has revealed differences between wild-type and EBER1 transgenic mice, especially in Peyer's patches, which is the highest expressing tissue of the line.
Several cross-breeding programmes are underway between EuEBER1 and EuEBNA1 and EuEBER1 and Eumyc transgenic mice. Tumour incidence will be monitored in order to determine if there is any cooperation between these genes in tumour onset or pathology. A triple cross-breed EuEBER1, EuEBNA1 and Eumyc is planned in order to generate an in vivo model of BL with the aim of providing further insights into the molecular mechanisms of the disease.

Comparison of EBV-specific IFN-γ responses in children with Burkitt's lymphoma to healthy children living in malaria holoendemic and sporadic regions of Kenya.

Moormann AM, Chelimo K, Tisch DJ, Sumba OP, Kazura JW, Rochford R

Contact:
Dr. Ann Moormann
Case Western Reserve University
Center for Global Health & Diseases (Cleveland, USA)
Email: moorms@case.edu

Abstract:
Objective: An association between holoendemic malaria (intense and stable transmission), EBV infection and endemic Burkitt's lymphoma (BL) is well established in Africa and Papua New Guinea. Early studies using regression assays to assess EBV-specific T cell function suggested that immune control of EBV is impaired in those living in malaria holoendemic regions.

Methods: In this study, we compared IFN-γ responses to EBV peptides in a cross-section of healthy children (age 1-14) living in two regions of Kenya with differing malaria transmission intensities: Kisumu District where malaria is holoendemic (n=105), and Nandi District where malaria transmission is sporadic (n=128). In addition, EBV-specific IFN-γ responses were also measured in children diagnosed with BL (n=93) prior to treatment at Nyanza Provincial Hospital in Kisumu, Kenya. Peripheral blood lymphocytes were cultured with HLA Class I-restricted peptide epitopes for EBV lytic and latent antigens and IFN-γ response was measured by ELISPOT assay. Only EBV-seropositive subjects were analyzed.

Results: Overall, no significant difference was observed in the frequency of responses to either lytic or latent epitopes between study groups. Because the peak age of BL is 6-7 years, we next categorized the children into three age groups (1-4 yrs, 5-9 yrs, and 10-14 yrs). The proportion of responders to EBV latent and lytic epitopes was significantly lower in children ages 5-9 living in the malaria holoendemic area compared to children from the region with sporadic malaria transmission ($p < 0.04$) and to BL patients who were mostly from the malaria holoendemic region ($p < 0.02$). In all populations, robust IFN-γ responses were observed to PHA, a common mitogenic stimuli, demonstrating that the reduced number of responders in the children from the malaria holendemic was antigen-specific.

Conclusions: Our data suggests that chronic and repeated malaria infections has immunopathologic consequences on EBV immunity and sets the stage for the emergence of BL. However, children presenting with BL have regained their EBV-specific responses suggesting a transient nature to the putative malaria-induced immunosuppression.

Does Epstein-Barr virus co-infection influence the outcome of Plasmodium falciparum malaria in African children?

Yone CLRP, Kube D, Kremsner PG, Luty AJF

Contact:
Clarisse L. R. P. Yone
Institute of Tropical Medicine
Human Parasitology (Tübingen, Germany)
Email: yonecla@yahoo.com

Abstract:
Objective: While Plasmodium falciparum malaria is thought to be a co-factor for development of Burkitt's lymphoma, little is known about the potential influence of Epstein-Barr virus (EBV) infection on malaria. We investigated the influence of EBV co-infection on the humoral response to P. falciparum malaria.

Methods: 60 Gabonese children, half of whom presented with mild, the other half with severe P. falciparum malaria, and matched for age, gender and provenance, were included in a longitudinal study. Whole blood samples were collected at admission to the hospital (acute phase), one month (convalescent phase), and at least 6 months (healthy phase) post-admission. We determined antibody responses to various P. falciparum asexual stage antigens. Then, we monitored circulating EBV-DNA in the same children by DNA-based real-time PCR using EBV-polymerase as the target.

Results: Circulating EBV-DNA decreased significantly in the mild malaria group ($P<0.020$), but persisted in the severe malaria group, with a significant difference between the groups when they were healthy ($P = 0.040$). Anti-VSA IgG4, anti- MSP-119 and anti-AMA-1 IgG responses were higher in EBV-negative children, and inverse associations were evident between the level of circulating EBV-DNA and anti-VSA IgG4 ($P = 0.009$), age ($P = 0.008$) and the time to first P. falciparum reinfection ($P = 0.010$).

Conclusions: The data suggest impaired control of EBV in children with severe malaria subsequent to an acute malaria episode. The impact of EBV co-infection on P. falciparum malaria epidemiology and immunity will be discussed.

Production of high-titer EBV recombinants derived from Akata cells by using a bacterial artificial chromosome system

Kanda T, Yajima M, Ahsan N, Tanaka M, Takada K

Contact:
Dr. Teru Kanda
Institute for Genetic Medicine, Hokkaido University
Center for Virus Vector Development (Sapporo, Japan)
Email: tkanda@igm.hokudai.ac.jp

Abstract:
Objective: We recently cloned the genome of Akata strain EBV (Burkitt's lymphoma-derived) as a bacterial artificial chromosome (BAC) clone (designated as AK-BAC), and demonstrated that transgenes could be inserted into AK-BAC by means of efficient homologous recombination method (called GET recombination) in E. coli. We now report a strategy to produce pure recombinant viruses in large quantities using Akata cell system.

Methods: Akata cells (EBV-negative, EBV-positive) were transfected with the purified DNAs of the AK-BAC-GFP (a BAC clone having Akata EBV genome with an inserted GFP transgene) to obtain G418-resistant cell clones harboring BAC clones. Episome formation efficiencies were examined by rescuing the BAC clones from the G418-resistant cell clones.

Results: We found that EBV-positive Akata cells were far more competent to incorporate transfected BACmids as intact episomes compared to EBV-negative Akata cells. Accordingly, we used EBV-positive Akata cells as recipient cells and derived a strategy to obtain cell clones harboring only AK-BAC-GFP episomes. The obtained cell clones could produce highly-transforming AK-BAC-GFP virus in response to anti-IgG treatment, and lymphoblastoid cell lines expressing GFP were readily established. We further facilitated the process of transgene insertion by generating a derivative of AK-BAC (designated AK-BAC-IPpoI), which contained a unique I-PpoI site just downstream of the SV40 enhancer promoter. The cDNA of tumor-associated antigen Muc1 was successfully inserted into AK-BAC-IPpoI by in vitro ligation. Infecting peripheral lymphocytes with the recombinant EBV resulted in establishing lymphoblastoid cell lines that were 100% Muc1-positive.

Conclusions: The results clearly demonstrate that AK-BAC system is a powerful tool to produce large quantities of pure recombinant EBVs. The system exhibits unprecedented flexibilities and should also be useful for various genetic analyses of EBV.

Integration of Epstein-Barr virus into chromosome 6q15 of Burkitt lymphoma cell line (Raji) induce loss of BACH2 expression

Takakuwa T, Luo WJ, Ham MF, Ishikawa E, Wada N, Aozasa K

Contact:
Prof. Tetsuya Takakuwa
Osaka University Graduate School of Medicine
Department of Pathology (Osaka, Japan)
Email: takakuwa@molpath.med.osaka-u.ac.jp

Abstract:
Epstein-Barr virus (EBV) initially isolated from cultured Burkitt lymphoma (BL) cells, is one of well-known oncogenic virus. The Raji cell line was established from BL tissue and used for research worldwide. Previous study showed that each Raji cell contains an average of 50-60 EBV genome equivalents, and a significant proportion of the EBV genome is linearly integrated into host genome through BamHI-W close to the BamHI-Y fragment. However, a definitive EBV integration site in the chromosome has not been identified as yet. In this study, direct evidence that EBV DNA is integrated into the host genome was provided through cloning of the fragments containing nucleotide sequence of Raji integration sites. Integrated EBV DNA consisted of the BamHI-W fragment at one end and BamHI-D fragment at another end. Both junction sites were highly G/C-rich. The BamHI-W fragment and the adjacent part of chromosome 6 showed 70% homology, while no homology was found between the BamHI-D and adjacent host sequences. EBV is present at intron 1 of the BACH2 gene which is located on chromosome 6q15. BACH2 mRNA was not expressed in the Raji cell line. Immunohistochemistry confirmed the loss of BACH2 expression at protein level. Because BACH2 is a putative tumor suppressor gene, loss of its expression through EBV integration might contribute to lymphomagenesis. Present findings might indicate a new paradigm for EBV oncogenesis.

An EBNA2-positive, LMP1-negative form of latency in Burkitt's Lymphoma cells

Kelly GL, Bell A, Rickinson AB

Contact:
Dr. Gemma Kelly
University of Birmingham
Cancer Research UK Institute for Cancer Studies (Birmingham, UK)
Email: gemmak@cancer.bham.ac.uk

Abstract:
Objectives: Epstein-Barr virus (EBV)-positive Burkitts Lymphoma (BL) cell lines provide important in vitro models in which to study restricted forms of virus latency. Here we study an unusual tumour, AWIA-BL, that contains cells with an EBNA2-positive, LMP1-negative pattern of antigen expression similar to that observed by antibody staining within EBV-infected tonsillar B cell populations in vivo.

Methods: EBV genome content and gene expression in the parental line and in derived clones were analysed by standard methods (see Hutchings et al., abstract 07.01).

Results: The AWIA-BL tumour carried both wild type and EBNA2-deleted genomes, as did recently described cases of 'Wp-restricted' BL, but was distinct in that EBNA2+, LMP1- cells were detectable both in the original biopsy and in the early passage BL cell line. Limiting dilution of the AWIA-BL line yielded three types of clones, all with the typical BL cell phenotype and growth characteristics (i) clones carrying both genomes or the EBNA2-deletion mutant virus only and showing classical 'Wp-restricted' latency with expression of EBNAs 1, 3A, 3B, 3C and truncated EBNA-LP in the absence of EBNA2 and LMPs, (ii) clones carrying only the wild-type genome and displaying classical Latency I infection with Qp-driven expression of EBNA1, and (iii) clones carrying both genomes or the wild type genome only and expressing the full complement of six EBNAs in the absence of detectable LMPs; the intensity of EBNA2 staining at the single cell level varies between clones but in some cases is equal to that seen in lymphoblastoid cell lines transformed with the wild type AWIA virus strain. Interestingly, in these unusual EBNA2+, LMP1- clones, EBNA transcription appears to be initiating from an unidentified site other than the Wp or Cp promoters.

Conclusions: We have established cell clones from an endemic BL which retain cellular and cytogenetic markers characteristic of this tumour but which display an unusual EBNA2+, LMP1- form of latency. These cells provide an opportunity to re-examine relationships between viral gene expression and the c-myc driven cellular phenotype in BL cells and may also provide an in vitro model of a hitherto poorly understood form of in vivo infection.

Dissecting the role of EBNA 1 oncogenic potential in EmEBNA 1 transgenic mice
AL-Sheikh YA, Wilson JB

Contact:
Yazeed Al-Shaikh
University of Glasgow
Molecular Genetics (Glasgow, UK)
Email: yazeedsa@yahoo.com

Abstract:
Epstein Barr Virus Nuclear Antigen 1 is the only viral protein expressed in all latent EBV associated tumours. EBNA-1 oncogenic potential has been suggested by the expression of an EmEBNA-1 transgene in C57/B6 mice. Mouse lines expressing the transgene succumb to monoclonal B-cell lymphoma (lines 26 and 59). These lines display very different latency periods and penetrance of tumour onset. 100% of line 26 mice succumb to tumour within 4 to 12 weeks where approximately 50% of mice in line 59 develop lymphoma by 2 years. In addition a sub line arose spontaneously from line 26 (line 26A) which had partially lost the transgene and with that the tumour phenotype.
We are exploring several avenues to dissect the role of EBNA-1 in leading to lymphoma and to characterise the tumour phenotype in these mouse lines. In order to examine any possible contribution to the phenotype from rearrangement at the transgene integration site, one aim has been to clone and examine these sequences. Cloning transgene repeat units and adjacent rearranged cellular sequence is notoriously complicated. As such a variety of means haven been used, including phage cloning, inverse PCR, cDNA cloning and more. In lines 59 the transgene has integrated into the D3 region of chromosome 4. Analysis of cellular genes at this integration site does not suggest a role of the integration process in the tumour phenotype with closest predicted gene being the Laptm 5 gene located 36 kb downstream to the site of integration. In the sub line 26A adjacent genomic sequence are derived from chromosome 3 band H2, with the closest predicted gene being the Rap1GDS1 located 1.2Mb upstream to the integration site. Cloning the transgene integration site in line 26 is proving recalcitrant but is currently under way.
Second, using Florescent Activated Cell Sorting (FACS) analysis to characterise the surface markers expressed on EBNA-1 tumour cells. We have found that most tumour B-cells express markers indicating an immature state. Third, selected cellular gene expression levels have been examined in these tumours to explore which contribute to the tumourigenic process in these mice. Finally, using Electro mobility shift assays (EMSAs) we present preliminary data that suggest c-myc enhances EBNA-1 binding to oriP sequences in EmEBNA-1 x Emc-myc mouse tumours.

Exposure to holoendemic malaria results in elevated Epstein-Barr viral loads in children
Rochford R, Sumba OP, Ploutz-Snyder R, Lutzke ML, Kazura J, Moormann AM

Contact:
Dr. Rosemary Rochford
SUNY Upstate Medical University
Microbiology and Immunology (Syracuse, USA)
Email: rochforr@upstate.edu

Abstract:
Endemic Burkitt's lymphoma (eBL) is the most common childhood malignancy in tropical Africa and occurs primarily in children between 2 and 15 years of age with a peak incidence in children ages 5-9 years. Epstein-Barr virus (EBV) infection and sustained and intense exposure to Plasmodium falciparum malaria (holoendemic malaria) are co-factors in the development of BL but the mechanisms by which these two pathogens interact remain unknown. To examine this question, we have compared EBV persistence in children living in two regions in Kenya with differing malaria transmission patterns: Kisumu district where malaria transmission is holoendemic and parasite prevalence in children is high and Nandi district where malaria transmission is sporadic and parasite prevalence in children is low. For comparison, blood samples were also obtained from US adults, Kenyan adults, and eBL patients. We observed a significant linear trend in mean viral load with the lowest viral load detected in US adults, and increasing viral loads in Kenyan adults, Nandi children, Kisumu children and eBL patients, respectively. Because malaria morbidity is age- dependent and malaria immunity is gradually acquired, we wanted to determine if there was an age-related difference in viral load between children living in Kisumu versus those living in Nandi. We categorized the children into three age groups (1-4 yrs, 5-9 yrs, and 10-14 yrs) then submitted their viral load data to a two-factor ANOVA, crossing age category and region (Nandi vs. Kisumu). There was a significantly higher viral load in Kisumu children age 1-4 years compared to the same age children living in Nandi. We are currently determining whether elevated viral loads are due to increases in the numbers of peripheral B cells in children living in holoendemic regions. Our results support the hypothesis that chronic malaria infections in very young children modulate EBV persistence and set the stage for the development of eBL.

A pilot study on African children with Burkitt's lymphoma: adjuvant therapy for non-responsive tumours

Griffin BE, Kazembe P, Wansambo C, Magwira C, Phillips J

Contact:
Prof. Dr. Beverly E. Griffin
Imperial College, Faculty of Medicine at St. Mary's
Viral Oncology Unit (London, UK)
Email: b.griffin@imperial.ac.uk

Abstract:
Objective: To develop a simple alternative for treating the app.50% African children with Burkitt's lymphoma (BL) that fail to respond to classical (cyclophosphamide monotherapy) chemotherapy protocols.

Methods: Adjuvant therapy, giving sodium phenylbutyrate (Buphenyl) to BL patients who have received several doses of cyclophosphamide, with no response, at Buphenyl doses approved for administration to children with urea-cycle disorders.

Results: In the pilot study carried out to date on two eligible Malawian BL patients, using the so-called 'orphan drug', Buphenyl, no toxic side effects have been observed. Their tumours are currently responding to the treatment.

Conclusions: The rationale behind this programme is to stimulate the expression of lytic cycle EBV genes, which may promote tumour response either through oncolysis or stimulation of surveillance by the host immune system, or both. The study has ethical approval by the relevant Malawian health and safety committee. Both patients entered to date into the trial are alive and well, and their tumours show signs of regression. The aim, if early studies - including enrolling several more patients - continue promising, is to establish a full-scale phase I trial.

EBV persistence in vivo - closing the circle.
Thorley-Lawson DA

Contact:
Prof. Dr. David A. Thorley-Lawson
Tufts University School of Medicine
Dept. of Pathology, Jaharis Building Boston (Boston, USA)
Email: David.Thorley-Lawson@tufts.edu

Abstract:
We have previously published a model of EBV persistence based on the observation that EBV is found restricted to resting memory B cells in the peripheral blood (Thorley-Lawson, D. A. Epstein-Barr virus: exploiting the immune system. Nat Rev Immunol 1, 75-82).The essence of this model is that EBV uses different transcription programs to regulate the behavior of latently infected B cells as they move through the differentiation process to ultimately end up as latently infected resting memory B cells and that this is the site of long term persistence. In this presentation the status of the model will be updated together with a discussion on the possible origins of EBV associated lymphoma (Thorley-Lawson, D. A., and Gross, A. (2004). Persistence of the Epstein-Barr virus and the origins of associated lymphomas. N Engl J Med 350, 1328-1337).

Non proliferating EBV-B lymphocyte interactions

Klein E, Kis LL, Liu A, Nagy N

Contact:
Prof. Eva Klein
Karolinska Institutet
Microbiology and Tumor Biology Center (Stockholm, Sweden)
Email: Eva.Klein@mtc.ki.se

Abstract:
Four EBV encoded protein expression patterns occuring in B lymphocytes can be detected in lymphoid tissues of IM patients. Among these, only one, Type III, induces proliferation in vitro without the contribution of additional factors. Burkitt Lymphoma exhibits the Type I program; only EBNA-1. These cells are driven for proliferation by the Ig/myc translocation. In cells of the B lineage 2 further patterns, type IIa and type IIb, express virally encoded proteins in different combinations. The characteristic H/RS cells carry EBV in about 50% of the cases. These cells failed in their regular differentiation and would have normally succumbed to apoptosis. When EBV positive, they are type IIa: EBNA-1, LMP-1 and -2. It is likely that the expression of LMP-1 is regulated by signals from the microenvironment. In spite of several attempts EBV carrying H/RS lines were not established.
The Type IIb pattern (all EBNAs but no LMP-1) has received little attention until now. It is exhibited by B-CLL cells infected with EBV in vitro. The morphology of the infected cells remains unchanged, the cells do not enter DNA synthesis and they do not elicit a T cell response. Even when the infected cells were activated by exposure to CD40L and entered the cell cycle, they did not express LMP-1 and did not proliferate. From very rare CLL clones immortalised lines could be obtained. We analysed one such line established by us (its origin proven by cytogenetical marker), it had the Type III pattern and except for the CD5 and CD14 CLL markers its general gene expression did not differ from a normal B cell derived LCLs. An EBV positive subclone was detected in one CLL clone, these cells grew in vitro (became Type III), but remained silent in the patient for several years.
It seems therefore that among the various types of viral expression strategies in B lymphocytes only Type III leads to proliferation without contribution of other factors and represents a threat as malignancy. However such cells are strongly immunogenic and are kept under control unless the immune system is impaired. In the healthy EBV carrier individual the normal B cells are Type I. What is the fate of the Type IIa and Type IIb cells that exist in IM? Under the influence of external signals Type IIa cells may give rise to EBV positive HD. It is likely that the Type IIb cells undergo apoptosis.

EBV Infection Induces the Expression of specific Splice Variants of IRF-5

Martin H, Kwon HC, Dong SM, Park JH, Hayward SD, Lee JM

Contact:
Heather Martin
Johns Hopkins University
Pharmacology and Molecular Sciences (Baltimore, USA)
Email: hmartin4@jhmi.edu

Abstract:
Interferon Regulatory Factors (IRFs) participate in the downstream regulation of interferon stimulated genes following interferon signaling. Many members of this family of nine transcription factors have roles not only in anti-viral defense, but also in cell cycle arrest and apoptotic pathways. Loss of IRF function can lead to cell proliferation and oncogenesis. Many viruses intercept the interferon pathway, but some viruses also use IRFs for their own purposes. One example of this is the relationship between IRF-7 and LMP1.
To evaluate potential interactions between EBV and the interferon system, we performed in vitro EBV infection time courses and RT-PCR analyses of IRF expression. EBV induced the expression of specific splice variants of IRF-5 during early infection of Ramos cells, a BL, EBV negative cell line. Infection in Ramos cells also induced expression of a novel putative dominant negative form of IRF-5. Immunofluoresence analyses indicate possible different functions of these isoforms based on their varied locations in the cell. Different isoforms of IRF-5 were expressed in PBMC when compared to LCLs and it was found that B cell lines express dramatically different levels of IRF-5, as well as different isoforms. Levels of expression correlated with methylation of the CpG-island promoter sequence of IRF-5 and treatment with the DNA methylation inhibitor 5-aza-2-deoxy cytidine reactivated the expression of IRF-5. All latency III, EBV positive cell lines had high levels of IRF-5 expression, while most EBV negative or latency I cell lines had low levels of expression. Preliminary experiments indicate an interaction between EBNA2 and IRF-5.
In conclusion, EBV infection influences the IRF-5 isoforms being expressed. High levels of IRF-5 in latency III B cell lines suggest that IRF-5 may play a role in EBV mediated B cell proliferation.

Cell cycle-associated chromatin modifications regulate DNA replication at OriP
Zhou J, Chau CM, Deng Z, Shiekhattar R, Lieberman PM

Contact:
Dr. Paul Lieberman
The Wistar Institute (Philadelphia, USA)
Email: lieberman@wistar.upenn.edu

Abstract:
Objective: The Epstein-Barr virus origin of plasmid replication (OriP) uses the cellular licensing machinery, including ORC and the MCM complex, to regulate replication during latent infection of human cells. Selection and licensing of viral and cellular DNA replication origins may be regulated by epigenetic changes in chromatin structure. We sought to determine whether changes in chromatin structure or histone modification regulates OriP-dependent DNA replication.

Methods: We examined the chromatin structure and histone modifications at the DS element of OriP. Chromatin structure was analyzed by micrococcal nuclease digestion assays and indirect end-labeling or primer extension assay of mononucleosomes. Histone modifications were determined by chromatin immunoprecipitation. Cells were arrested at various stages of the cell cycle to monitor changes in histone modifications and chromatin structure. siRNA was also used to verify the role of ATP-dependent chromatin remodeling factors in OriP-dependent DNA replication.

Results: We show that nucleosomes are positioned at the boundaries of the minimal replicator sequence of OriP, referred to as the dyad symmetry (DS). These nucleosomes were subject to cell cycle- dependent chromatin remodeling that coincided with histone deacetylation and MCM3 loading during G1. The chromatin remodeling factor SNF2h and histone deacetylase HDAC2 physically and functionally associated with DS in G1 arrested cells. We also found that constitutively high histone H3 K4 methylation at DS was important for OriP replication and binding to SNF2h and MCM3.

Conclusions: We conclude that an SNF2h-HDAC1/2 complex coordinates cell cycle-regulated chromatin remodeling and histone deacetylation required for DNA replication of chromosomal origins like OriP.

The EBNA1-dependent promoter-enhancer element FR in oriP is regulated by oct-proteins and their co-factors: implications for a viral switch

Almqvist J, Zou J, Ernberg I

Contact:
Jenny Almqvist
Karolinska Institute
Microbiology and Tumourbiology Center (Stockholm, Sweden)
Email: jenny.almqvist@mtc.ki.se

Abstract:
Objective: The activity of different viral promoters determines which of several EBV latency programs will be expressed. In latency III, C promoter (Cp) transcription leads to expression of all nine latency proteins resulting in a proliferating phenotype. Q promoter (Qp) transcription (latency I) results in selective EBNA1 transcription and a resting phenotype. Indirect evidence suggests that a switch between Cp- and Qp-promoter driven transcription can occur. We have addressed how cellular proteins can interact with EBNA1 to regulate these promoters.

Methods: A FR-repeat from oriP was used as a probe in an EMSA ("band shift"), to identify FR-binding proteins in extracts from latency I or III cells. By base substitutions in the probe the binding sites were mapped in detail. A luciferase reporter vector containing FR upstream of a heterologous promoter was used for assaying FR-driven transcription. Proteins in different cell types were detected by Western blotting.

Results: Nine complexes were identified to interact specifically with FR by EMSA; one was composed of EBNA 1, while two of the other complexes were due to the binding of the cellular transcription factors Oct-1 and Oct-2, respectively. Moreover, Oct-1 and Oct-2 were shown to have an additive effect when co-transfected with EBNA1 compared to the activity of the individual proteins. Finally, we have recently discovered one cellular protein that can repress EBNA1- FR-transcription in the presence of Oct-proteins. Interestingly we have detected a dramatic difference in the amounts of endogenous Oct-proteins in latency I versus latency III B-cell lines, opposite to the high abundance of EBNA 1 in latency III and low in latency I cells.

Conclusions: We have demonstrated that the EBNA1 responsive element family of repeats (FR) also can be regulated by members of the Oct-transcription factor family. We suggest a model where EBNA 1 driven Cp-transcription controlled by FR can be switched off by Oct-binding and recruitment of a repressor, and controlled by the levels of EBNA 1 and Oct in different cell types. This model also has interesting implications for switch-on-off of Qp.

EBNA-1 Enhances Transcription Through Ying-Yang 1 (YY1)

Perrigoue J, Nanbo A, Sugden B

Contact:
Dr. Asuka Nanbo
University of Wisconsin
Oncology, McArdle Laboratory for Cancer Research (Madison, USA)
Email: nanbo@oncology.wisc.edu

Abstract:
The EBV latent protein EBNA-1 activates transcription from transfected templates. Recently, EBNA-1 was found also to activate transcription of an integrated template consisting of the family of repeats upstream of a thymidine kinase promoter driving luciferase (FR-TK-luciferase) within BJAB cells. While wild-type EBNA-1 was able to activate transcription of integrated templates up to 25-fold, a derivative of EBNA-1 with a deletion in the unique region of linking region 1 lacking amino acids 65-89 (∆UR1) was unable to activate transcription and inhibited the activation mediated by wild-type EBNA-1 upon co-expression (Kennedy and Sugden, Mol Cell Biol 23: 6901-6908, 2003). We performed a yeast-two hybrid screen using two copies of wild type ∆UR1 as bait and subsequently ∆UR1 as a counter screen to identify cellular proteins that interact with UR1. Ying-Yang 1 (YY1), a transcription factor capable of both repressing and activating transcription of a diverse number of promoters interacts with the UR1 domain of EBNA-1 specifically. YY1 also augments the ability of EBNA-1 to enhance transcription from integrated copies of FR-TK-luciferase in BJAB cells. A fusion of the DNA binding and dimerization domain of EBNA-1 to full-length YY1 also activates transcription of these integrated templates. Additionally, preliminary chromatin immunoprecipitation results indicate that YY1 may be bound to FR, presumably through an interaction with EBNA-1 since there are no known YY1 binding sites within FR. These findings together indicate that YY1 by binding EBNA-1 can positively regulate transcription. It is possible that if EBNA-1 binds sites in human chromosomes it may either positively or negatively affect transcription by tethering YY1 to those sites.

Transcriptional Activation of EBER Expression

Arrand JR, Chen X, Dawson CW, Felton-Edkins Z, Murray PG, O'Neil JD, Reynolds GA, Wei W, White RJ, Wood VHJ, Yao Y, Young LS

Contact:
Dr. John Arrand
University of Birmingham
Institute for Cancer Studies (Birmingham, UK)
Email: arrandj@cancer.bham.ac.uk

Abstract:
Cells that are latently infected with EBV generally express high levels of the RNA polymerase III (pol III) transcripts EBER1 and EBER2. However following infection of B-cells by EBV the EBERs are temporally the last latent gene products to be expressed implying that their expression is not constitutive but is regulated. We show that EBER expression appears to be modulated by an EBV latent function that upregulates pol III-specific cellular transcription factors.
Pol III-mediated transcription is enhanced by a number of viruses and also in some tumour cells by increased levels of particular components of pol III-associated transcription factors. We show that infection by EBV of the epithelial cell lines HeLa or AdAH, the EBV-negative NPC line HONE1 or the EBV-negative variant of the BL cell line Akata results in upregulation of specific subunits of the pol III transcription factor complex TFIIIC.
Microarray analysis of epithelial cell lines engineered to express individual EBV latent proteins reveals that a single latent gene product can induce enhanced expression of particular TFIIIC components. Semi-quantitative PCR confirms the array results and extends them by analysing low-abundance TFIIIC mRNAs that are called as absent by the array analysis software. In vivo confirmation of the cell-line data is obtained by immunohistochemical staining of EBV-associated tumours with antibodies against TFIIIC. The results demonstrate that the strongly EBER-positive tumour cells express elevated levels of TFIIIC components.
The data suggest that EBV autoinduces high level EBER expression through upregulation of RNA polymerase III-specific transcription factors by a virus-encoded latent protein.

Epstein-Barr virus latent membrane protein 2A regulates viral and cellular gene expression via modulation of the STAT and NF-κB transcription factor pathways

Stewart SE, Dawson CW, Takada K, Moody CA, Sixbey JW, Young LS

Contact:
Suzanne Stewart
University of Birmingham
CR UK Institute for Cancer Studies (Birmingham, UK)
Email: suzannes@cancer.bham.ac.uk

Abstract:
Epstein-Barr virus (EBV) associated malignancies display distinct patterns of virus latent gene expression that reflect the complex interplay between the virus and its host cell. In the EBV-associated epithelial tumour, nasopharyngeal carcinoma (NPC), the virus-encoded latent membrane protein LMP2A is consistently expressed while the oncogenic LMP1 protein appears to be restricted to only a proportion of tumours. In an attempt to understand the contribution of LMP2A to the pathogenesis of NPC, we established carcinoma cell lines stably infected in vitro with either a wild type recombinant EBV (rEBV) or a mutant rEBV in which LMP2A is deleted (rEBV-2A). An NPC like pattern of EBV gene expression including LMP2A but not LMP1 was consistently observed in carcinoma cells infected with rEBV. However, carcinoma cells infected with rEBV-2A expressed high levels of LMP1 from the STAT regulated L1-TR promoter. Consistent with this effect, basal STAT activity was reduced in rEBV-infected carcinoma cells and this repression was relieved in the absence of LMP2A. This modulation of STAT activity correlated with the ability of LMP2A to inhibit the autocrine secretion of IL-6 from carcinoma cell lines. Exogenous IL-6 was able to induce expression of LMP1 via STAT3 activation in both rEBV-infected carcinoma cell lines and in the EBV-positive C666-1 NPC cell line. The LMP2A-mediated suppression of IL-6 was a consequence of NF-kB inhibition. These data reveal that LMP2A modulates two key transcription factor pathways in carcinoma cells and suggests that this may be important in the pathogenesis of EBV-associated tumours.

Epstein-Barr virus Protein Kinase Phosphorylates EBNA2 and Suppresses EBNA2 Transactivation of the LMP1 Promoter

Yue W, Gershburg E, Pagano JS

Contact:
Dr. Wei Yue
University of North Carolina at Chapel Hill
Lineberger Cancer Center (Chapel Hill, USA)
Email: wei_yue@med.unc.edu

Abstract:
Objective: EBV nuclear antigen 2 (EBNA2) is a phosphoprotein, it is essential for immortalization of B-lymphocytes and transactivation of viral and cellular promoters including the promoter for LMP1, the principal EBV oncoprotein. EBV protein kinase (PK) expresses during lytic cycle and phosphorylates numerous viral and cellular proteins. To date, there is no evidence of influence of PK on its target proteins function. Her we studied whether PK phosphorylates EBNA2 and influences EBNA2 transactivation of the LMP1 promoter.

Methods: EBNA2 was expressed and purified from bacteria, EBV PK and kinase-dead PK were from insect cells; PK in vitro kinase assays were carried out with purified EBNA2 as substrate. In order to study phosphorylation of EBNA2 by PK in vivo and co-immunoprecipitation of these two proteins, EBNA2 and EBV PK were co-expressed in HeLa cells. λ-PPase treatment was used to confirm the phosphorylation of EBNA2 by PK. LMP1 promoter reporter assay were used to determine the effect of PK on EBNA2 transactivation of LMP1 promoter.

Results: We found that purified EBNA2 was phosphorylated by PK, but not kinase-dead PK in vitro. Migration of EBNA2 was retarded when co-expressed with PK in HeLa cells; λ-PPase treatment shifted both hypo- and hyperphosphorylated EBNA2 to its unphosphorylated form indicating that EBNA2 is hyperphosphorylated by PK in vivo. EBNA2 was co-immunoprecipitated with FLAG-tagged PK suggesting physical association of these two proteins. In the LMP1 reporter assay, neither PK nor kinase-dead PK alone activates LMP1 promoter activity. However, EBV PK suppresses EBNA2's ability to transactivate the LMP1 promoter ($p<0.01$), while the kinase-dead PK has no such effect.

Conclusions: The results demonstrate that: 1) EBV PK phosphorylates EBNA2 in vitro. 2) PK phosphorylates and physically associates with EBNA2 in vivo when co-expressed in HeLa cells. 3) EBV PK suppresses the EBNA2 function in transactivation LMP1 promoter most likely through hyperphosphorylation of EBNA2. Here is the first report of influence of EBV PK on its target protein's function.

EBNA-LP preferentially co-activates EBNA2-mediated stimulation of latent membrane proteins expressed from the viral divergent promoter

Ling P

Contact:
Dr. Paul Ling
Baylor College of Medicine
Department of Molecular Virology and Microbiology (Houston, USA)
Email: pling@bcm.tmc.edu

Abstract:
Objective: To determine whether EBNA-LP is a global coactivator of EBNA2 and if coactivation requires interactions with EBNA2.

Methods: RT-PCR, Coimmunoprecipitation assays, deconvolution microscopy, and mammalian two-hybrid assays

Results: In type I Burkitts lymphoma cells, we found that EBNA-LP strongly coactivated EBNA2 stimulation of only LMP-1 and LMP2B RNAs, which are expressed from the viral divergent promoter. Surprisingly, the viral LMP2A gene and cellular CD21 and Hes-1 genes were induced by EBNA2 but no further induction was observed after EBNA-LP coexpression. To identify possible mechanisms by which EBNA-LP might mediate coactivation of EBNA2, we investigated whether these proteins might interact with each other. We found that EBNA-LP did not stably interact with EBNA2 in coimmunoprecipitation assays even though the conditions were adequate to observe specific interactions between EBNA2 and its cellular cofactor, CBF1. Colocalization between EBNA2 and EBNA-LP was not detectable in EBV transformed cell lines or transfected type I Burkitts cells. Finally, no significant interactions between EBNA2 and EBNA-LP were found using mammalian two-hybrid assays. In contrast, we have identified a cellular nuclear protein that interacts with EBNA-LP in co-immunoprecipitation assays. A coactivation mutant of EBNA-LP which still localizes to the nucleus fails to interact with this cellular factor.

Conclusions: From this data, we conclude that EBNA-LP preferentially coactivates EBNA2 stimulation of the viral divergent promoter. While this may require specific transient interactions between these proteins that only occur in the context of the divergent promoter, our data strongly suggests that EBNA-LP might also cooperate with EBNA2 through mechanisms that do not require direct or indirect complex formation between these proteins. Preliminary data suggest that we have identified a candidate cellular cofactor that mediates EBNA-LP coactivation function.

The atypical bZIP domain of EBNA 3C forms oligomers not homodimers but has a novel role in maintaining the RBP-Jk interaction

West MJ, Webb HM, Sinclair AJ, Woolfson DN

Contact:
Dr. Michelle West
University of Sussex
School of Life Sciences (Brighton, UK)
Email: M.J.West@sussex.ac.uk

Abstract:
Objective: To determine whether the putative bZIP domain of EBNA 3C functions as a bona fide α-helical dimerization interface and to study the role played by this domain in the regulation of transcription by EBNA 3C.

Methods: Circular dichroism spectroscopy and analytical ultracentrifugation using EBNA 3C zipper peptides and domain-swap analysis using EMSAs. Analysis of the effects of mutagenesis of the bZIP domain on the ability of EBNA 3C to inhibit EBNA 2 activation in reporter assays and to co-precipitate with RBP-Jk in vivo.

Results: EBNA 3C contains a stretch of basic residues followed by a run of leucine residues spaced seven amino acids apart that is widely believed to represent a leucine-zipper dimerization motif (bZIP). We have performed the first structural and functional analysis of this motif and demonstrate that it cannot direct the formation of stable homodimers. EBNA 3C zipper peptides are 54-67% α-helical in solution but cannot form dimers at physiologically relevant concentrations. Moreover, the EBNA 3C zipper cannot functionally substitute for another homodimerizing zipper in domain-swap experiments. Our data indicate however that the EBNA 3C zipper behaves atypically and is capable of self-associating to form higher-order α-helical oligomers containing 18 or more zipper domains. Using mutagenesis we have identified a new role for the bZIP domain in maintaining the interaction between EBNA 3C and RBP-Jk. Disruption of the helical nature of the zipper domain by the introduction of proline residues reduces the ability of EBNA 3C to inhibit EBNA 2 activation and interact with RBP-Jk in vivo by 50% and perturbation of the charge on the basic region completely abolishes this function of EBNA 3C.

Conclusions: The EBNA 3C bZIP cannot form stable homodimers or substitute for a genuine homodimerizing zipper domain. Since the basic residues in the EBNA 3C bZIP do not appear to direct sequence-specific DNA binding we conclude that this bZIP has none of the properties exhibited by genuine members of the bZIP protein family. We have shown however that residues in this domain are important in maintaining the interaction between EBNA 3C and RBP-Jk and suggest that the conservation of the basic charge and the hydrophobic repeat between different viral isolates has been driven by this function of the bZIP.

Array Analysis of Transcription Program of Epstein-Barr Virus

Lu CC, Jeng YY, Tsai CW, Liu MY, Chen MR

Contact:
Chih-Chung Lu
Graduate Institute and Department of Microbiology, College of Medicine, National Taiwan University
Department of Microbiology, College of Medicine, National Taiwan University (Taipei, Taiwan)
Email: d91445002@ntu.edu.tw

Abstract:
Epstein-Barr virus (EBV) is a gammaherpesvirus associated with several human malignancies, including B and T lymphoma, Hodgkin's disease and nasopharyngeal carcinoma. To characterize the viral gene expression in EBV replicating cells simultaneously, a nylon-membrane based DNA array system containing nearly all the known and predicted open reading frames (ORFs) was established. The kinetics of the viral gene expression upon induction of lytic replication were studied in two EBV positive Burkitt's lymphoma cell lines: Akata, which contains the full-length viral genome, and Raji, in which viral replication cycle cannot be completed due to the lack of major DNA binding protein. The transactivation ability of Rta on genome-wide EBV gene expression in the absence of Zta was analyzed by transfecting Rta expressing plasmid into Raji cells. The transcription control of individual genes that can be activated by Rta was revealed. The similarities of gene expression profile were grouped by clustering algorithm analysis. Possible roles of EBV genes with unknown function were suggested according to the correlation between gene expression during lytic replication and protein function. The split expression pattern of genes involving DNA replication, which appeared to be conserved among gamma herpesviruses, suggests possible sequential transcription control during the progression of viral DNA replication.

Microarray profiling of latent and lytic EBV gene expression in tumour cells

Bernasconi M, Sigrist JA, Berger C, Ladell K, Niggli FK, Nadal D

Contact:
Dr. Michele Bernasconi
University Children's Hospital Zurich
Experimental Infectious Diseases and Cancer Research (Zurich, Switzerland)
Email: michele.bernasconi@kispi.unizh.ch

Abstract:
Objective: To profile the simultaneous expression of latent and lytic EBV genes in a panel of Burkitt's lymphoma cell lines during exponential growth in culture (steady state) and during induction of the lytic cycle using a customized microarray chip.

Methods: We developed an oligonucleotide (ODN) chip with probes specific for latent (EBNA1, 2, 3A, 3C, LMP1, LMP2) and lytic genes (BZLF1, gp85). A set of cellular housekeeping genes was selected for normalization of the results. Long ODN (70mers) were selected based on their specificity and sensitivity of detection. Total RNA was amplified by linear amplification, indirectly labelled with Cy3 or Cy5 and after competitive hybridization on customized microarray data were normalized to the housekeeping gene set.

Results: The BL cell lines Akata, Jijoye, Raji, P3HR1, Namalwa were competitively hybridized against B95-8 (reference cell line). Expression of latent and lytic genes was detectable and generally higher in B95-8. Type III latency gene expression was detected in all cells, exception made for P3HR1 where EBNA2 is deleted. Lytic gene expression was higher in B95-8 reflecting the basal lytic activity of the virus. In Namalwa cells no BZLF1 expression could be detected. Following induction of the lytic cycle in B95-8 by PMA, expression of the latent genes remained stable, while expression of BZLF1 peaked at 72hrs. In Akata cells induction of the lytic cycle by IgG cross-linking resulted in an increase of expression of lytic genes as early as 6hrs after induction (BZLF1) and of latent genes (EBNAs as well as LMPs) at 24hrs.

Conclusions: We have developed a novel tool to simultaneously study the expression of different EBV genes. We have established the quantitative expression profile of several BL cell lines. The expression levels for a given gene varied between the cell lines investigated. We could monitor the dynamics of gene expression upon induction of the EBV lytic cycle. Induction of the EBV lytic cycle in Akata cells resulted in increased expression levels of lytic and latent genes. Here, increase was more pronounced than in B95-8, owing to the lower latent gene expression before induction. This custom microarray may be expanded to cover the whole EBV genome, and to include probes for cellular genes to investigate the interplay between EBV gene products and host cellular regulatory components.

Epstein-Barr Virus DNA and gene expression levels in in vitro-infected lymphocytes and in lymphoma cells

Berger C, Bonanomi A, Ladell K, Kochli N, Nadal D

Contact:
Dr. Christoph Berger
University Children's Hospital Zürich
Division of Infectious Diseases (Zürich, Switzerland)
Email: christoph.berger@kispi.unizh.ch

Abstract:
Objective: Epstein-Barr virus (EBV) is associated with lymphoproliferation and lymphomas that express different EBV genes. The quantitative dynamics of EBV gene expression during different phases of tumor formation are not known.

Methods: We developed real-time PCR assays targeting 6 EBV key genes, including 4 genes expressed in latent infection (EBNA1, EBNA2, LMP1, LMP2) and two genes expressed in lytic infection (bzlf1, gp85). Quantitative viral DNA and RNA expression levels were analyzed in lymphoblastoid cell lines (LCL) established from peripheral blood or cord blood lymphocytes following infection with EBV (B95-8) in vitro, in comparison with EBV-containing Burkitt lymphoma (BL)-derived tumor cell lines, and following lytic EBV cycle induction in B95-8 and Akata cells.

Results: Comparative analysis of viral gene expression levels related to the housekeeping gene hydroxymethylbilane synthase (HMBS) revealed an LCL-like pattern with strongest and predominant relative expression of EBNA2 and LMP1 (LCLs, Raji, IB4) and another pattern with highest levels of LMP2 and gp85 (B95-8, Jijoye, P3HR1, AG876). In the latter, present in most BL-derived cell lines and B95-8, the expression levels of gp85 were higher than those of EBNA2 and LMP1. Among different cell lines the EBV copy numbers per cell varied by 3 orders of magnitude. Expression levels of latent genes, in particular of EBNA2, were highest in cell lines with low viral copy numbers per cell and vice versa. Expression levels of EBNA2, EBNA1 and LMP1 showed a significant inverse correlation with the copy number per cell in LCLs, but in BL-derived cell lines only weakly for EBNA2. Lytic cycle induction in B95- 8 cells resulted in an increase in lytic, and in Akata cells in a more extensive increase in both latent and lytic gene expression levels.

Conclusions: Comparative analysis of differential EBV gene expression revealed predominant high level transforming EBNA2 and LMP1 latent gene expression in LCLs but not in BL cell lines. Moreover, it disclosed an inverse relation between viral copy number and latent gene expression levels, suggesting that EBV ensures its latency and thus survival through regulation of latent gene expression levels.

Regulation of CD95 in EBV infected B-cells

Le Clorennec C, Youlyouz I, Adriaenssens E, Coll J, Bornkamm GW, Feuillard J

Contact:
Prof. Dr. Jean Feuillard
Faculté de Médecine et CHU Dupuytren de Limoges
UMR CNRS 6101 et Laboratoire d'Hématologie (Limoges, France)
Email: jean.feuillard@chu-limoges.fr

Abstract:
Expression of CD95 is regulated by various agents such as TNFα, IFNγ, or Daunorubicine, able to induce the transcriptional factors NF-kB, STAT1 and p53 respectively. The CD95 promoter is known to harbour binding sites for these factors. These factors are modulated both in expression and activity during EBV infection of B-cells. EBV is known to induce CD95 expression. The objective of this work was to understand the contribution of p53, STAT1 and NF-kB in the regulation of CD95 expression by EBV.
To control the activity of NF-kB, STAT1, p53 and LMP1, we have developed and used various episomal inducible vectors. One of the characteristics of these vectors is that the gene of interest is expressed from a bidirectional doxycycline regulatable promoter allowing simultaneous expression of truncated NGF receptor, used as a surrogate marker of inducibility. We have subcloned the following cDNAs into these vectors: dominant negative IkBa, active (STAT1a) and dominant inactive natural isoform of STAT1 (STAT1b), p53, a dominant negative mutant of LMP1 (LMP1CT) and LMP1 wild type. After stable transfection of an LCL, induction of the cDNA of interest was performed with doxycycline for 24H. Positive cells for NGFR were purified using magnetic beads and were additionally treated with TNFα, IFNγor Fludarabine for 24H. To control the latency III program, we have used the EREB2-5 cell line harboring an estrogen-regulatable EBNA2 gene. The function of CD95 was studied by quantifying apoptosis induction after CD95 cross linking with a specific antibody.
We found that induction of latency III program in EREB 2-5 cells was associated with an increase of both p53 and CD95 expression.Inhibition of LMP1 by LMP1CT decreased the expression of CD95. CD95 expression was hardly regulated by p53. Inhibition of both NF-kB and STAT1 decreased CD95 expression. Only IFNγ was able to increase expression of CD95. Finally, we show that induction of CD95 expression by EBV render the infected B-cell sensitive to the induction of CD95-mediated apoptosis.
Our results suggest that the latencyc III program of EBV sensitize the infected B-cells to CD95-mediated apoptosis via STAT1 and NF-kB activated by LMP1. In the view of the virus/host equilibrium, this process may render EBV-infected B-cells in latency III susceptible to elimination by the immune system.

The in vitro EBV-infected Hodgkin lymphoma (HL)-derived cell line KMH2 expresses only EBNA-1; CD40-ligand and IL-4 induces LMP-1 in the absence of EBNA-2

Kis LL, Nishikawa J, Takahara M, Nagy N, Klein G, Klein E

Contact:
Dr. Loránd L. Kis
Karolinska Institutet
Microbiology and Tumor Biology Center (Solna, Sweden)
Email: Kis.Lorand.Levente@mtc.ki.se

Abstract:
Objective: EBV is present in about 50% of HLs and the viral latent gene expression pattern is type II (EBNA-1 and the latent membrane proteins LMP1, LMP2A and LMP2B). The regulation of LMP's expression in the absence of EBNA-2 in the EBV-carrying Hodgkin-Reed Sternberg (HRS) cells is not known. Since no EBV-positive HL-derived cell line or type II B-cell line exists, we investigated the interaction of EBV and HRS cells in vitro by establishing an EBV-infected subline of the well-characterized HL-derived cell line KMH2.

Methods: The EBV negative, CD21-positive KMH2 cell line was infected with the B95-8 and Akata-GFP EBV strains. The expression of EBV-encoded latent genes was monitored by immunoblotting and by immunoflourescence. Using the neomycin-resistance gene carrying recombinant Akata virus (Akata-NeoR) we established an EBV-infected subline of KMH2, called KMH2-EBV. The expression of EBV genes was investigated in the untreated KMH2-EBV cells and after exposure to the demethylating agent 5-AzaC, to the histone deactylase inhibitor TSA and sodium butyrate, to the phorbol ester PMA, and to CD40-ligand (CD40L) and IL-4 stimuli.

Results: Only EBNA-1 expression was detected in a few cells in spite of the fact that all cells could be infected as indicated by the GFP expression. In the established KMH2-EBV cells LMP-1 could be induced by TSA, n-butyrate, 5-AzaC, or PMA. None of these treatments induced EBNA-2 and only PMA induced the lytic cycle in 5% of the cells. Importantly exposure to the physiological stimuli CD40L and IL-4 induced LMP-1. Neither EBNA-2 nor lytic cycle was induced.

Conclusions: In the EBV-positive HRS cells the latent gene expression is type II. It was not possible to establish an EBV carrier subline of KMH2 unless the recombinant virus imposed neomycin resistance on the cells. The EBV expression was restricted to EBNA-1. However LMP-1 could be induced both by chemical agents and by CD40L and IL-4. The latter result strongly suggests that in vivo the surrounding activated T-cells in HL tissues contribute to the latent EBV gene expression seen in HRS cells.

Mapping of potential internal ribosome entry sites in the EBNA transcripts.
Larsson U, Isaksson Å, Ahmed Mohamed R, Berggren M, Ricksten A

Contact:
Ulrica Larsson
Laboratory Medicine
Clinical Chemistry and Transfusion Medicine (Gothenburg, Sweden)
Email: ulrica.larsson@clinchem.gu.se

Abstract:
Objective: To investigate potential internal ribosome entry sites (IRESs) in the 5'-untranslated region of Epstein- Barr virus nuclear antigen (EBNA) transcripts. Transcriptional initiation by alternative promoters, promoter strength and long-range splicing events are important mechanisms in the regulation of EBNA gene expression. Earlier work by our group has identified a functional IRES in the U leader exon of the EBNA1 gene. IRESs are cis-acting elements located in the 5'-untranslated region of the message, and capable of recruiting the translation machinery to an internal initiation codon in the mRNA. Results from investigations on IRES-activity have demonstrated a dependence on complex RNA secondary structure with conserved motifs. A number of viruses, such as hepatitis C and Kaposi`s sarcoma herpes virus use IRES-mediated translation for synthesis of viral proteins. The EBNA transcripts initiated from the Cp/Wp promoters have long 5'-UTR consisting of several leader exons, C1-2, W1-2, Y1-3 and U, which are rich in G and C nucleotides. Here we report the investigation of mapping potential IRES elements in the untranslated W and Y mRNA regions.

Methods and Results: The Mfold program composed a model for secondary structure of the 5'-UTR of the EBNA transcripts. Complex and energetic stable RNA models with several IRES-specific motifs were predicted in the W1W2 and Y1Y2Y3 regions, respectively. The leader exon regions were inserted between two reporter genes and the ability of these sequences to promote internal ribosome entry on the bicistronic message was analyzed in transiently transfected cell lines. Our data show that the regions W1W2 and Y1Y2Y3 contain translation elements that are capable of increasing expression of the second cistron. In different Burkitt lymphoma cell lines, expression of the second cistron was stimulated 2-7 fold relative to the control vector. The cell-type specific activities suggest favourable interactions between the 5'-untranslated regions and factors present in EBV-positive cells.

Conclusions: Our present findings further indicate IRES-mediated translation as a mechanism for regulation of the EBNA gene expression. Utilizing of an internal entry site for translation will ensure EBNA protein synthesis during cell conditions where cap-mediated translation is impaired.

Deletion of the Epstein-Barr virus IRES by alternative splicing of the EBNA1 gene in organ transplant patients

Berggren M, Isaksson Å, Larsson U, Miriami E, Ekman T, Nilsson F, Ricksten A

Contact:
Malin Berggren
Inst. Laboratoriemedicin
Klinisk Kemi och Transfusionsmedicin (Göteborg, Sweden)
Email: malin.berggren@vgregion.se

Abstract:
Objective: Epstein-Barr virus (EBV)-induced post transplant lymphoprolipherative disorder (PTLD) is a severe complication after solid organ transplantation. Lesions of PTLD are EBV positive with a majority of the cells expressing Epstein-Barr nuclear antigen (EBNA) 1. EBNA1 is absolutely required for maintaining viral latency and it is expressed during all three types of latent EBV infections. Transcriptional initiation from alternative promoters (Cp, Wp, Fp and Qp), promoter strength and splicing events are important mechanisms in the regulation of EBNA1 expression. Here, we report the finding of an alternatively spliced EBNA1 transcript expressed in peripheral blood cells in EBV-positive organ transplant patients with and without diagnosed PTLD. This transcript lacks the U leader exon in the 5'- untranslated region (UTR). Previously we identified a functional internal ribosome entry site, the EBNA IRES, in the U leader exon of the EBNA1 gene.

Methods and Results: Transfection experiments showed that the EBNA IRES promotes cap-independent translation and increases the level of Fp- and Qp initiated transcripts in a reporter system with high efficiency. The influence of the EBNA IRES activity on EBNA1 translation of the EBV-negative cell line DG75 where the different EBNA1 cDNA -splices was inserted in vectors driven by the CMV-promoter. Western blot on protein extracts showed increased levels of EBNA1 protein when the U exon was present confirming the suggested EBNA IRES function. The alternative EBNA1 transcript is expressed in the majority of the investigated patient samples as well as in EBV-positive B-cell lines, which gives EBV potential of translational regulation by modifying 5'-UTR.

Conclusions: The EBNA IRES in the EBV U exon is shown to be functional in EBNA1 translation in transient transfections. We have identified alternatively spliced EBNA1 in transplant patients and in EBV-positive cell lines suggesting a translational regulation. Our findings provide further insight into the complex regulation of EBNA1 expression that may contribute to development of human B-cell proliferation.

The effects of artificially up-regulated EBV LMP2 expression in an EBV-transformed B-cell line

Patsos G, Wilson AD, MacArthur GJ, Morgan AJ

Contact:
Dr. Andrew Morgan
University of Bristol
Pathology and Microbiology (Bristol, UK)
Email: andy.morgan@bristol.ac.uk

Abstract:
Objective: The Epstein-Barr virus (EBV) latent membrane protein (LMP) 2A is expressed at barely detectable levels in lymphoblastoid cell lines (LCL) and these cells are not normally susceptible to killing by autologous LMP2A-specfic cytotoxic T-cells (CTL) in vitro unless pre-loaded with the appropriate synthetic peptide or treated with the E. coli enterotoxin subunit, EtxB (Ong et al., 2003, J. Virol. 77: 4298-4305). Our aim was to obtain artificially high levels of LMP2A expression in an LCL and to measure subsequent effects on cell growth, on expression of other EBV genes, and susceptibility to LMP2A-specific CTL.

Methods: An LCL was created (P2) in which an ecdysone-inducible LMP2A plasmid expression system was stably transfected. This system allows tight control over LMP2A expression and can be switched on simply by the addition of ponasterone A to cultures.

Results: The level of LMP2A in P2 cells could be increased at least ten fold using ponasterone induction. Increased LMP2A expression resulted in the up-regulation of LMP1, and the down-regulation of BZLF1 and BRLF1 while the expression of EBNA2 was unaffected. Expression of cell surface markers MHC class I, MHC class II, B7 integrin, CD80 and CD96 showed no change while levels of CD40 decreased and ICAM1 increased. Elevation of LMP2A levels had no effect on cell growth nor on susceptibility to Fas-induced apoptosis. P2 cells in which LMP2A expression was increased at least ten-fold did not become susceptible to LMP2A-specific CD8+ T-cell mediated killing unless pre-treated with EtxB. Furthermore both untransfected LCL and P2 cells could be killed following LMP2A peptide pulsing of the target cell showing that there was no defect in either the CTL used or the susceptibility of the target cell to killing per se.

Conclusions: Overall the effect of LMP2A upregulation was to inhibit progression to lytic cycle. The results also indicate that EBV-infected LCLs are unable to process and present LMP2A effectively to functional LMP2A-specific CTLs. It remains to be seen whether this is an intrinsic property of the LMP2A molecule that contributes to the survival of EBV-associated tumour cells expressing this molecule in the face of competent LMP2A-specfic CTL responses.

Epstein-Barr virus latent membrane protein 2A downregulates telomerase reverse transcriptase (hTERT) in epithelial cell lines

Chen F, Liu C, Lindvall C, Xu DW, Ernberg I

Contact:
Dr. med. Fu Chen
Karolinska Institute
Microbiology and Tumour Biology Centre (Stockholm, Sweden)
Email: Fu.Chen@mtc.ki.se

Abstract:
Objective: The LMP2A has been implicated in the maintenance of viral latency and appears to function by inhibiting B-cell receptor (BCR) signaling through its ITAM motifs and it may also have a role in tumorigenesis. We investigated regulatory effects of LMP2A on the telomerase reverse transcriptase (hTERT) expression.

Methods: 1) Reverse transcription and quantitative determination of hTERT expression. 2) LMP2A derivatives containing Y74F-, Y85F- and Y74FY85F-LMP2A mutations were generated by PCR mutagenesis and authenticated by DNA sequencing.3) Telomerase activity assay using a commercial telomerase PCR ELISA kit.4) Transient transfection and reporter gene assay by insertion of the hTERT promoter deletion mutants p3996 and p330 cloned upstream of the firefly luciferase reporter.

Results: 1) We observed a significant and constant reduction of hTERT mRNA in a bladder carcinoma 5637 cell line expressing LMP2A. This was accompanied by decreased telomerase activity. 2) We further showed that LMP2A inhibited the hTERT promoter activity. 3) The ITAM motif of LMP2A was required for this inhibition.

Conclusions: LMP2A expression leads to the transcriptional repression of the hTERT gene through specific pathways. This function may contribute to the control of EBV latency.

Epstein-Barr virus latent membrane antigen 2A (LMP2A) can induce the lytic cycle
Schaadt E, Adler B, Bornkamm GW

Contact:
Prof. Dr. med. Georg W. Bornkamm
GSF-Research Center for Environment and Health
Institute of Clinical Molecular Biology and Tumor Genetics (München, Germany)
Email: bornkamm@gsf.de

Abstract:
Objective: Epstein-Barr Virus (EBV) establishes a persistent latent infection in peripheral memory B-lymphocytes. The only viral gene shown to be expressed in these cells is the gene for the latent membrane protein 2A (LMP2A). LMP2A carries an ITAM motif and has been shown to mimick B cell receptor signaling by providing a survival signal for mature B cells in transgenic mice. Conversely, LMP2A has been reported not to stimulate but to inhibit B cell receptor signaling and to block lytic virus induction in EBV-infected B cells by anti-Ig treatment. This apparent paradox prompted us to re-examine the role of LMP2A in lytic cycle induction in B cells conditionally immortalized by EBV.

Methods: Transfection of LMP2A and LMP1 into estrogen-deprived EREB2-5 cells, Intracellular BZLF1, early antigen and VCA staining.

Results: Here we show that in the absence of other stimuli LMP2A expression alone can lead to induction of the viral lytic cycle. Similarly to B cell receptor stimulation by anti-Ig treatment, this LMP2A mediated reactivation is dependent on the MAPK pathway and can be inhibited by the viral latent membrane protein 1 (LMP1).

Conclusions: Our data resolve the apparent paradox in the literature that LMP2A was reported to inhibit and not to induce the lytic cycle. The data reinforce the notion that LMP2A is a functional homologue of the B cell receptor not only with respect to B cell survival but also with respect to regulation of the lytic cycle.

The EBV SM protein interacts cooperatively with cellular sp110b to stabilize target mRNAs and enhance EBV lytic gene expression

Nicewonger J, Suck G, Bloch D, Swaminathan S

Contact:
Dr. Sankar Swaminathan
University of Florida
UF Shands Cancer Center (Gainesville, USA)
Email: sswamina@ufl.edu

Abstract:
Background: Several herpesviruses induce expression of interferon stimulated genes (ISGs) by interferon-independent mechanisms. Although ISG expression may represent a host defense response, it is also possible that some ISGs may be co-opted by the virus to enhance virus replication. Interferons increase the size and number of PML nuclear bodies and stimulate transcription of several genes encoding PML nuclear body proteins. PML bodies are disrupted during replication of many DNA viruses, suggesting that they play an antiviral role. However, some PML nuclear body proteins colocalize at sites of viral DNA synthesis and transcription, suggesting that they may be utilized by DNA viruses. The EBV lytic protein is a post-transcriptional regulator of EBV and host cell gene expression. We have previously shown that the EBV lytic protein SM specifically increases expression of several genes (ISGs) via STAT1. We recently identified a component of the PML nuclear body, Sp110b, as a potential SM-interacting protein.

Objective: To investigate the interactions between (EBV) SM protein and Sp110b, a PML nuclear body protein.

Results: SM bound to Sp110b in an RNA-independent manner. SM also specifically induced expression of Sp110b during lytic EBV replication and in several cell types. Exogenous expression of Sp110b synergistically enhanced SM-mediated accumulation of intronless and lytic EBV transcripts. This synergistic effect was shown to be promoter-independent, post-transcriptional, and associated with increased stabilization of target transcripts. Finally, inhibiting Sp110b expression decreased accumulation of an SM-responsive lytic EBV transcript in EBV-infected cells.

Conclusions: These findings demonstrate that SM induces Sp110b expression, binds to Sp110b protein, and utilizes the recruited Sp110b protein to increase the stability of lytic EBV transcripts, indicating that Sp110b is a component of the cellular machinery that EBV utilizes to enhance lytic EBV replication. Our findings therefore suggest that some ISGs that comprise the PML body, such as Sp110b, may be induced by replicating viruses and used to enhance viral replication.

RBP-J signalling of the EBNA-2 CR4 region is critical but not essential for the proliferation of EBV infected B-cells

Grabusic K, Hartmann A, Maier S, Mantik A, Santak M, Popp C, Kremmer E, Hammerschmidt W, Kempkes B

Contact:
Andrea Hartmann
GSF - Hämatologikum
KMOLBI -AG Kempkes (München, Germany)
Email: andrea.hartmann@gsf.de

Abstract:
Epstein-Barr Virus (EBV) nuclear antigen 2 (EBNA-2) is a transactivator of viral and cellular genes which lacks an intrinsic DNA binding domain and is recruited to the promoter of target genes by the sequence specific DNA binding protein RBP-J. EBV infects and growth transforms primary B-cells in culture leading to outgrowth of lymphoblastoid cell lines (LCLs). EBNA-2 is absolutely required for initiation and maintenance of this process since it induces cell cycle entry. Nine conserved regions (CR1-9) within the primary structure of EBNA-2 have been defined by homology of different EBV strains as well as baboon and rhesus lymphocryptoviruses.
Recently, EBNA-2 has been shown to bind to Nur77, an orphan member of steroid receptor super family, targeting to mitochondria and hence block the pro-apoptotic function of Nur77 in response to Sindbis virus infection.
We have investigated the contribution of the Nur77/EBNA-2 interaction to B-cell growth transformation by EBV. EBV mutants, which express EBNA-2 mutants deleted for the Nur77 interaction domain CR4 promote the proliferation of infected B-cells less efficiently than wild-type EBV growth transformed B-cells.
We have now started to characterize in more detail EBNA-2 associated proteins which bind to the EBNA-2 CR4 region.

Inactivation of RBP-J in the somatic human B-cell line DG75 by homologous recombination

Maier S, Mantik A, Kremmer E, Hammerschmidt W, Kempkes B

Contact:
Dr. Sabine Maier
GSF-Hämatologikum
KMOLBI - Ag Kempkes (München, Germany)
Email: sabine.maier@gsf.de

Abstract:
RBP-J (CBF1), a member of the CSL family (CBF1 in mammals, suppressor of hairless in Drosophila, Lag-1 in C. elegans) of DNA binding factors is a ubiquitous highly conserved protein. It mediates either transcriptional repression or transcriptional activation. The RBP-J binding motif can be found in cellular as well as viral genes (ex. C-promoter of EBV). After binding of RPB-J to its recognition sequence co-repressors like CIR, SMRT and HDAC are recruited to the promoters of target genes followed by repression of these genes. On the other hand RBP-J is used as an adaptor molecule for transactivators without intrinsic DNA-binding domains like activated Notch or the viral protein EBNA2. Their binding to RBP-J replaces the co-repressor complex and leads to a recruitment of co-activators and activation of downstream target genes.
We are interested in the B-cell specific functions of RBP-J in proliferation, activation and differentiation and in the molecular mechanisms of RBP-J function. In order to have a tool, that allows the functional and biochemical analysis of RBP-J in somatic human B-cells we have generated an RBP-J negative derivative of the cell line DG75. DG75 is an EBV-negative human Burkitt's Lymphoma cell line. Exon 4 of RBP-J has been deleted by homologous recombination. In the resulting cell line DNA binding activity and RBP-J expression are lost, as we show by electrophoretic mobility shift assays (EMSA) and western blot. Functional analysis of these cells with reporter constructs confirmed the complete loss of RBP-J function.
RBP-J knockout cells proliferate, showing no alterations in their growth characteristics. So, RBP-J is not essential for the normal growth of these cells. Expression of EBNA2 induced CD21 and CCR7 expression only in the presence of RBP-J, not in the knockout cells. In contrary the EBNA2 mediated downregulation of IgM and c-myc is only partially RBP-J dependent.

Generation and characterisation of transgenic mice expressing a fusion protein containing the transmembrane domain of LMP1 and the signalling domain of CD40

Homig C, Rastelli J, Casola S, Rajewsky K, Muller W, Zimber-Strobl U

Contact:
Cornelia Hömig
GSF-National Research Center for Environment and Health
Institut für Klinische Molekularbiologie und Tumorgenetik (Munich, Germany)
Email: hoemig@gsf.de

Abstract:
Objective: The Epstein-Barr virus (EBV) latent membrane protein1 (LMP1) signals ligand independently through recruitment of tumour necrosis factor receptor (TNF-R) associated factors (TRAFs). Several in vitro data suggest that the signalling domain of LMP1 has extensive functional homology with CD40, a member of the TNF-R family. Both LMP1 and activated CD40 induce activation and proliferation of B-cells. In CD40 deficient mice LMP1 expression restored class switching, but failed to restore the formation of germinal centres (GC) as well as the production of high affinity antibodies. Surprisingly, LMP1 expression also blocked the formation of GC in response to immunisation in the presence of functional CD40, whereas production of high affinity antibodies was not affected. In this study we addressed the question if a constitutive CD40 signal acts like LMP1 in vivo and if the inhibition of GC formation by LMP1 is due to its constitutive signalling activity or due to the provided signals.

Methods: Since it has been shown in vitro that a chimeric protein containing the transmembrane domain of LMP1 and the signalling domain of CD40 can mimic a constitutive CD40 signal, we generated a conditional transgenic mouse which expresses the LMP1/CD40 fusion protein upon Cre-mediated recombination. The conditional LMP1/CD40 mice were crossed to mice expressing the Cre recombinase in a B-cell specific manner and the influence of LMP1/CD40 on B-cells was analysed by flow cytometry and histological methods.

Results: Preliminary data show that similar to LMP1 expressing mice, B-cell restricted LMP1/CD40 expression results in an activated phenotype of mature B-cells as shown by up-regulation of activation markers like FAS, CD80 and ICAM. Furthermore the formation of GC in spleen upon immunisation with NP16-CGG is blocked in LMP1/CD40 expressing mice. In contrast to LMP1 mice, LMP1/CD40 expression exhibits a splenomegaly with a strong increase in the numbers of mature B- and T-cells.

Conclusion: Our data suggest that the block of GC formation induced by LMP1 results from its constitutive signalling activity rather than from a functional difference in signalling capacity between LMP1 and CD40. Currently we investigate whether the LMP1/CD40 can take over the function of CD40 in CD40 deficient mice.

The role of Notch2, the cellular homologue of the viral protein EBNA2 in B cell development

Strobl L, Marschall G, Homig C, Bornkamm GW, Rajewsky K, Zimber-Strobl U

Contact:
Dr. Ursula Zimber-Strobl
GSF-National Research Center for Environment and Health
Institut für Klinische Molekularbiologie und Tumorgenetik (Munich, Germany)
Email: strobl@gsf.de

Abstract:
Objective: Notch1-4 represents a family of transmembrane receptors, which is physiologically involved in cell fate decisions. After activation of the Notch-receptor by binding one of its ligands the receptor is intracytoplasmatically cleaved (Notch-IC) and translocated to the nucleus, where it interacts with the DNA-binding protein RBP-J leading to the transcriptional activation of genes with RBP-J binding sites. Since EBNA2 interacts like Notch-IC with RBP-J and thereby leads to ligand-independent gene activation it is regarded as a functional homologue of Notch-IC. To get deeper insight into the role of Notch signalling during lymphocyte development and to understand whether the Epstein-Barr viral protein uses the Notch-signalling pathway to immortalize B-lymphocytes we generated transgenic mice, where the Notch2 receptor can be conditionally inactivated.

Methods: The conditional Notch2 knock-out mice were crossed with CD19-Cre mice to inactivate the Notch2-gene in B cells and B cell development was analysed by FACS-analysis and histology.

Results: We could show that after inactivation of the Notch2-gene marginal zone B-cells are missing. In addition, we defined another B-cell population, which is also missing in these mice and corresponds most likely to the precursor cells of marginal zone B-cells. After inactivation of the Notch2-gene CD21 was clearly downregulated, whereas the expression of IgH and CD23, two other EBNA2-regulated genes was not affected.

Outlook: It will be interesting to see if EBNA2 can rescue the phenotype of Notch2-deficient mice. This would answer the question, if EBNA2 is really mimicking an active Notch-receptor in vivo.

AP-2 alpha cooperates with Epstein Barr-Virus Nuclear antigen 2 (EBNA2) in activating the Latent Membrane Protein 1 (LMP1) promoter in B cells.

Johansson P, Jansson A, Yang W, Rymo L

Contact:
Pegah Johansson
Institute of Laboratory Medicine
Sahlgrenska University Hospital (Gothenburg, Sweden)
Email: pegah.johansson@clinchem.gu.se

Abstract:
Objective: The Epstein-Barr virus (EBV) LMP1 gene expression is transactivated by EBNA) in human B cells. Preliminary results have suggested that the LMP1 promoter inducibilty by EBNA2, is partially dependent on an AP-2 binding site located at -95/-107 region of the promoter. Further, it has been shown by electrophoretic mobility shift assay that AP-2a binds this site. Here, we aimed to investigate the effect of AP-2a expression on LMP1 promoter regulation, in the absence and presence of EBNA2. We also investigated the endogenous levels of AP-2a in B cells, since it has not been reported previously.

Methods: Fragments with 3'-ends corresponding to position +40 in LMP1 regulatory sequence (LRS) and 5'-end positions corresponding to the -107 and -634 have been cloned in front of a CAT reporter. Mutations have also been made in these plasmids in the AP-2 site (-103/-95). The regulation of these LRS fragments was studied by transient transfections into DG75 cells (EBV negative BL) together with different concentrations of EBNA2 and AP-2 expression vectors. EBNA2 and AP-2 expression vectors were also transiently transfected into P3HR1 cells. This cell line is EBV positive but deficient in EBNA2 resulting in very low-level expression of LMP1. LMP1 expression was analysed after transfection by western blots. AP-2a expression was examined by mRNA quantification and western blots.

Results: Co-transfected EBNA2 and AP-2alpha, cooperated in the activation of LMP1 promoter reporter plasmids through an AP-2 site (-103/-95). Also, exogenously expressed AP-2alpha and EBNA2 upregulated LMP1 expression in the P3HR1 cell line. Interestingly, AP-2a expression alone could activate the LMP1 promoter, suggesting a mechanism for EBNA2 independent expression of LMP1. On the other hand, EBNA2 alone could not activate the promoter when the AP-2 site was mutated, and upstream EBNA2 response elements were deleted. We also show that AP-2a is expressed in the B cell lines that we have examined, albeit at low levels.

Conclusions: Together, the results suggest a possible role for EBNA2 as a co-activator of AP-2. Interestingly there appears to be a correlation between a higher AP-2a expression and EBV positive cells. This would present an important positive regulatory pathway, whereby EBV upregulates the AP-2a transcription factor that in turn upregulates LMP1 expression.

Functionally important sequence variation within the ATF/CRE site in the LMP1 promoter between the P3HR1 and the B95-8 viral strains.

Jansson A, Johansson P, Li X, Rymo L

Contact:
Ann Jansson
Institute of Laboratory Medicine
Gothenburg University (Gothenburg, Sweden)
Email: ann.jansson@clinchem.gu.se

Abstract:
Objective: The ATF/CRE element in the Epstein-Barr virus LMP1 promoter has been shown to mediate transcriptional activation of LMP1. Preliminary data suggest differences in the ATF/CRE site in the LMP1 promoter in the P3HR1 virus compared to the B95-8. The aim was to investigate the significance of this sequence variation.

Methods: The LMP1 regulatory sequence, LRS(-634/+40) was sequenced from the P3HR1 viral genome and compared to the B95-8 viral sequence. EMSAs and competition experiments were performed with two double stranded oligonucleotides corresponding to position -50 to -19 in the LRS from P3HR1 and B95-8 virus strains. P3HR1 derived LRS (-634/+40) as well as B95-8 derived LRS (-634/+40) with the P3HR1 specific mutation in the CRE-site were cloned into the luciferase reporter vector. Transient cotransfections with reporter plasmids in the presence and absence of EBNA2 in combination with dominant negative 254CREB1 and 181ATF1 were done in DG75 cells.

Results: There were 25 nucleotide substitutions and one insertion in LRS (-634/+40) in P3HR1 compared to the corresponding B95-8 sequence. One of the substitutions, a C to an A, was within the ATF/CRE motif. The other mutations were not located in known important LRS elements. While there were no qualitative differences in the binding pattern to the ATF/CRE element between P3HR1 and B95-8 derived LRS sequences, the binding affinity of the ATF1/CREB1 factors to the P3HR1 ATF/CRE element was decreased. The mutation in the ATF/CRE site in P3HR1 lowered the EBNA2-induced activity of the reporter plasmid by approximately 50% compared to the B95-8 wildtype reporter. The C to A mutation in the ATF/CRE site in B95-8 context led to the same level of reduced reporter activity. Dominant negative CREB1 and ATF1 reduced the EBNA2-induced activation of LRS reporter plasmids from both the B95-8-derived and the P3HR1 derived LMP1 promoters to about the same level.

Conclusions: We conclude that ATF1/CREB1 factors are important activators of the LMP1 promoter and that sequence variation within the ATF/CRE site between different viral strains may affect the binding of these factors as well as promoter activity.

The oncogenic Epstein-Barr virus latent membrane protein 1 (LMP1) regulates its own expression: the opposite role of the JNK and NF-kB pathways.

Goormachtigh G, Adriaenssens E, Mougel A, Manet E, Fafeur V, Joab I, Feuillard J, Auriault C, Coll J

Contact:
Dr. Jean Coll
CNRS - Institut de Biologie de Lille
UMR8527 (Lille, France)
Email: jean.coll@ibl.fr

Abstract:
Epstein-Barr virus (EBV) contributes to oncogenesis as evidenced by its ability to transform normal B lymphocytes into permanently growing lymphoblastoid cell lines (LCLs) and by its frequent detection in a number of human tumors, often displaying a type II latency program. In our laboratory, we have obtained two cell lines with such a type of latency. These cells are of monocytic (TE1) and T-lymphocytic (NC5) origin and are dependent on LMP1 for their survival and proliferation. LMP1, one of the proteins expressed during this latency (and type III latency), has been described as the major oncogene of EBV. This integral membrane protein is functionally related to the tumor necrosis factor receptor family. The main signal transduction pathways triggered by this protein are NFkB and JNK. However, their influence has never been tested on the expression of LMP1 itself.
In order to test these two major pathways, we first studied the expression of LMP1 in the presence of NFkB or JNK inhibitors in two different types of EBV-infected and LMP1-positive cells (LCLs and TE1). In this way, we could show that these two pathways worked in opposite manner: inhibition of NFkB permit s a better expression of LMP1, whereas the inhibition of JNK results in a strong diminution of LMP1. To further detail this balance between these two pathways implicated in the regulation of LMP1 expression, we performed reporter gene transactivation assays by transient transfections in HEK293 cells. Using various LMP1 mutants, dominant negative and positive of NFkB and JNK pathways, we have demonstrated that the LMP1 TES2 domain, particularly via the MKK7 module of the JNK pathway, allowed the autoactivation of LMP1. On the other hand, the LMP1-induced NFkB pathway mediated the autoinhibition. Finally, a dominant negative approach with a truncated LMP1 (LMP1-TM) in one of our EBV type II latency model (NC5) followed by a FACS analysis permit us to show that the global inhibition of all the LMP1 induced pathways leads to a better expression of LMP1 at the cell membrane.
Altogether, our results suggest that the LMP1-induced NFkB and JNK pathways function as a balance to regulate the LMP1 oncogene. However, since this balance seems to result in an autoinhibition of LMP1, the exact mechanism responsible for the sustained LMP1 expression in the EBV type II latency infected cells is still to be elucidated.

Interaction of Epstein-Barr virus encoded EBNA-3 protein with the Aryl hydrocarbon receptor-XAP2 complex

Kashuba E, Kazlauskas A, Szekely L, Poellinger L, Klein G

Contact:
Dr. Elena Kashuba
Microbiology and Tumor Biology Center
Karolinska Institute (Stockholm, Sweden)
Email: Elena.Kashuba@mtc.ki.se

Abstract:
Objective: The specific interaction of EBNA-3 with AhR-XAP2 comlex

Methods: Co-immunoprecipitation, transfection, immunostaining

Results: EBNA-3 is one of the EBV encoded nuclear antigens that is indispensable for transformation and maintenance of B-lymphocytes. Molecular mechanisms that underlie EBNA-3 function are poorly understood. Recently we have shown that EBNA-3 interacts with an immunophilin-like protein XAP2/ARA9/AIP, which is found in mammalian cells in association with the latent aryl hydrocarbon/dioxin receptor (AhR/DR). AhR is a ligand-inducible transcription factor that mediates cell response to environmental pollutants such as dioxins. In this study we have demonstrated the specific interaction of the EBV protein EBNA-3 with AhR. The stability of this interaction is determined by the activation state of AhR and by expression levels of XAP2 protein. XAP2 seems to have a higher affinity to AhR than EBNA-3. Therefore, the intracellular localization pattern of the non-activated AhR complies with the dominant effect of XAP2, which re-distributes AhR to the cytoplasm. However, in the presence of ligand (dioxin, TCDD), XAP2 effects on AhR are overcome by EBNA-3, which facilitates AhR nuclear translocation. Searching for the physiological significance of the interaction between EBV and AhR-dependent cellular pathways, we found that EBNA-3 counteracts the inhibitory TCDD effect on cell growth in EBV-infected B-lymphocytes.

Conclusions: The results allowed us to speculate that the EBNA-3-AhR interaction may be part of EBV's growth transformation strategy, established to divert TCDD-AhR-dependent negative effects on cell proliferation and survival.

EBNA-6 regulates Rb levels through the interaction with the new Rb binding S18-2 protein

Kashuba E, Snopok B, Yurchenko M, Szekely L, Klein G

Contact:
Dr. Elena Kashuba
Microbiology and Tumor Biology Center
Karolinska Institute (Stockholm, Sweden)
Email: Elena.Kashuba@mtc.ki.se

Abstract:
Objective: Identification of the EBNA-6 binding proteins

Methods: Yeast two-hybrid system, GST pull down assay, surface plasmon resonance (SPR), transfections, immunostainings.

Results: Using the yeast two-hybrid system we have identified an EBNA-6 binding protein S18-2. We have confirmed the interaction between EBNA-6 and S18-2 by GST-pull down assay and surface plasmon resonance (SPR) method. We observed that over-expression of mainly cytoplasmic S18-2 led to cell death. EBNA-6 co-expression partially inhibited the cell death. EBNA-6 recruited the portion of S18-2 to the nucleus and these two proteins co-localized in the nuclear speckles. We have shown that S18-2 binds to Rb using GST pull down assay and SPR. The level of Rb protein was decreased significantly in the S18-2 over-expressing cells. When EBNA-6 is co-expressed with S18-2, the level of Rb remains quite high. We have observed the EBNA-6 - Rb - S18-2 triple protein complex formation, however, EBNA-6 bound to S18-2 lost its ability to interact with Rb.

Conclusions: We have found new EBNA-6 binding protein S18-2. We have shown the triple protein complex formation, in which EBNA-6 and S18-2 are bound to Rb. We propose that EBNA-6 may regulate Rb levels and cell cycle progression through the binding to S18-2.

RNA polymerase II C-terminal domain kinases play a role in the regulation of transcription by EBNA 3C and EBNA 2

Bark-Jones SJ, West MJ

Contact:
Sophie Bark-Jones
University of Sussex
School of Life Sciences (Brighton, UK)
Email: S.J.Bark-Jones@sussex.ac.uk

Abstract:
Objective: To investigate whether the RNA polymerase II (pol II) C-terminal domain (CTD) kinases CDK7, 8 and 9 play a role in the regulation of transcription by the EBNAs.

Methods: Co-expression and immunoprecipitation of CTD kinases and EBNAs in vivo, CTD kinase assays and an examination of the effects of expressing dominant negative mutants of CTD kinases on EBNA 2-activated transcription.

Results: Phosphorylation of the CTD of RNA polymerase II is required for efficient transcriptional initiation and elongation and an increasing number of studies have shown that CTD kinases can be recruited and/or activated by viral and cellular transcriptional activator proteins. We have shown that the EBV transcriptional regulator EBNA 3C associates with CDK7, 8 and 9 in vivo. Our studies to date indicate that the N-terminus of EBNA 3C including the bZIP domain is not required for these interactions and that the site of interaction lies C-terminal to amino acid 397. EBNA 3C does not appear to affect the CTD kinase activity of these CDKs making it likely that this association reflects the ability of EBNA 3C to recruit these kinases to the transcription complex. In addition, although EBNA 2 does not appear to directly associate with these kinases, using dominant negative mutants we have shown that the kinase activity of CDK9, but not that of CDK8, is required for the activation of transcription by EBNA 2. We are currently using chromatin immunoprecipitation techniques to examine the composition of transcription complexes and the phosphorylation status of the CTD at EBNA 2-activated promoters in vivo.

Conclusions: Our data provide the first evidence to implicate CTD kinases in the regulation of transcription by the EBNAs. The association of EBNA 3C with CDK7, 8 and 9 suggests that EBNA 3C may be able to recruit these kinases to EBNA 3C-regulated promoters and modulate the phosphorylation state of the CTD of pol II. We also show that EBNA 2-activated transcription is CDK9-dependent suggesting that CDK9 forms part of the transcription complex assembled in the presence of EBNA 2. Since CDK9 is the only CTD kinase that has been shown to have a role in the activation of transcription elongation in addition to initiation these data raise the possibility that EBNA 2 acts to promote the processivity of transcription complexes in addition to increasing initiation rates.

Complex EBV genome study with a murine model system.
Zychlinska M, Herrmann H, Strobl U, Hammerschmidt W

Contact:
Magdalena Zychlinska
GSF-National Research Center for Environment and Health
Gene Vectors (Muenchen, Germany)
Email: zychlinska@gsf.de

Abstract:
The aim of this project is to gain information on how EBV influences B cell differentiation, with the special interest in how it reaches and modifies memory B cells, which is a controversial issue nowadays. The project consists of two parts: in vitro and in vivo. The first part allows one to investigate the virus-cell interactions in EBV-infected B-cells at different stages of differentiation. The second part enables a study on EBV in the context of the whole murine organism, and it could provide an animal model for EBV-related lymphoma.

Epstein-Barr Virus (EBV) is involved in many human malignancies via its latent gene products, which interact with cellular proteins and mimic discrete functions of cellular signaling pathways. Up to now, studies on EBV have been carried in vitro and ex vivo only (LCLs - human lymphoblastoid cell lines). This limited tool set is due to the fact that EBV cannot infect animal cells, which lack the receptor proteins for EBV on their surface. It is already known that the main target of EBV infection is the human B cell compartment.

The project relies on the conditional regulation of the almost all known viral latent genes, which are currently believed to be involved in the growth transformation (or in vitro immortalization) of a host B cell. There are two loxP sites located such that when Cre recombinase inverts the fragment in between them, the functional expression of latent EBV genes is blocked by disruption of exon/intron boundaries or interruption of transcriptional units. Such plasmid is introduced into murine Embryonic Stem (ES) cells, which are further either differentiated to B-cells in vitro, or introduced into a blastocyte to create a mouse having EBV genes in each cell. At this stage, expression of latent proteins is switched on by re-inversion of the loxP flanked fragment (delivery of Cre again). Such system allows one to study virus-host interactions in variety of aspects: at different stages of B cell development, in specific cell compartments, at desired stages of organ development.

LMP1 expression induced by cytokines in EBV positive NK malignant cell lines

Takahara M, Kis LL, Nagy N, Liu A, Klein E

Contact:
Dr. Miki Takahara
Microbiology and Tumorbiology Center
Karolinska Institute (Stockholm, Sweden)
Email: miki.takahara@mtc.ki.se

Abstract:
Objective:Most nasal NK/T cell lymphoma are EBV positive, therefore it can be assumed that the virus is an important factor in tumor development. The EBV expression pattern in the cell population is usually mixed, the tissue contains Type I and Type II cells. While LMP-1 expression is known to be regulated by EBNA-2 and it is pivotal for immortalization of B-cells, regulation of LMP-1 and its role in NK/T tumors is not clarified. The cell lines developed from NK tumors express LMP1 and require IL2 for propagation except one line YT which has Type I pattern and can be grown in absence of IL2. Since it is known that STATs bind to the LMP-1 promoter, it is possible that cytokines regulate LMP-1 expression. Cytokines can be provided to the infected cells by the granulomateous tissue in vivo, these may contribute therefore to the development of malignancy. We studied the influence of various cytokines on the proliferation and LMP-1 expression of 3 NK cell lines.

Methods: SNK6, established from nasal NK cell lymphoma, KAI3, originated from chronic active EBV infection, and YT, obtained from acute lymphoblastic lymphoma were used in the experiments. The lines were cultured for 48 hrs in medium without cytokines or with IL2, IL4, IL10, IL13, or IL15. Total cell lysates were analysed for LMP-1, EBNA-2, and Bcl-2 by Western blotting. The proliferative response of the cell lines was examined by thymidine incorporation for the last 12h of 48 hrs incubation period. In SNK6 and KAI3, the proliferation assay was performed after 24 hrs starvation of IL2.

Results: IL2, IL4, IL10, and IL15 increased LMP-1 expression in SNK6 and KAI3. They did not induce EBNA-2. Bcl-2 was expressed in all lines, it was not influenced by the cytokines. IL2 and IL15 but not IL4 and IL10 enhanced level of thymidine uptake in SNK6 and KAI3. In YT, cytokines did not induce LMP-1, and did not influence the uptake.

Conclusions: Several cytokines could enhance LMP-1 expression, and may contribute therefore to the type II latency pattern in EBV positive NK malignant lines. It could be expected that external stimulation maintain the type II latency also in vivo. LMP-1 expression enhanced by IL4 and IL10 did not affect proliferation in NK-cell lines. It is suggested that LMP-1 alone is insufficient to drive the proliferation in NK lines.

Distinct Subsets of Primary Effusion Lymphoma Can Be Identified Based on Their Cellular Gene Expression Profile and Viral Association

Fan W, Bubman D,Chadburn A, Harrington WJ, Knowles DM, Cesarman E

Contact:
Dr. Ethel Cesarman
Weill Medical College of Cornell University
Pathology and Laboratory Medicine (New York, USA)
Email: ecesarm@med.cornell.edu

Abstract:
Objective: Primary effusion lymphomas (PEL) are associated with infection by KSHV. While KSHV is essential for the development of PEL it is not sufficient. It does not transform B cells in vitro. Most PELs have concomitant EBV infection, suggesting that EBV is an important co-factor in PEL development, although little or no expression of EBV transforming genes is found in PEL. Other cofactors, such as cellular genetic alterations are likely to play a role in PEL development but are currently unknown. Lymphomatous effusions also occur that lack KSHV infection, but in the setting of HIV infection are frequently infected by EBV. This study was designed to address the viral impact on cellular gene expression and in the pathogenesis of lymphomatous effusions.

Methods: We compared the gene expression patterns of KSHV-positive PELs with that of KSHV-negative effusion lymphomas. We further subdivided the KSHV-positive category into EBV-positive and EBV-negative subcategories, and compared their gene expression patterns. RNA from nine cell lines, three from each group, was examined in duplicate for gene expression using the Affymetrix microarray technique. RNA extracted from three primary samples was also tested and compared to that obtained from cell lines.

Results: The greatest divergence was found between KSHV-positive and -negative lymphomatous effusions, where close to 500 genes were differentially expressed. Among these genes were cell cycle regulators, transcription factors and signal transduction regulators. Among the KSHV-positive PELs, there were also clear differences between those containing and those lacking EBV-coinfection, as identified by unsupervised clustering. We found 40 genes that are significantly differentially expressed according to several different analytical procedures. Among these genes, five genes that are regulators of the MAP kinase pathway were up-regulated in the KSHV-positive, EBV-negative PEL group.

Conclusions: Our results indicate that KSHV plays an important role in the pathogenesis of PELs, as its presence selects for a very distinct cellular gene expression category and a clearly different lymphoma type. Within the KSHV-positive PELs, the effect of EBV is more subtle but nevertheless clear. Our results suggest that in the absence of EBV infection, activation of the MAP kinase pathway.

Expression Of MHC Class I And II In Gastric Carcinomas N.O.S. In Relation To T-Cell Infiltrate: Differences Between Epstein Barr Virus Positive And Negative Tumours

van Beek J, Bloemena E, Snel A, Vos W, van de Velde CJ, Kranenbarg EK, Meijer CJLM, Middeldorp JM

Contact:
Prof. Dr. Jaap M Middeldorp
VU Medical Center
Pathology (Amsterdam, The Netherlands)
Email: j.middeldorp@vumc.nl

Abstract:
Epstein Barr Virus (EBV) is associated with several benign and malignant diseases, amongst which gastric adenocarcinomas (GCs). We have previously demonstrated, in a large Dutch cohort of GCs, collected between 1989 and 1993 in the course of a large randomised multicenter surgical study (D1D2), that the incidence of EBV-positive GCs was 7.2% (41 of 566 patients; J. van Beek et al., J. Clin. Oncol. (2004) 22, 664-670). Remarkably, EBV-associated tumours had a significantly lower frequency of lymph node metastases (63.4% N0) compared to EBV-negative tumours (38,7% N0) (P=0.034). In the present study we investigated whether the lower frequency of lymph node metastases could be attributed to differences in expression of MHC molecules and/or T cell infiltrate between EBV-positive and negative tumours, reflecting possible immune stimulation by the presence of non-self, virus related antigens.
Formalin-fixed, paraffin embedded material of primary tumours (EBV+: n=20; EBV-: n=28) were stained with the antibodies HCA2, HC10 (MHC I), beta-2-microglobulin (MHC I), LN3 (MHC II), CD3 (pan T), CD4, CD8, and GB7 (granzyme B). Results were scored semi-quantitatively.

EBV-associated GCs were significantly more often positive for MHC cl II (EBVpos=12/20 vs EBVneg=8/28) (P=0.029), had a more extensive T cell infiltrate (P= 0.0001), with a higher relative contribution of CD8+ cells (P=0.022) of which a higher percentage displayed an activated (GB7 positive) phenotype (P=0.028).
These results reveal an active local inflammation in EBV bearing GC compared to EBV negative GC, leading to MHC cII upregulation on the tumour cells and being paralleled by a significant T-cell infiltration/activation yielding a local cytotoxic immune response, presumably directed against EBV related antigens on the neoplastic cells. These findings are in line with the improved disease-free survival rates in these patients and suggest an active role of local anti-EBV immunity in preventing GC metastasis to regional lymph nodes.

Loss of EBV-specific CD4+T cells during progression to EBV-related AIDS non-Hodgkin Lymphoma: restoration by highly active antiretroviral therapy (HAART)

Piriou E, van Dort K, Nanlohy N, van Oers M, Miedema F, van Baarle D

Contact:
Erwan Piriou
Sanquin at CLB
Clinical Viro-Immunology (Amsterdam, The Netherlands)
Email: e.piriou@sanquin.nl

Abstract:
Objective: EBV-specific CD8+ T cells have been extensively studied in various settings, and appear to play a major role in the control of EBV-related malignancies. In contrast, it is still unclear whether EBV-specific CD4+ T cells play a role in vivo. To study this question, an assay was developed to measure the CD4+ T-cell response towards two EBV antigens, in both healthy and HIV-infected subjects. In addition, both HAART-treated and untreated HIV+ individuals - including progressors to EBV-related lymphoma - were studied longitudinally.

Methods: EBV-specific CD4+ T cells were stimulated with peptide pools from latent protein EBNA1 and lytic protein BZLF1, and detected by measurement of IFNg-production.

Results: After direct ex vivo stimulation, EBNA1 or BZLF1-specific IFNg- (and/or IL2) producing CD4+ T cell numbers were low, and measurable in less than half of the subjects studied (either HIV- and HIV+). Therefore, PBMC were cultured for 12 days in the presence of peptides and IL2 (from day 3), and then restimulated with peptides, allowing specific and reproducible expansion of EBV-specific CD4+ T cells, independent of HLA type and ex vivo antigen processing. Interestingly, numbers of EBV-specific CD4+ T cells inversely correlated with EBV viral load, implying an important role for EBV-specific CD4+ T cells in the control of EBV in vivo. Untreated HIV-infected individuals had a lower CD4+ T cell response to both EBNA1 and BZLF1 as compared to healthy EBV carriers and HAART-treated HIV+ subjects. In longitudinal samples, EBNA1-specific, but not BZLF1-specific T-cell numbers increased after HAART, while EBV load was not affected by treatment. In all the progressors to EBV-related lymphoma, EBV-specific CD4+ T cells were lost at least 24 months before lymphoma diagnosis.

Conclusions: Both cross-sectional and longitudinal data suggest an important role for EBV-specific CD4+ T cells in the control of EBV-related malignancies. Furthermore, it seems that HAART treatment leads to recovery of EBNA1-specific, but not BZLF1-specific CD4+ T-cell responses, implying changes in the reactivation pattern of EBV, despite an unaltered cell-associated EBV DNA load. Thus, early HAART treatment might prevent loss of specific CD4+ T-cell help and progression to NHL.

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The Frequency and Characterization of Ig-null CD19+ B Cells in Pediatric Solid Organ Transplant Recipients

Schauer E, Green M, Webber S, Rowe DT

Contact:
Elizabeth Schauer
University of Pittsburgh
Infectious Diseases and Microbiology (Pittsburgh, USA)
Email: emsst63@pitt.edu

Abstract:
Background: The pediatric solid organ transplant recipient is at an elevated risk for post-transplant lymphoproliferative disease.Transplanted children who exhibit no symptoms of PTLD often carry significantly elevated viral loads of EBV in their peripheral blood. The viral DNA is carried in B cells which may have high and low numbers of EBV genomes per cell. Low load carriers typically have only low copy cells while high load carriers have low and high copy cells. Furthermore, the majority of high copy cells appear to be Ig-null, bearing no detectable surface expression of immunoglobulin.

Methods: In this study, we further examine the Ig-null population of B cells in high, low and non-detectable load-carrying transplant recipients. The frequency of Ig-null cells in these 3 populations was determined by flow cytometry.Rare high copy cells from high load patients were sorted and further examined by QC-PCR and ISH. PBMCs from the high load patient population were examined by 4-color flow cytometry for the surface expression of MHC Class I, CD5, CD20, and CD27.

Results: Ig-null cells were detected in every patient examined. The frequency of Ig-null cells was highest in the high load patients, with an average of 4.03% of all CD19+ B cells having no detectable sIg.Low load patients had the second highest frequency, with 1.37%, followed by ND patients, who had just 0.6% of their CD19+ B cells represented as Ig-null cells. QC-PCR results on sorted cells from high load patients suggest that the bulk of the EBV load is indeed carried in the Ig-null population. Four-color flow cytometry for MHC Class I, CD20, CD5, and CD27 was used to further examine the Ig-null cells in the circulation of high load patients. On average, one third of CD19+ Ig-null cells were also MHC Class I negative. The CD19+, sIg- cells were shown to be CD27-, CD5-, and approximately one half of these cells were CD20-.

Conclusions: In normal latency, the EBV load is carried in the resting memory B2 compartment. We suggest that in immunocompromised patients, the bulk of the elevated viral load is carried in an aberrant B cell with the phenotype CD19+, sIg-, CD5-, CD27-, ClassI+/-, and CD20+/-. Efforts aimed at targeting this cell type for elimination may help reduce the risk of PTLD. Additional studies to more thoroughly characterize the origins of high copy cells are ongoing.

Mechanisms underlying the antiproliferative effects of retinoic acid in EBV-immortalized B lymphocytes: further support for a therapeutic role in the management of EBV-related lymphoproliferations

Dolcetti R, Zancai P, Guidoboni M, Dal Col J, Cariati R, Rizzo S, Boiocchi M

Contact:
Dr. Riccardo Dolcetti
C.R.O. - National Cancer Institute, Immunovirology and Biotherapy Unit
Dept. of Pre-Clinical and Epidemiological Research (Aviano, Italy)
Email: rdolcetti@cro.it

Abstract:
We have previously shown that retinoic acid (RA) potently inhibits the proliferation of EBV+ LCLs by up-regulating the CDK inhibitor p27. To evaluate more thoroughly the therapeutic potential of RA, we have investigated the mechanisms by which RA up-regulates p27 in B-cells. Moreover, considering that IL-6-mediated B-cell growth promotion is involved in the pathogenesis of EBV+ lymphoproliferations of immunosuppressed patients, the effects of RA on IL-6 signalings were also analyzed. Here we show that RA inhibits ubiquitination and proteasome-dependent degradation of p27, a phenomenon that is associated with down-regulation of Thr187 phosphorylation of the protein whereas the phosphorylation on Ser10 is unaffected. Furthermore, RA down-regulated the expression of p45Skp2 and Cks1, two essential components of the SCF ubiquitin ligase complex that targets p27Kip1 for degradation. Down-regulation of p45Skp2 and Cks1 is due to enhanced proteasome-mediated proteolysis of these proteins. Moreover, overexpression of p45Skp2 prevented p27 protein accumulation and promoted resistance to the antiproliferative effects of RA, indicating that down-regulation of p45Skp2 is a key element underlying RA-induced p27 stabilization in B cells.

RA was also able to down-regulate IL-6-receptor (IL-6R) components with agonist activity (membrane and soluble gp80) and increase the levels of soluble gp130, an IL-6 antagonist, effects mainly mediated by RARI±. Moreover, RA increased IL-6 production as a result of enhanced IL-6 mRNA expression. RA did not abolish IL-6-mediated phosphorylation of gp130, whereas JAK1 phosphorylation induced by IL-6 were markedly inhibited. Consistently, IL-6-induced STAT3 phosphorylation and activation were also inhibited by RA. Overall, the effects of RA resulted in the induction of a complete resistance of LCLs to IL-6-mediated growth promotion. Conversely, RA did not inhibit the constitutive activation of JAK1, TYK2, STAT3 and ERK1/2, ruling out that the JAK/STAT and MAPK pathways may mediate the antiproliferative activity of RA. The findings that RA severely impairs IL-6-dependent signalings in LCLs and inhibits their growth despite the presence of constitutively active JAK/STAT and MAPK cascades provide additional support for a role of RA in the prevention and treatment of EBV-related lymphoproliferative disorders of immunosuppressed patients.

Monitoring of Epstein-Barr virus (EBV) DNA load after hematopoietic stem cell transplantation for prevention, early diagnosis as well as antiviral and immune therapy of EBV-associated lymphoproliferative diseases

Meerbach A, Gruhn B, Hafer R, Zintl F, Wutzler P

Contact:
Dr. Astrid Meerbach
Friedrich Schiller University Jena
Institute of Virology and Antiviral Therapy (Jena, Germany)
Email: Astrid.Meerbach@med.uni-jena.de

Abstract:
Objective: The development of a life-threatening EBV-associated lymphoproliferative disease (PTLD) is a serious complication in patients after hematopoietic stem cell transplantation (HSCT). Monitoring of viral load is a useful and sensitive parameter in the surveillance of EBV reactivation for prevention and treatment of EBV-associated PTLD.

Methods: A semiquantitative PCR for evaluating EBV-genome copy numbers in plasma and peripheral blood mononuclear cells (PBMC) was established. The method bases on a nested PCR using primers of the structural protein region p23 and an end-point dilution. Using this assay in 65 patients undergoing HSCT EBV DNA load was prospectively screened weekly after transplantation.

Results: EBV reactivations (>1,000 EBV-genome copies measured in 100,000 PBMC) were observed in 11 patients (16.9%). Three patients developed PTLD with extremely high EBV-genome copy numbers in PBMC (>100,000) and plasma. The rapid increase of EBV-genome copies occurred 1-4 weeks before the onset of the disease. After combined antiviral and immune therapy 2 of 3 patients showed a dramatic decrease of EBV load and survived, while the third patient died of lymphoma. A subclinical EBV reactivation was observed in 5 cases with EBV-genome copies ranging from 1,000 to 10,000. After reduction of immunosuppression the EBV levels normalised. In three patients the high copy number of >10,000 and plasma positivity prompted the physicians to start pre-emptive therapy with rituximab and cidofovir for prevention of EBV-associated PTLD. After drug administration the high EBV load in plasma and PBMC reduced dramatically. The decrease of EBV-genome copies was associated with a decrease of B lymphocytes and an increase of CD8+ and CD4+ T lymphocytes. The 54 patients who had copy numbers of <1,000 did not develop EBV-associated PTLD.

Conclusions: Monitoring of EBV DNA load is a useful method for early diagnosis and treatment of EBV-associated PTLD as well as for the follow-up of therapy efficacy.

Epstein-Barr virus type I EBNA3A and EBNA3C natural sequence variants in primary infections and in immunocompromised patients

Gorzer I, Niesters HGM, Cornelissen J, Steininger C, Puchhammer-Stockl E

Contact:
Dr. Irene Görzer
Medical University of Vienna
Institute of Virology (Vienna, Austria)
Email: irene.goerzer@meduniwien.ac.at

Abstract:
Objective: After primary infection with Epstein-Barr virus (EBV), the virus persists lifelong and has the ability to transform B cells, which can result in the development of B cell lymphoma. In EBV-associated posttransplant lymphoproliferative disorders (PTLDs) a characteristic set of latent genes, six nuclear antigens (EBNAs) and three membrane antigens (LMPs) are expressed. In this study, we have determined naturally occurring sequence variants in defined regions of EBNA3A and EBNA3C. These are both transcriptional regulatory proteins and are immunodominant CTL (cytotoxic T-cell)-targets during persistent EBV infection. We have also identified the associated LMP1 variations to assess linkages among these latent genes and a possible association to PTLD.

Methods: DNA sequences of defined regions of the EBNA3A, EBNA3C, and LMP1 genes were analyzed in EBV strains obtained from the blood of 40 cases of primary infections, of 50 posttransplant patients with (n=13) and without (n=37) PTLD, and of 9 non-transplant patients with EBV-associated lymphoma.

Results: The identification and linkage of distinct EBNA3A and EBNA3C variants resulted in the definition of four EBNA3 subtypes present in primary infection as well as during reinfection/reactivation in immunocompromised patients. The distribution of EBV strains occurred at similar frequency. Specific substitution mutations are localized in immunodominant CTL-epitopes of EBNA3A and EBNA 3C. Among EBNA3 subtypes and LMP1 groups preferential linkages were observed. A possible linkage of EBNA3/LMP1 combinations to PTLD is shown.

Conclusions: The combined effects of specific amino acid changes identified in certain variants of EBNA3A, EBNA3C, and LMP1 might influence the development of EBV-associated malignancies.

Gastritis and Gastric Cancer Tissues Commonly Harbor Latent EBV

Ryan JL, Morgan D, Shen YJ, Thorne LB, Elmore SH, Gulley ML

Contact:
Julie Ryan
University of North Carolina School of Medicine
Pathology and Laboratory Medicine (Chapel Hill, USA)
Email: jryan@med.unc.edu

Abstract:
Background: Gastric adenocarcinoma is the second most common type of cancer and the second leading cause of cancer death worldwide. Incidence varies by geographic region, and Epstein-Barr virus (EBV) is present in the malignant cells of about 10% of cases. It is unclear whether EBV infects benign gastric epithelium or lesions that predispose to cancer, such as gastritis.

Methods: To explore these issues, we examined 75 gastric adenocarcinomas (including 36 from the United States and 39 from Honduras where incidence is much higher) and 91 non-neoplastic gastric specimens (2 normal gastric mucosa, 89 gastritis) using a battery of sensitive and specific quantitative real-time PCR (Q-PCR) assays targeting disparate parts of the EBV genome (BamH1W, EBNA1, LMP1, LMP2, BZLF1, EBER1). PCR-positive specimens were further evaluated by histochemical assays targeting latent (EBER1) and lytic (BZLF1, BMRF1) viral proteins to localize the viral infection.

Results: EBV DNA was detected by Q-PCR in 21/36 (58%) US gastric cancers and 39/39 (100%) Honduran gastric cancers. EBER1 was localized to malignant epithelial cells in 3/36 (8%) US gastric cancers and 3/39 (8%) Honduran gastric cancers, suggesting that the high incidence of cancer in Honduras is not attributable to a high prevalence of EBV-positive cancers. Viral loads were considerably higher in the EBER1-positive cancers (mean level 133,000 versus 80 EBV copies per 100,000 cells). One infected cancer selectively failed to amplify the LMP2 gene due to a point mutation in the primer sequence targeted by the Q-PCR assay. EBV DNA was not detected in the two normal gastric mucosa specimens, but low level EBV (mean level 40 EBV copies per 100,000 cells) was detected in 41/89 (46%) gastritis lesions by Q-PCR. Histochemical assays localized latent virus to lymphocytes, not benign epithelial cells. No lytic viral protein expression was detected in benign or malignant lesions.

Conclusions: These findings suggest that EBV commonly infects inflamed gastric mucosa and localizes to lymphocytes where it expresses EBER1 but not replicative viral proteins. EBV viral load is much lower in gastritis lesions than in EBV-related gastric cancers.

Real-Time PCR Measures Epstein-Barr Virus DNA in Archival Breast Adenocarcinomas
Thorne LB, Ryan JL, Elmore SH, Glaser SL, Gulley ML

Contact:
Dr. Leigh Thorne
University of North Carolina - Chapel Hill
Pathology (Chapel Hill, USA)
Email: lthorne@unch.unc.edu

Abstract:
The role of Epstein-Barr virus (EBV) in breast cancer pathogenesis remains controversial. Fifty-five cases of paraffin-embedded, formalin-fixed invasive breast cancer were screened for the presence of EBV using quantitative polymerase chain reaction (PCR) directed at five different targets within the EBV genome (BamH1W, LMP1, EBNA1, LMP2, and BZLF1 regions). In four tumors (7%), low level EBV DNA was detected by at least one of the assays, with levels of up to 11 copies of EBV DNA per 100,000 cells. Immunohistochemistry for viral BMRF1 and BZLF1, and in situ hybridization for lytic gene transcripts showed no evidence of replicative EBV gene expression. Lymphocytes and malignant cells were also negative for latent infection by EBER in situ hybridization. Laser capture microdissection followed by quantitative real-time PCR was not useful in localizing EBV DNA to malignant cells or bystander lymphocytes. In conclusion, EBV DNA is detectable in a fraction of breast cancer specimens using real-time PCR as a screening tool, albeit at quite low levels which suggests that only rare cells are infected. The low levels probably confounded our ability to localize the virus to particular cell types or to characterize viral gene expression.

Epstein-Barr Virus and Cytomegalovirus in Marrow of Patients with Myelodysplastic Syndrome

Fan H, Ryan JL, Dunphy CH, Elmore SH, Gulley ML

Contact:
Dr. Hongxin Fan
University of North Carolina
Pathology (Chapel Hill, USA)
Email: hfan@unch.unc.edu

Abstract:
Objective: The etiology of myelodysplastic syndrome (MDS) is uncertain and is likely to be multifactorial. Epstein-Barr virus (EBV) and cytomegalovirus (CMV) have been implicated in the pathogenesis of several other hematological diseases, yet little is known about their prevalence in marrow of MDS patients. Recent technologic advances now permit accurate measurement of viral DNA levels by quantitative real-time polymerase chain reaction (Q-PCR). The goal of this study was to examine EBV and CMV viral load in paraffin-embedded bone marrow samples of MDS patients.

Methods: Twenty-eight MDS patients were studied including 19 de-novo and 9 secondary MDS. EBV and CMV viral loads were assayed in the paraffin-embedded marrow clot and core biopsy samples using a battery of TaqMan Q-PCRs targeting six disparate, but highly conserved segments of the EBV genome (the BamH1W reiterated segment, and the EBNA1, LMP1, LMP2, BZLF1 and EBER1 genes) and two highly conserved CMV segments (immediate early gene and polymerase genes). Viral load was calculated as the number of viral genome copies per 100,000 cells, with cell number estimated based on human APOB gene copy number by Q-PCR.

Results: The recovery of extracted DNA was higher in marrow clots than in the decalcified marrow core biopsies, most likely due to interference by the decalcification step required for the marrow core biopsy processing. EBV DNA was detected at low levels in marrow clots of 13/28 MDS patients (range up to 611, mean 45 EBV copies per 100,000 cells). CMV was not detected in any of the marrow clots of the 28 MDS patients.

Conclusions: Bone marrow clots were superior to the decalcified marrow core biopsies in terms of recovery of adequate amounts of extracted human DNA from paraffin-embedded tissues. The low EBV viral loads detectable in about half of MDS marrow samples are consistent with what might be expected if there were latent EBV infection of a small fraction of lymphocytes. This outcome, combined with the lack of CMV DNA in any of the 28 cases, suggests that EBV and CMV do not play a major role in the pathogenesis of MDS.

Effect of Epstein Barr virus on mammary epithelial cells

Arbach H, Cochet C, Ramirez V, Lefeu F, Jauliac S, Mourah S, Hutt-Fletcher L, Mauviel A, Calvo F, Joab I

Contact:
Haratech Arbach
Laboratoir de Pharmacologie Experimentale et Clinique (Paris, France)
Email: Haratech.Arbach@stlouis.inserm.fr

Abstract:
Objective: Epstein- Barr virus (EBV), a human herpesvirus associated with some epithelial tumors, such as poorly differentiated nasopharyngeal carcinoma and a proportion of gastric carcinoma. Several studies have reported the presence of EBV genome and transcripts in a fraction of tumor cells in a subset of breast cancers. Our objective is to evaluate the possible impact of EBV upon mammary tumor development in the in vitro model which we have developed.

Methods: We have established an in vitro EBV infected mammary epithelial cell line using recombinant EBV-neo-gfp. In order to investigate the effect of EBV infection on gene expression profile in the cells, cDNA array experiments were performed using Atlas Human Cancer 1.2 K array (Clontech). The expression of candidate genes affected by EBV was confirmed by semi quantitative RT-PCR. Moreover, functional transforming tests were performed, such as anchorage-dependent growth, migration and invasion assays.

Results: Our results show that EBV persist in the mammary epithelial cells, and that this persistence is associated with overexpression of genes considered as poor prognostic markers of breast cancer (Ki67, CD73 and MMP14), in addition to c-Jun, cyclooxygenase-2 and a deleted isoform of p63. Moreover, clones of EBV infected cells exhibited large growing colonies in soft agar compared to non-infected cells. The supernatant from these EBV-infected cells stimulated cell migration and invasion of non infected cells.

Conclusions: Our data indicate that EBV is able to latently infect mammary epithelial cells and modulate the expression of genes implicated in tumor progression. Moreover, EBV infection of a particular subset of mammary cells led to enhancement of cell invasion and migration.

Circulating EBV DNA and RNA in patients with asymptomatic HIV-infection

Stevens SJC, Verkuijlen SAWM, Smits PHM, Middeldorp JM

Contact:
Dr. Servi Stevens
Vrije Universiteit Medical Center
Department of Pathology (Amsterdam, The Netherlands)
Email: s.stevens@vumc.nl

Abstract:
Quantitative monitoring of circulating EBV-DNA has little diagnostic value for predicting EBV-driven ARNHL, as approx. 25% of asymptomatically infected HIV-carriers show elevated EBV-DNA loads in whole blood, even on HAART. This study aimed 1) to determine the distribution of elevated EBV loads over the blood compartments of HIV-carriers and 2) to investigate differences in EBV mRNA transcription in blood of ARNHL versus HIV-carriers as putative tumour marker. The nature of circulating EBV DNA was investigated by quantifying EBV loads in simultaneously obtained whole blood and plasma samples from 14 asymptomatic HIV-carriers using a 213 bp and 99bp EBNA1-derived amplicon in LightCycler-based real-time PCR. EBV DNA loads ranged from 2,800-89,400 copies/ml blood. Only the HIV carrier with highest EBV load in whole blood had detectable EBV DNA in plasma (4,200 copies/ml). All other plasma samples were below cut-off in either 213 or 99 bp assay. This indicates that circulating EBV DNA is not derived from virions or damaged cells, but is B-cell-associated. Using RT-PCR and NASBA, expression of BamHI-A rightward transcripts (BARTs) was found in 11/14 blood samples from HIV-carriers. One also had mRNA encoding EBNA1 and LMP2, but mRNA for the EBV oncogene LMP1 was never found. In 16 follow-up whole blood samples from 3 ARNHL patients (EBV DNA loads ranging from 2,000-120,000 copies/ml), BARTs were detected in 6/16 samples, 2/16 had EBNA1 expression and 2/16 showed LMP2 expression. Again, LMP1 mRNA was not present. We conclude that 1) circulating EBV DNA in HIV-carriers is largely cell-associated, 2) (qualitative) EBV mRNA monitoring in WB has no diagnostic value for ARNHL prediction and 3) despite elevated EBV DNA loads, viral latent mRNA expression in blood of asymptomatic HIV-carriers and ARNHL-patients is restricted, with only frequent expression of BARTs. This resembles EBV mRNA expression in healthy EBV-carriers, where circulating cells are transcriptionally quiescent but abundantly express non-coding BARTs. We are currently initiating studies on EBV mRNA profiling and immunocytochemistry in isolated B-cell fractions for more sensitive determination of viral latency gene expression in the circulation of these patients in relation to B-cell tropism.

Risk of post-transplant lymphoma in Italian kidney transplanted patients.

Serraino D, Piselli P, Busnach G, Angeletti C, Farchi F, Bellelli S, Colombo V, Minetti E, Perrino ML, Civati G, Pozzetto A, Rezza G, Citterio F

Contact:
Dr. Diego Serraino
INMI L. Spallanzani
Epidemiology (Rome, Italy)
Email: serraino@inmi.it

Abstract:
Objective: Patients who underwent organ transplantation, because of immunosuppressive therapies, are at increased risk of cancer. The present study statistically compared incidence rates (IR) of post-transplant lymphoproliferative disorders (PTLD) in patients kidney transplanted and in the general population of Italy. The aim was to quantify the excess risk associated with immunosuppressive therapies.

Methods: We studied 1,843 patients (1,203, 65,3%, males) who underwent kidney transplantation between 1970 and 2003 in two main Italian Transplantation Centers: Niguarda Ca' Granda Hospital, Milan, northern Italy; and the Policlinico A. Gemelli, Rome, central Italy. The follow-up period started from the date of transplantation and ended on the earliest of the following dates: tumour diagnosis, death, or last follow-up. Overall, 15,820 person years (PYs) of follow-up were recorded. Standardized incidence ratios (SIRs) and their 95% confidence intervals (95% CI) were computed to compare observed IR of PTLD with IR in the Italian general population of the same age and sex reported by Italian Cancer Registries (Cancer Incidence in five Continents, vol VII, IARC).

Results: 18 cases of PTLD were diagnosed during the 15,820 PYs of follow-up (15 cases of non-Hodgkin's lymphoma -NHL, 2 cases of multiple myeloma and 1 case of myeloid leukemia) against 3.4 cases expected. Such difference resulted in a statistically significant 5.3-fold increased risk (95% CI: 2.8-9.4) of PTLD in kidney tranplanted patients, as compared to the general population of Italy. SIRs were similar in the two sexes but higher in younger patients.

Conclusions: These findings confirm that transplanted patients are at higher risk for EBV-related cancers like PTLD, particularly when immunosuppression occurs at younger ages. Interestingly, this pattern is similar to the one registered among HIV-infected patients. Cofactors like duration, type and degree of immunosuppression are key elements in such pattern, and further investigation is needed to highlight the relationship between immunosuppression, EBV infection and cancer risk.

Relationship between fludarabine*, p53, and stat1 activation b lymphocytes

Youlyouz I, Le Clorennec I, Baran-Marszak F, Najjar I, Fagard R, Bornkamm GW, Feuillard J

Contact:
Prof. Dr. Jean Feuillard
Faculté de Médecine et CHU Dupuytren de Limoges
UMR CNRS 6101 et Laboratoire d'Hématologie (Limoges, France)
Email: jean.feuillard@chu-limoges.fr

Abstract:
Fludarabine is a nucleotide analog used in the treatment of various haematological malignancies. This chemotherapeutic drug induces cell cycle arrest and apoptosis via activation of p53. Several studies have suggested the existence of a relation between Fludarabine and regulation of STAT1, a transcription factor activated by Interferons. STAT1 has been shown to interact physically with p53. But, the mechanism of STAT1 regulation by Fludarabine is not well established. Here, we raise the question of the role of Fludarabine-induced p53 activation in STAT1 activation.
To control the activity of p53, we have developed and used various episomal inducible vectors. One of the characteristics of these vectors is that the gene of interest is expressed from a bidirectional doxycycline regulatable promoter allowing simultaneous expression of truncated NGF receptor, used as a surrogate marker of inducibility. We have subcloned the following cDNAs into these vectors: p53 wild type (p53wt), p53 mutated on Arginine 248 from the BL41 cell line (p53mt) and MDM2 as dominant negative. After stable transfection of an LCL, induction of the cDNA of interest was performed with doxycycline for 24H. Positive cells for NGFR were purified using magnetic beads and were additionally treated with Fludarabine. Apoptosis was studied by measuring Annexin V binding on cells and sub-G1 peak by flow cytometry.

Our first results showed that Fludarabine is inducing apoptosis in both LCL and BL2 cells (with p53wt) but not in BL41 cells (p53mt). On one hand, kinetic experiments showed that Fludarabine induces STAT1 activation at 30 - independently of the p53 mutational status. On the other hand, induction of over-expression of p53 is associated with both over-expression of STAT1 protein and STAT1 phosphorylation. Moreover, p53 activation by Fludarabine is associated with a specific increase of INFgamma mRNA, an effect that was increased by induction of over-expression of p53.
Our data suggest that activation of STAT1 by Fludarabine correspond to a complex mechanism that is partly independent of p53 and partly related to p53 activation, probably via regulation of IFNgamma gene expression. The effect of negative regulation of p53wt by p53mt or MDM2 on STAT1 activation, and the existence of a putative autocrine loop involving secretion of interferons is under current investigation.

Tumor suppressor function of RASSF1A in nasopharyngeal carcinoma
Lo KW

Contact:
Prof. Dr. Kwok Wai Lo
Department of Anatomical & Cellular Pathology
The Chinese University of Hong Kong (Hong Kong, HK)
Email: kwlo@cuhk.edu.hk

Abstract:
The tumorigenesis of nasopharyngeal carcinoma (NPC) is a multi-step process involving various factors including Epstein-Barr virus infection, and accumulation of epigenetic and genetic alterations. Deletion of the short arm of chromosome 3 is one of the most common and earliest changes in NPC development. Detailed physical mapping and functional studies have further targeted a critical tumor suppressor gene(s) for NPC to the region 3p21.3. Recently, we confirmed that RASSF1A is the critical target at 3p21.3 while loss of expression and aberrant methylation of the other eight candidate genes/transcripts (HYAL2, FUS1, RASSF1C, BLU, NPRL2, 101F6, PL6 and CACNA2D2) in this region were rare in NPC samples. The high incidence of RASSF1A methylation in the precancerous lesions and primary NPCs suggest that inactivation of RASSF1A is an early event in NPC tumorigenesis. The tumor suppression activity of RASSF1A in NPC has been demonstrated by restoring its expression in a RASSF1A deficient cell line, C666-1. Transfection of wild-type RASSF1A resulted in marked growth inhibition in NPC cells while expression of RASSF1A did not alter the cell cycle or induce apoptosis in NPC cells. Soft-agar assay showed decreased number and sizes of colony formed in RASSF1A-transfected clones. The tumorigenic potential in vivo was also dramatically reduced.By high-density oligonucleotide array, multiple potential novel target genes that are regulated by RASSF1A were identified. These potential target genes are involved in multiple pathways including G-protein regulation, TGF-beta signaling, stress induced apoptosis and cell cycle control. The findings provide strong evidence to support RASSF1A as a target tumor suppressor gene on 3p21.3 in NPC.

Characterization of BARF1 protein: its mitogenic activity

Sall A, Caserta S, Jolicoeur P, Franqueville L, de Turenne-Tessier M, Ooka T

Contact:
Prof. Dr Tadamasa Ooka
Laboratoire de Virologie Moléculaire, UMR5537, CNRS
Faculté de Médecine Laennec, Université Lyon-1 (Lyon, France)
Email: ooka@laennec.univ-lyon1.fr

Abstract:
Objective: We previously reported that BARF1 gene has either an immortalizing effect, when expressed in primary primate epithelial cells, or a malignant transforming activity, when expressed in established and non-tumoral rodent fibroblast or human B cell lines. The p29 protein encoded by this gene is secreted in culture medium of divers cell type. We ask whether secreted p29 BARF1 protein has cell stimulating activity by paracrine mechanism. As BARF1 was able to activate specifically the bcl2 gene, we ask also whether Bcl2 expression is activated by the same mechanism.

Methods: Production of p29 protein with a tetracycline-regulatable recombinant adenovirus. Purification of p29 by sequential methods: ammonium sulfate precipitation, sucrose gradient and ion-exchange chromatography. MTT test and [H3] thymidine incorporation assay were used for examining its cell stimulating activity.

Results: As predicted from sequence analysis, we found that BARF1 coded protein can be secreted from 293 epithelial infected by recombinant adenorius and BARF1-transfected Balb/c3T3 rodent fibroblasts. Since efficient BARF1 expression could be obtained from 293-tTA cells infected, secreted BARF1 product could be purified from the culture medium of such cells by ammonium sulfate precipitation, ion exchange chromotography, and sucrose gradient sedimentation. The addition of a purified product of secreted BARF1 protein to serum-free culture medium of Balb/c3T3 rodent fibroblasts, human Louckes B cell line and primary monkey kidney epithelial cells, resulted in a cell cycle activation which was inhibited by affinity-purified anti-BARF1 antibody. Moreover when purified p29 was added in Balb/c3T3 culture, bcl2 was activated.

Conclusions: Our demonstration of a specific stimulation of cell cycle in vitro by BARF1 secreted product suggests that this EBV encoded BARF1 protein could act as a growth factor in vivo. Bcl2 protein was probably stimulated by a paracrine mechanism.

EBV LMP-1 Half-Life and Signaling in Epithelial Cells is Down-Regulated by lyLMP-1

Pandya J, Walling DM

Contact:
Dr. Dennis M. Walling
University of Texas Medical Branch
Internal Medicine, Infectious Diseases (Galveston, USA)
Email: dwalling@utmb.edu

Abstract:
Objective: LMP-1 influences cell proliferation, differentiation, apoptosis, and immune response. The LMP-1 gene manifests remarkable natural sequence heterogeneity, and LMP-1 sequence variation appears to influence LMP-1 oncogenic activity. This study investigated the functional consequences of LMP-1 sequence variation in epithelial cells to identify molecular mechanisms that may influence LMP-1 oncogenesis.

Methods: Thirteen recombinant LMP-1 expression clones were created, each with a different specific sequence variation superimposed upon an otherwise identical B958 sequence background. In RHEK-1 epithelial cells, LMP-1 half-life was determined by pulse-chase labeling experiments, and LMP-1-activated signaling activity was measured using dual-luciferase reporter assays. The effect of lyLMP-1 co-expression on LMP-1 half-life and signaling activity was also determined.

Results: LMP-1 half-life was not influenced by sequence variation in amino acids 250-307 (repeat region) or amino acids 343-352 (deletion/duplication region). LMP-1 half-life was short and LMP-1 signaling activity was low when codon 129 encoded ATG (Met129), the initiation codon of lyLMP-1. Mutation of codon 129 to ATT (Ile129), and ablation of intrinsic lyLMP-1 expression, significantly increased both LMP-1 half-life and LMP-1-activated NF-kB, AP-1, and STAT signaling. Co-transfection and expression of lyLMP-1 with the mutated LMP-1 (Ile129) down-regulated both LMP-1 half-life and LMP-1-activated NF-kB, AP-1, and STAT signaling in a dose-dependent manner, restoring a short half-life and low signaling phenotype to the mutated LMP-1 (Ile129).

Conclusions: These results demonstrate that expression of lyLMP-1 down-regulates in parallel both LMP-1 half-life and LMP-1 signaling activity in epithelial cells, suggesting that lyLMP-1 may act to attenuate the oncogenic potential of LMP-1 in epithelial EBV infection. Consequently, these results may explain the previously described apparent negative selection of lyLMP-1 in the pathogenesis of NPC.

Induction of id1 and id3 by latent membrane protein 1 of Epstein-Barr virus and regulation of p27/kip and cyclin dependent kinase 2 in rodent fibroblast transformation

Everly DN, Mainou BA, Raab-Traub N

Contact:
Dr. David Everly
University of North Carolina-Chapel Hill
Lineberger Comprehensive Cancer Center (Chapel Hill, USA)
Email: everlyd@med.unc.edu

Abstract:
Objective:To use genomic analysis of LMP1 expressing cells to identify new pathways regulated by LMP1 and gain insight into mechanisms by which LMP1 transforms cells.

Methods: Microarray analysis, quantitative PCR, transformation assays, and western blotting.

Results: In order to gain greater understanding into the mechanism of transformation of epithelial cells by LMP1, genomic analysis of C33A cells expressing LMP1 was performed.Two members of the inhibitor of DNA binding or inhibitor of differentiation (Id) family of proteins, Id1 and Id3, were upregulated by LMP1. Induction of Id1 and Id3 mRNA was confirmed by quantitative PCR and correlated with increased protein levels.Increased Id1 protein was detected by immunofluorescence in Rat-1 foci induced by transformation with LMP1 and Id1 and Id3 protein levels were increased in Rat-1 LMP1 stable cell lines. The Id proteins are potent regulators of cellular differentiation and cell cycle progression and contain helix-loop-helix domains that allow them to bind to helix-loop-helix transcription factors, called E-box proteins. However, the Id proteins lack DNA binding domains and act as dominant-negative transcription factors.Id proteins negatively regulate expression of cyclin dependent kinase inhibitors (cdkis) and in Rat-1 stable cell lines cdki p27 protein levels were reduced while levels of cyclin dependent kinase (Cdk) 2 and phosphorylated retinoblastoma (Rb) protein were increased. The LMP1-mediated effects upon Id proteins and cell cycle proteins required the CTAR1 signaling domain of LMP1.

Conclusions: Cyclin dependent kinase inhibitors (cdki) and the Rb pathway are important regulators of cell cycle G1/S entry and replicative senescence. In Rat-1 cells transformed by LMP1, expression of Id1 and Id3 was induced with decreased amounts of p27 and increased phosphorylated Rb. The cdki p16 is often not expressed in EBV-associated nasopharyngeal carcinoma. The effects of LMP1 on cdki p27 in Rat-1 cells identified in this study and cdki p16 in NPC cells possibly via induction of Id proteins likely represents an important mechanism of transformation by LMP1. The data presented in this study identify key properties of LMP1 that affect cell cycle progression and contribute to transformation and oncogenesis.

What do we learn from EB virus encoded LMP1 story? (I) Effect of LMP1 on the Cell Cycle
Cao Y

Contact:
Dr. Ya Cao
Cancer Research Institute (Changsha, China)
Email: ycao98@public.cs.hn.cn

Abstract:
Tumor development and progression are intensely involved in the deregulation of cell cycle. Our group has been focusing on elucidating the relationship between disturbance of cell cycle and mechanism of EBV LMP1. Our study shows that LMP1 represents the dual biological effect of disordered proliferation and inhibited apoptosis. Apoptosis is a cell cycle event. Coordination of proliferation and apoptosis (CAP) offer the platform for both cell cycle and apoptosis. We have thoroughly discussed the cell cycle checkpoint signaling pathways and how different components of these pathway being regulated by EBV LMP1.
(1) LMP1 stimulates the acceleration of G1/S phase
We firstly reported that cyclin D1 expression could be activated by LMP1 at the transcriptional level and protein level in both dose-dependent and time-dependent manner. The novel findings may thus represent a direct link between LMP1 and cell cycle regulator cyclin D1. Our studies also show that LMP1 could upregulate CDK4 expression, promote phosphorylation of pRB, and activate E2F transcription factor.We have firstly found that LMP1 could mediate a heterodimer form of c-Jun and JunB, which could be bound to the AP-1 sequence on the promoter of p16 and downregulated both the promoter activity and expression of p16. Aberrant expression of cyclin D1,CDK4 and p16 would enhance the cell cycle driving mechanism and weaken the checking mechanism, inducing more cells of G0 to re-enter the cell cycle and more cellsto progress from G1 to S phase rapidly. All these may lead to cell over-proliferation and maladjustment of G1 point, which would increase the genome instability and enhance the tumor development.
(2) LMP1 arrests G2/M phase
p53 is essential for the control of cell entry into mitosis when cells enter G2 phase with damaged DNA. We found that LMP1 could induce the accumulation of p53 protein and upregulated its transactivity, which decreased the kinase activity of cdc2/cyclinB complex and induced the arrest of cells in G2/M phase. These data suggest that LMP1 may have effects on many cell cycle regulators, activating the driver and surveillance mechanism of cell cycle at the same time, resulting in loss of G1/S checkpoint and the promotion of G1 cells into S phase and arrest of G2/M checkpoint.

EBV Nuclear Antigen 3C Modulates Cyclin A-Associated Kinase Activity
Knight J, Robertson E

Contact:
Jason Knight
University of Pennsylvania
Department of Microbiology (Philadelphia, USA)
Email: jaknight@mail.med.upenn.edu

Abstract:
Objective: EBV nuclear antigen 3C (EBNA3C/EBNA6) facilitates passage through the G1/S restriction point. This study seeks to identify EBNA3C binding partners that may contribute to cell cycle regulation by EBV.

Methods: Previously it was demonstrated that a stop codon introduced at amino acid 365 of the EBNA3C gene abrogates the transforming potential of EBV. To identify critical protein binding partners with a potential role in cell cycle regulation, a truncated form of EBNA3C corresponding to amino acids 365-992 was fused with the GAL4 DNA binding domain. This fusion was used as bait in a yeast two-hybrid screen to identify EBNA3C-interacting proteins expressed by an LCL-derived cDNA library.

Results: The aforementioned yeast-two hybrid screen yielded multiple positive clones encoding Cyclin A, an activator of S-phase progression.EBNA3C interacted with Cyclin A in vitro and associated with Cyclin A complexes in EBV-infected lymphoblastoid cell lines.Importantly, EBNA3C stimulated Cyclin A-associated kinase activity and rescued p27 suppression of this activity by decreasing the molecular association between Cyclin A and p27 in cells. Cyclin A activity was targeted by a region at the carboxy terminus of EBNA3C, shown to be important for both stimulation of kinase activity and cell cycle progression.Although the carboxy terminus of EBNA3C regulated kinase activity, a domain with greater binding affinity for Cyclin A was identified near the amino terminus of EBNA3C. Detailed mapping studies implicated amino acids 130-159 within the EBNA3 homology domain as having the greatest affinity for Cyclin A. This region of EBNA3C bound to the first helix of the highly conserved cyclin box of Cyclin A, with Cyclin A amino acids 206-226 required for strong binding to EBNA3C amino acids 130-159. Surprisingly, heterologous expression of the amino terminus of EBNA3C did not rescue p27 suppression of Cyclin A-associated kinase activity as did full-length EBNA3C and actually suppressed Cyclin A kinase activity in LCLs.

Conclusions: While further experimentation will help to clarify the timing of Cyclin A regulation by EBNA3C, the role of distinct EBNA3C domains in this process, and the recruitment of other regulatory molecules, this study links, by protein-protein interaction, an essential EBV latent antigen to cell cycle regulation in human cells.

The EBNA-3 gene family proteins disrupt the G2/M checkpoint

Sculley T, Krauer K, Burgess A, Buck M, Flanagan J, Gabrielli B

Contact:
Dr. Tom Sculley
Queensland Institute of Medical Research (Brisbane, Australia)
Email: tomS@qimr.edu.au

Abstract:
The Epstein Barr Nuclear Antigens (EBNA), EBNAs -3a, -3b and -3c, have previously been shown to act as transcriptional regulators, however, this study identifies another function for these proteins, disruption of the G2/M checkpoint. Lymphoblastoid cell-lines (LCLs) treated with a G2/M initiating drug azelaic bishydroxamine (ABHA) did not show a G2/M checkpoint response. Cell cycle analysis demonstrated that the individual expression of EBNAs -3a, -3b or -3c were capable of disrupting the G2/M checkpoint response, whereas EBNAs -2, and -5 were not. EBNA-3 gene family protein expression also disrupted the G2/M checkpoint initiated in response to the genotoxin etoposide and the S phase inhibitor hydroxyurea. The G2 arrest, in response to these drugs, was sensitive to caffeine, suggesting that ATM/ATR signaling in these checkpoint responses may be blocked by the EBNA3 family proteins. EBNA-3a was shown to immunoprecipitate with the checkpoint kinase chk2/cds1. This kinase is activated in response to DNA damage and replicative arrest in both yeast and humans and is capable of phosphorylating cdc25C which leads to inhibition of its ability to activate cdc2/cyclin B complexes necessary for G2/M transition. These results indicate that EBNA-3 associates with chk2 and suggest that this interaction could be responsible for the G2/M checkpoint disruption. The function of EBNAs -3a, -3b and -3c proteins appears to be more complex than anticipated and these data indicate a role for these proteins in disrupting the host cell cycle machinery.

The Epstein-Barr virus immediate early gene BRLF1 influences the in-vitro and in-vivo growth of lymphoblastoid cell lines.

Hong GK, Kenney SC

Contact:
Greg Hong
University of North Carolina
Microbiology & Immunology (Chapel Hill, USA)
Email: Gregory_Hong@med.unc.edu

Abstract:
Objective: EBV is associated with human malignancies. EBV exists in either a latent or lytic state, with latent gene expression mediating virally induced transformation and lytic gene expression resulting in shedding of infectious virus. The lytic cycle of EBV is triggered by expression of the EBV immediate early proteins BZLF1 and/or BRLF1; BZLF1 and BRLF1 function as transcriptional activators of viral lytic genes and cellular genes. A role for lytic gene expression in the development of malignancy has not been conclusively demonstrated and remains controversial. Here, using recombinant EBV in which BRLF1 has been deleted (EBV R-KO), we have investigated whether lytic gene expression influences the in-vitro or in-vivo growth of EBV transformed cells.

Methods: Peripheral blood leukocytes were transformed with either EBV R-KO or the corresponding wild-type virus (EBV WT) to yield continuously proliferating lymphoblastoid cell lines (R-KO LCL and WT LCL, respectively). R-KO and WT LCLs were then assayed for growth in-vitro under low serum (1% FBS) conditions. In-vivo growth was examined by recording tumor size following subcutaneous injection of LCLs into SCID mice. Gene expression between the R-KO and WT LCLs was compared using western blot and RT-PCR analysis.

Results: R-KO LCLs exhibited slightly impaired growth in-vitro in low serum conditions when compared to WT LCLs. However, R-KO LCLs exhibited markedly impaired growth following sub-cutaneous injection into SCID mice. In-vivo growth of WT LCLs was not affected by treatment with acyclovir, an antiviral drug which selectively inhibits early and late phases of lytic replication, suggesting that viral transmission does not account for the in-vivo difference between R-KO and WT LCLs. Western blot analysis of R-KO and WT LCLs growing in-vitro revealed similar levels of the viral transforming genes LMP1 and EBNA2. However, RT-PCR analysis revealed that R-KO LCLs express decreased levels of the cellular genes fatty acid synthase and the tyrosine kinase c-mer, both of which are activated by BRLF1 and have been implicated as being important for tumorigenesis of some malignancies.

Conclusions: The in-vivo growth defect of the R-KO LCL suggests that expression of lytic genes may contribute to the development of EBV associated malignancies, presumably through a paracrine mechanism.

Cooperation of exogenous and endogenous viruses: Human endogenous retrovirus K (HERV-K) Np9 protein downregulates Epstein-Barr nuclear antigen (EBNA2) activated promoters

Spurk A, Pfuhl T, Armbruster V, Sauter M, Muller-Lantzsch N, Grasser FA

Contact:
Andreas Spurk
Institut für medizinische Mikrobiologie und Hygiene
Abteilung Virologie (Homburg, Germany)
Email: spurk@web.de

Abstract:
Objective: Cooperation of EBNA2 and the HERV-K Np9 protein

Methods: Luciferase reporter gene assay, GST pulldown

Results: HERV-K Np9 protein downregulates EBNA2 activated promoters. Np9 directly binds to EBNA2

Conclusions: The Epstein-Barr virus (EBV) is involved in induction of a variety of human tumours of lymphoid and epithelial origin. The virus-encoded nuclear antigen 2 (EBNA2) regulates viral and cellular gene expression by binding to the transcriptional repressor RBPJk. EBNA2 is a key mediator of transformation of B-lymphocytes by EBV. We have found that EBNA2 is complexed to the Np9 protein encoded by the HERV-K env. The Np9 protein indirectly interacts with NUMB, an antagonist of RBPJk. In our luciferase reporter gene assays Np9 downregulates the EBNA2 mediated activation of the C-promoter and an artificial promoter containing 12 RBPJk binding sites. In contrast, Notch-IC mediated activation of the Hes1 promoter is not downregulated by Np9. Using a non-nuclear mutant of Np9 the downregulation was not observed. By GST-pulldown we demonstrate direct binding of Np9 to EBNA2. Likewise, we observe colocalisation by confocal laser scanning microscopy.

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Dominant negative derivatives of EBNA1 inhibit survival of EBV(+) and KSHV(+) PEL-derived cells

Mack AA, Sugden B

Contact:
Amanda Mack
McArdle Laboratory for Cancer Research, University of Wisconsin-Madison
Cancer Biology (Madison, USA)
Email: mack@oncology.wisc.edu

Abstract:
EBNA1 makes several contributions to a successful infection by EBV. It is the only viral protein required for replication and maintenance of the viral DNA and is found in all EBV-associated tumors.EBNA1 also supports survival of infected cells as demonstrated by its inhibition leading to apoptosis in normal and Burkitt 's lymphoma-derived cells infected with EBV (Kennedy et al., PNAS 100:14269-14274, 2003). We have extended these latter observations to include primary effusion lymphoma-derived cells (PELs). PELs are often infected with KSHV or dually infected with KSHV and EBV. We found the survival of the dually infected lines, BC-2 and JSC-1, decreased ($p < 0.05$) when they were infected with retroviruses expressing dominant negative derivatives of EBNA1 but not a control retrovirus. Expression of these EBNA1-derivatives did not affect survival of the hematopoietic cell lines K562, Jurkat, and MOLT-4 which are EBV(-) and KSHV(-).Previous, parallel experiments indicate that these derivatives of EBNA1 also do not affect survival of the EBV(-) and KSHV(-) B cell lines, BJAB and DG75 (Kennedy et al., ibid). These findings are consistent with the hypothesis that EBNA1 contributes to the survival of EBV(+) and KSHV(+) PEL cell lines as it does to EBV(+) Burkitt's lymphoma cell lines. It is also possible that the dominant negative derivatives of EBNA1 inhibit survival of EBV(+) and KSHV(+) PELs by inhibiting LANA1 which is at least functionally related to EBNA1.We are now distinguishing between these two possibilities by testing for any affects of these dominant negative derivatives of EBNA1 on EBV(-) and KSHV(+) PEL cell lines.

Interaction of Marek's Disease Virus Meq protein with CtBP in the pathogenesis of Marek's disease: lessons from and for EBV

Brown A, Hickabottom M, Nair V, Allday MJ

Contact:
Prof. Martin Allday
Imperial College London
Virology (London, UK)
Email: m.allday@imperial.ac.uk

Abstract:
EBV nuclear oncoproteins EBNA3A and EBNA3C can bind to the protein CtBP. This is a highly conserved cellular co-repressor of transcription involved in diverse processes (including developmental programming, cell cycle control and apoptosis). The ability of EBNA3A and EBNA3C to bind CtBP correlates partially with their ability to repress transcription when targeted to DNA and correlates very well with their ability to rescue primary rodent fibroblasts from premature senescence induced by oncogenic ras.
A potential CtBP-binding motif (PXDLS) exists in the Meq nuclear protein of Marek's disease virus (MDV). MDV is an α-herpesvirus of chickens that induces malignant T cell hyperplasia responsible for the fatal disease known as Marek's disease (MD). Although MDV is an α-herpesvirus, biologically it more closely resembles the lymphotropic γ-herpesviruses such as EBV, KSHV and HVS.
We show that human and chicken CtBP bind to Meq through a PLDLS motif in the N-terminus of Meq (aa 20-24). Meq is a major transforming protein of MDV and there are two copies of the Meq gene per MDV genome since it is located in each of two repeat elements (TRL and IRL). In order to determine the significance of the interaction between Meq and CtBP in the pathogenesis of MD, recombinant viruses were generated using an MDV-BAC based on the virulent RB1B strain. Several mutant viruses were generated including one with a single copy of Meq mutated at its CtBP-binding site, one with both copies of Meq mutated and revertant viruses. Stocks of these viruses were prepared in chicken embryo fibroblasts and injected into chickens. The surprising but highly reproducible result was that the double Meq mutant was consistently non-oncogenic. While the majority of chickens infected with WT virus, or a single copy mutant or the revertant viruses developed MD with characteristic lymphoma in visceral organs within 60 days; in contrast, the control birds and those infected with the double Meq mutant all remained perfectly healthy. Quantitative-PCR analysis of the viral load in PBL from all the infected birds showed similar growth curves for all the viruses, suggesting that the CtBP-binding mutant virus replicates in vivo as efficiently as the wild type MDV. The role of Meq and its interaction with CtBP in latency and in oncogenesis will be discussed.

Differential deregulation of DNA damage responses by EBV
O'Nions J, Allday MJ

Contact:
Dr. Jenny O'Nions
Imperial College
Virology (London, UK)
Email: j.onions@imperial.ac.uk

Abstract:
Objective: EBV is involved in the aetiology of a variety of cancers and has been shown to disrupt cell cycle checkpoints triggered by DNA damage in some tumour-derived cell lines. Comparison of EBV-infected primary B cells with an isogenic, mitogenically-stimulated population enables us to investigate whether EBV deregulates checkpoints in normal human B cells and hence add to our understanding of the virusO contribution to B cell malignancy. In this study, EBV and mitogen-driven B cells were exposed to genotoxic stress to determine whether viral latent gene expression alters the normal B cell response to DNA damage.

Methods: Primary B cells isolated from peripheral blood were either infected with EBV or co-cultured with the T cell-derived mitogens CD40L and IL4 (CD40L/IL4). Cells were treated with genotoxins that generate cross-links in DNA (cisplatin (CP) and melphalan) and those that generate double strand breaks (DSBs) (IR and bleomycin), and their response analysed by flow cytometry, RNase protection, immunoprecipitation, immunoblotting and kinase assays.

Results: B cells stimulated with CD40L/IL4 accumulated p21WAF1 following CP-induced DNA damage. This produced a block in DNA synthesis and arrest in G1. However, in B cells driven to proliferate by EBV, we found no increase in p21, despite stabilisation and activation of p53. As a result, Rb remained hyperphosphorylated and the cells continued to enter S phase. Conversely, in cells responding to IR, p21 accumulated and cell cycle arrest was activated irrespective of the presence of EBV. B cells were then exposed to other genotoxins that produce DNA cross-links or DSBs to investigate whether the type of DNA damage determines the ability of EBV to modify the B cell response. Consistent with this hypothesis, in cells treated with melphalan, p21 accumulated in CD40L/IL4-stimulated but not EBV-driven B cells, whereas an increase in p21 was detected in both populations following treatment with bleomycin.

Conclusions: EBV is able to suppress a key checkpoint activated in response to DNA cross-linking agents in B cells. This disruption of cell cycle fidelity could contribute to tumourigenesis. Interestingly, EBV does not influence the activation of cell cycle arrest in response to genotoxins that produce DSBs in DNA. Why EBV only modifies the response to certain types of DNA damage will be discussed.

Effects of EBV DNase and LMP1 on the Genome Stability

Liu MT, Chen YR, Lin CS, Chang YT, Wang WB, Chen JY

Contact:
Prof. Dr. Jen-Yang Chen
National Health Research Institutes
Extramural Research Affairs Dept. (Taipei, Taiwan)
Email: cjy@nhri.org.tw

Abstract:
Background: All cancers have been demonstrated to contain chromosomal aberrations. Accumulated data propose that genome instability is the cause rather than consequence of carcinogenesis. The human T cell leukemia virus Tax hepatitis B virus HBx, human papilloma virus E6 and E7, and hepatitis C virus core protein have been shown to contribute to the genome instability and proposed to be responsible for the development of respective tumors. Since EBV has been closely associated with many human malignancies including Burkitt's lymphoma and nasopharyngeal carcinoma, we looked for EBV gene products which may contribute to the genome instability and found that EBV DNase may induce DNA strand breaks and LMP1 may repress DNA repair and subsequently result in the genome instability.

Methods: Comet assay was used to examine the DNA strand break. Micronucleus formation was taken as a marker of genome instability. 6-Thioguanine resistance was used to score the HPRT gene mutation. Host cell reactivation (HCR) assay was employed to detect damaged DNA repair.

Results: Using an inducible system, expression of EBV DNase in Raji cells was able to induce DNA strand breaks detected by comet assay. Furthermore, micronuclei formation and HPRT gene mutation frequency were increased. Through HCR assay, LMP1 was found to repress damaged DNA repair in both p53-wild-type and p53-deficient human epithelial cells. Further mapping employed LMP1 deletion mutants revealed that CTAR1 and CTAR2 are the domains responsible for this repression activity. To elucidate the mechanism of p53-dependent repression, we found that the repression may act through the NFKB pathway and LMP1 is able to inhibit the transactivation ability of p53.

Conclusion: We have been able to demonstate that EBV DNase and LMP1 contribute to genome instability. We propose that, in addition to HTLV, HBV, HPV and HCV, EBV is another human tumor virus which may play a role in the carcinogenesis through the introduction of genome instability.

Regulation of Survivin and CDK4 by Epstein-Barr Virus Encoded Latent Membrane Protein 1 in nasoparyngeal carcinoma cell lines

AI M, Li L, Tao Y, Ding L, Zheng H, Cao Y

Contact:
Dr. Midan AI
Cancer Research Institute
Xiangya School of Medicine, Central South University (Changsha, China)
Email: aimidan@hotmail.com

Abstract:
Objective: Epstein Barr virus (EBV) encoded latent membrane protein 1 (LMP1) plays an important role in the pathogenesis and development of nasoparyngeal carcinoma (NPC). It has a dual effect of increasing cell proliferation and inhibiting cell apoptosis in NPC.In this study, we will investigate the regulation of LMP1 on Survivin and CDK4 to study the mechanism of dual effect of LMP1 .

Methods: Cell lines we used in this study: CNE1 is an LMP1-negative highly differentiated nasopharyngeal carcinoma cell line; CNE-LMP1 is a stably transfected cell line, established by introducing LMP1 cDNA into CNE1 cell, in which LMP1 is highly expressed; Tet-on-LMP1-HNE2 is an established cell line using a newly developed Tet-on gene expression system in which LMP1 is high-effectively expressed in a dosage-dependent manner.Methods we used: Preparation of fractionated proteins; Westernblotting analysis; Immunofluorescence analysis; Immunoprecipitation analysis; Flow cytometry analysis; Flow cytometry cell sorting; Post-sorting western blotting analysis.

Results: In the current study, we showed a higher expression level of Survivin protein, CDK4 protein and phosphorylated CDK4 protein in LMP1 positive NPC epithelial cell CNE-LMP1 than in LMP1 negative NPC epithelial cell CNE1, and the increasing expression of Survivin protein, CDK4 protein and phosphorylated CDK4 protein are LMP1 dosage-dependent in a Dox-inducible system, Tet-on-LMP1-HNE2. We further found that LMP1 can promote Survivin protein expression at all the three cell cycle phases: G0/G1 phase, S phase and G2/M phase through comparing CNE1 and CNE-LMP1. We also observed that LMP1 could promote Survivin and CDK4 to translocate into nuclei. Moreover, we identified that survivin could interact with CDK4 in CNE-LMP1 nuclei after translocation, but the complex wasn 't observed in the CNE1. The results demonstrated that Survivin translocated into the nucleus and interacted with CDK4 under the induction of LMP1.

Conclusions: Our study indicated that LMP1 regulating on Survivin and CDK4 might be involved in the dual effect of LMP1 promoting cell proliferation and inhibiting apoptosis.

Latent membrane protein 1 modulates cell adhesion and motility: a role for Peta-3/CD151
Laverick L, Young LS, Dawson CW

Contact:
Dr. Christopher Dawson
University of Birmingham
CR UK Institute for Cancer Studies (Birmingham, UK)
Email: c.w.dawson@bham.ac.uk

Abstract:
Objectives: Members of the tetraspanin superfamily of proteins (TM4 proteins) are known to play essential roles in cell differentiation, cell motility and tumour cell metastasis. Peta-3/CD151 is particularly interesting, as it regulates the activities of a number of integrin receptors and participates in maintenance and assembly of cadherin based intercellular contacts. CD151 has been shown to influence adhesion-dependent downstream signalling pathways via modulation of receptor tyrosine kinases (RTK's), non-RTK's, and activation of focal adhesion kinase (FAK). Previous work has identified an ability of LMP1 to promote cell scattering and motility, and to increase cell invasion. This study set out to determine whether any of the phenotypic effects associated with LMP1 expression were due to modulation of CD151 expression and/or function.

Methods: MDCK cell lines stably expressing LMP1, various LMP1 mutants, and CD151, were generated by retroviral transduction. Transduced cells were subsequently analysed for (i) their overall morphology, (ii) the expression and sub-cellular localisation of CD151, E-cadherin and adherens junction (AJ) proteins, and (iii) their ability to migrate in transwell migration assays.

Results: MDCK cells stably expressing LMP1 displayed a gross alteration in cell morphology reminiscent of epithelial cells undergoing epithelial-mesenchyme transition (EMT). This morphological change was accompanied by the loss of E-cadherin/AJ proteins from the cell membrane, a loss of CD151 from cell-cell contacts, and actin filament reorganisation. The ability of LMP1 to induce this phenotype mapped to the CTAR1 domain of LMP1, as an LMP1 mutant crippled for CTAR1 failed to induced these changes. Interestingly, reconstitution of CD151 expression in wild-type LMP1-expressing cells resulted in reversal of the EMT phenotype, with re-establishment of cell-cell contacts, re-localisation of E-cadherin and AJ proteins to cell-cell contacts, and decreased cell movement.

Conclusions: The ability of LMP1 to modulate CD151 expression/distribution clearly has an impact on epithelial cell behaviour. The breakdown of E-cadherin and adherens junction formation that accompanies CD151 loss from the cell membrane endows cells with increased migratory properties. Future studies will aim to identify the mechanism by which LMP1 modulates CD151 function.

Epstein-Barr virus Latent Membrane Protein 1 promotes secretion of fibroblast growth factor 2 by a novel mechanism

Ceccarelli S, Visco V, Wakisaka N, Pagano JS, Torrisi MR

Contact:
Dr. Simona Ceccarelli
Univ. Roma La Sapienza
Dept. Experimental Medicine and Pathology (Roma, Italia)
Email: simona.ceccarelli@uniroma1.it

Abstract:
Objective: FGF2 is a potent angiogenic factor known to be involved in tumor invasion and released by a non-classical secretory pathway. The EBV oncoprotein LMP1 is able to promote the expression of FGF2 and the release of the 18KDa isoform of FGF2 into the extracellular fluid. Aim of our study was to analyze in detail the mechanism of FGF2 secretion induced by LMP1.

Methods and Results: Confocal immunofluorescence microscopy in LMP1/18KDa FGF2 cotransfected AdAH cells revealed that the FGF2 signal is not only diffuse in the central cytosol, as expected, but also concentrated in small dots located in proximity of the plasma membrane and along cellular projections, where it colocalizes with LMP1. These peripheral dots double positive for LMP1 and 18KDa FGF2 appeared also positively stained for cathepsin D, a marker of late endosomes or multivesicular bodies (MVBs) and lysosomes. Because recent reports indicate that LMP1 secretion occurs by exosomes, released by several cell types and corresponding to the inner vescicles of MVBs, we hypothesized that the mechanism of FGF2 release could involve these structures. We purified exosomes by ultracentrifugation of the culture medium from AdAH cells cotransfected with LMP1 and 18KDa FGF2, and both biochemical analysis and immunoelectron microscopy of the exosomal fraction demonstrated the increased release of exosomes and the presence of LMP1 and FGF2. Moreover, cotransfection with 18KDa FGF2 and LMP1 appeared to induce partial redistribution of Na+/K+ ATPase, which is known to participate to FGF2 release, from the plasma membrane to the intracellular LMP1/FGF2 positive dots, and treatment with ouabain, a drug which is known to inhibit Na+/K+ ATPase activity, partially suppressed FGF2 secretion via exosomes in a dose-dependent manner.

Conclusions: Taken together, our results suggest that exosomes could represent a novel mechanism for LMP1-mediated FGF2 release, and that this pathway involves the activity of Na+/K+ ATPase.

LMP1 Transformation of Rodent Fibroblasts Requires CTAR1 but not CTAR2
Mainou BA, Ward G, Everly DN, Raab-Traub N

Contact:
Bernardo Mainou
University of North Carolina at Chapel Hill
Microbiology and Immunology (Chapel Hill, USA)
Email: mainou@med.unc.edu

Abstract:
Objective: To analyze LMP1-induced transformation of fibroblasts and determine the contribution of carboxy-terminal activating region 1(CTAR1) and carboxy terminal activating region 2(CTAR2).

Methods: Western blot analysis, immunofluorescence, focus formation, colony formation in soft agar, and tumor formation in nude mice.

Results: LMP1 is considered the EBV oncogene as it can transform Rat-1 fibroblasts and has major effects on lymphocyte and epithelial cell growth. To analyze the contribution of the two major signaling domains of LMP1 in transformation, transformation assays were carried out with full length LMP1, LMP1 deleted for CTAR1 (del187-351), and LMP1 deleted for CTAR2 (1-231) in Rat-1 fibroblasts. Transduction with full length LMP1 and LMP(1-231) but not LMP(del187-351) induced foci formation. Immunofluorescence staining for LMP1 and LMP(1-231) was restricted to foci and revealed that LMP1 and LMP(1-231) expression always induces focus formation. Focus formation was blocked by treatment with the phosphoinositide-3 kinase (PI-3K) inhibitor, LY29004. As with Rat-1 cells, full length LMP1 and LMP(1-231) but not LMP(del187-351) supported focus formation in human fibroblasts. Stable Rat1 cell lines that express LMP1 and the deletion mutants were established and analyzed for their ability to grow colonies in soft agar as well as form tumors in nude mice. As with focus formation, full length LMP1 and LMP(1-231) cells grew in soft agar while LMP(del187-351) and control cells did not grow under anchorage independent conditions. Cell lines expressing LMP1 and LMP(1-231) rapidly formed tumors with slow development of tumors with LMP(del187-351).

Conclusions: LMP1 CTAR1 but not CTAR2 is required for transformation of rat and human fibroblasts and this transformation is dependent upon activation of PI-3K. The role of the PI-3K targets, Akt, glycogen synthase kinase beta (GSK3B) and beta-catenin in LMP1-mediated transformation will be evaluated.

Effect of LMP-1 suppression in EBV-associated lymphoma cell survival.
Guasparri I, Cesarman E

Contact:
Dr. Ilaria Guasparri
Weill Cornell Medical College
Pathology (New York, USA)
Email: ilg2001@med.cornell.edu

Abstract:
Objective: LMP1 is essential for the immortalization of B cells by EBV, and is considered to be one of the main viral oncoproteins. LMP-1 activates the transcription factor NF-kB, and inhibition of NF-kB leads to the apoptosis of EBV-infected lymphoblastoid and lymphoma cell lines. Our goal was to determine whether elimination of LMP1 is sufficient to induce apoptosis of lymphoma cells expressing this protein, which would validate LMP-1 as a specific therapeutic target.

Methods: We developed and tested a siRNA targeting LMP1. We delivered it by transfection into a lymphoblastoid cell line (LCL9001), two EBV-positive AIDS immunoblastic lymphoma cell lines (BCKN-1 and IBL-1; type III latency), one EBV negative AIDS-related large B cell lymphoma cell line (BCHN-1), BJAB and Namalwa. We performed immunoblot analyses to assess expression of LMP-1 and selected NF-KB-dependent genes and the effect of LMP1 suppression on constitutive NF-KB activity using a luciferase reporter assay. Apoptosis was measured by flow cytometry analysis for annexin V.

Results: Treatment of EBV-infected cells with siRNA to LMP-1 effectively suppressed production of this protein to levels undetectable by immunoblotting and resulted in inhibition of NF-kB-regulated genes and NF-kB transcriptional activity. Transfection of LMP-1 siRNA into the LCL9001, BCKN-1 and IBL-1 cell lines resulted in apoptosis of 30 to 45% of the cells. No apoptosis was seen when EBV-negative or type I-latency cell lines were treated with siRNA to LMP-1, or when LMP-1-expressing lines were transfected with scramble and irrelevant (vFLIP) siRNAs.

Conclusions: These data indicate that EBV-infected lymphoma cells depend partially on LMP-1 expression for survival. As only approximately one third of the cells undergo apoptosis upon LMP-1 suppression, other sources of NF-kB activity may exist in these cells, and cellular genetic abnormalities could result in generation of alternative anti-apoptotic signals. These data are consistent with the observation that LMP-1 is not expressed by all EBV-infected lymphoma cells in latency type II or III, but rather only by a proportion of neoplastic cells. Suppression of LMP-1 may be effective as a therapeutic approach in combination with other treatment modalities including cytotoxic therapy and suppression of additional EBV-encoded proteins.

EBER Promotes Growth of EBV-infected T-cells through Interleukin-9 Induction

Yang L, Aozasa K, Oshimi K, Takada K

Contact:
Prof. Dr. Kenzo Takada
Institute for Genetic Medicine, Hokkaido University
Department of Tumor Virology (Sapporo, Japan)
Email: kentaka@igm.hokudai.ac.jp

Abstract:
Epstein-Barr virus (EBV) associates with various T-cell-proliferating diseases such as chronic active EBV infection and nasal lymphoma. In contrast to B-cells, which are highly susceptible to EBV infection in vitro, T-cells are refractory to EBV infection in vitro, and it has been difficult to examine the effects of EBV infection on T-cells. We recently generated EBV recombinants with a selectable marker, which made it possible to select EBV-infected cells even when the efficiency of infection was low. Using the recombinant virus, we found that a human T-cell line, MT-2, was susceptible to EBV infection, and succeeded in isolating EBV-infected cell clones with type II EBV latency, which was identical with those seen in EBV-infected T-cells in vivo. EBV-infected MT-2 cell clones had shorter cell doubling times and higher saturation density than EBV-uninfected counterparts. We found that EBV-positive MT-2 cells expressed higher levels of interleukin (IL)-9 than EBV-negative MT-2 cells at the transcriptional level. It was also demonstrated that EBV-encoded small RNA (EBER) was responsible for IL-9 expression. Addition of recombinant IL-9 accelerated the growth of MT-2 cells, whereas growth of the EBV-converted MT-2 cells was blocked by treatment with an anti-IL-9 antibody. These results suggest that IL-9 induced by EBER acts as an autocrine growth factor for EBV-infected T-cells. Analysis of nasal lymphoma biopsies indicated that three of four specimens expressed IL-9. The present findings suggest that EBV directly affects the pathogenesis of EBV-associated T-cell diseases.

Growth promotion of Epstein-Barr virus-infected epithelial cells mediated by an Epstein-Barr virus-encoded small RNA

Iwakiri D, Eizuru Y, Tokunaga M, Chen JY, Sheen TS, Huang DP, Takada K

Contact:
Dr. Dai Iwakiri
Institute for Genettic Medicine, Hokkaido University
Dept. of Tumor Virology (Sapporo, Japan)
Email: iwakiri@igm.hokudai.ac.jp

Abstract:
Objective: Epstein-Barr virus (EBV) has been reported to be associated with epithelial malignancies including nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC). Our previous study has demonstrated that gastric epithelial cells infected with EBV in vitro expressed a limited number of latent genes as EBV-positive GC cells do in vivo, and were able to proliferate faster than uninfected cells. In this study, we tried to clarify how EBV promotes growth of epithelial cells.

Methods: EBV-negative gastric carcinoma cell line NU-GC-3, EBV-negative NPC line CNE1 and HONE1 were infected with recombinant EBV carrying the neomycin-resistant gene, and EBV-infected cells were cloned by G418 selection. EBV-positive NPC cell line C666-1 was also used in this study. We carried out RT-PCR or ELISA to evaluate expression of epithelial growth factors, and also performed immunoblot analysis to study intracellular signal transduction. Transient transfections of reporter plasmid constructs were carried out using lipofection method followed by luciferase assay.

Results: A comparison of growth between EBV-infected and -uninfected cell lines revealed that EBV promotes cell proliferation. RT-PCR analysis and ELISA demonstrated that insulin-like growth factor (IGF)-1 was upregulated in EBV-infected cells compared to uninfected cells. In addition, IGF-1 was also highly expressed in C666-1 cells. Studies of cellular growth using recombinant IGF-1 or IGF-1 neutralizing antibody demonstrated that IGF-1 is an autocrine growth factor for EBV-infected cells. Constitutive activation of IGF-1 receptor signaling in EBV-infected cells was observed by Western blot analysis. RT-PCR analysis revealed that IGF-1 was highly expressed in EBV-positive GC tissues compared to EBV-negative tissues, and all EBV-positive NPC tissues showed high IGF-1 expression. Transfection studies using expression plasmids carrying individual latent gene (EBNA1, EBER, and BARF0) revealed that EBER was responsible for IGF-1 induction. Reporter analysis using luciferase-IGF-1 promoter plasmid showed that EBER activated transcription of IGF-1 gene.

Conclusions: EBER is responsible for EBV-mediated growth promotion of epithelial cells. Our results suggested that EBER plays a significant role in EBV-associated epithelial malignancies.

Transcriptional profiling reveals a role for EBNA1 in regulating cellular gene transcription

Wood VHJ, O'Neil JD, Dawson CW, Young LS

Contact:
Victoria Wood
University of Birmingham
CR UK Institute for Cancer Studies (Birmingham, UK)
Email: victoriawood@cancer.bham.ac.uk

Abstract:
In addition to its role in maintaining the integrity of the EBV episome, EBNA1 is known to function as a transcriptional transactivator, up-regulating activity of viral Cp and LMP1 promoters. In view of these findings, it has been hypothesised that EBNA1 may also target cellular promoters and thus influence cellular gene transcription. Previous work from our group has demonstrated that stable EBNA1 expression can only be tolerated in epithelial cells of undifferentiated or glandular origin (Ad/AH, Hela) but not in differentiation-competent squamous epithelial cells (SCC12F, SVK) (Jones et al., Virology, 313: 663, 2003). In SVK and SCC12F cells, EBNA1 expression is associated with a G2-M cell-cycle arrest, cellular cytotoxicity, and degradation of the EBNA1 protein. These effects are not apparent in Ad/AH or Hela cells, where expression of EBNA1 is tolerated. One possible explanation for these findings is that EBNA1 has variable effects on cellular gene transcription in different epithelial cell environments. To explore the possibility that EBNA1 may alter or influence cellular gene expression, transcriptional profiling using Affymetrix Focus Arrays was employed to compare gene transcription in Ad/AH cells stably expressing EBNA1 compared to control Ad/AH cells. In response to EBNA1 expression, 113 genes were up-regulated and 49 genes were down-regulated, with a significant fold difference of 1.5 or greater. Validations at the mRNA and protein level have been performed by semi-quantitative RT-PCR and immunoblotting, respectively. These assays have confirmed that EBNA1 is able to regulate the expression of genes associated with diverse cellular functions including signal transduction, transcriptional regulation and protein degradation. The functional consequences of these effects are currently under investigation and will be presented.

Structural and Functional Studies on the EBNA1 Interaction with USP7/HAUSP
Sarkari F, Holowaty M, Saridakis V, Sheng Y, Edwards A, Arrowsmith C, Frappier L

Contact:
Feroz Sarkari
University of Toronto
Medical Genetics and Microbiology (Toronto, Canada)
Email: feroz.sarkari@utoronto.ca

Abstract:
We have previously used an in vivo TAP-tagging approach and an EBNA1 affinity column approach to identify cellular proteins that specifically bind EBNA1, and both methods revealed a stable interaction with the ubiquitin specific protease, called USP7 or HAUSP. USP7 has also been shown to bind and stabilize p53 as well as the p53 E3 ubiquitin ligase, MDM2, with the overall effect of either increasing or decreasing p53 levels under different circumstances. We have shown that EBNA1 binds to the N-terminal domain of USP7, which is the same domain that binds p53, but does so with 10-fold higher affinity than p53, and efficiently competes with p53 for USP7 binding. We have now solved the 2 Å crystal structure of the USP7 N-terminal domain bound to the interacting EBNA1 peptide, revealing the mechanism of this interaction. We have also investigated the effect of EBNA1 on the expression level and turnover of p53 and MDM2 and on p53-mediated apoptosis. We find that EBNA1, but not a USP7-binding mutant of EBNA1, can counter act the stabilizing effect of USP7 on MDM2. We also find that EBNA1, but not the USP7-binding EBNA1 mutant, can decrease p53-mediate apoptosis. Taken together, the results suggest that EBNA1 can alter MDM2 and p53 levels through USP7 binding, perhaps in different ways under different circumstances.

EBNA-1 induces B-cells to respond to IL-2 survival signals

Tsimbouri P, Drotar M, Hill E, Wilson JB

Contact:
Dr. Joanna B. Wilson
University of Glasgow
IBLS Molecular Genetics (Glasgow, UK)
Email: joanna.wilson@bio.gla.ac.uk

Abstract:
Whether EBNA-1 contributes to tumourigenesis by means other than its function in viral DNA propagation is controversial. In order to address this, we have explored the consequences of EBNA-1 expression in the B-cells of two independently derived lines of transgenic mice (Wilson et al., 1996, EMBO J., 15, p3117). With the aim of examining the direct effects of EBNA-1 expression and not secondary mutations occurring through tumourigenesis, our studies have been conducted using explanted transgenic lymphocytes prior to the development of any tumour pathology. Transgenic lymphocytes show enhanced proliferation compared to controls and prolonged survival when cultured in the presence of IL-2. Surviving cells are B-cells and continue to express EBNA-1. This phenotype is demonstrated by lymphocytes derived from both transgenic mouse lines, developed independently and with distinct transgene integration events. As such the properties of prolonged cell survival and enhanced growth cannot be due to insertion site effects and can only be attributed to the actions of EBNA-1. These properties are characteristic oncogenic activities and in this system are context dependant, upon IL-2 signalling, a cytokine normally produced by activated T-cells supporting both T- and B-cell immune responses.
In order to explore the consequences upon tumour development of co-expression of EBNA-1 (inducing B-cell responsiveness to IL-2 as described above), LMP1 (a partial mimic of CD40 constitutive signalling) and LMP2A (mimicking B-cell receptor survival signals) a tritransgenic mouse crossbreed was developed. As previously reported, EμEBNA-1 mice develop B-cell tumours. Co-expression of EBNA-1 and LMP1 showed no impact upon the latency to tumour development while co-expression of EBNA-1 and LMP2A showed a slight inhibition in tumour development. However, co-expression of all three latent proteins significantly delayed the EBNA-1 induced tumour onset. Whether together the LMPs activate opposing mechanisms to EBNA-1 in B-cell development and differentiation will be discussed.

The Epstein-Barr virus nuclear antigen 2 transcriptionally activates the cellular anti-apoptotic bfl-1 gene by an RBPJk/CBF1 dependent pathway

Pegman P, Smith S, D'Souza B, Loughran S, Rowe M, Kempkes B, Gelinas C, Walls D

Contact:
Dr. Dermot Walls
Dublin City University
School of Biotechnology (Dublin, Irland)
Email: dermot.walls@dcu.ie

Abstract:
EBV establishes a latent infection and promotes the long-term survival of the infected host cell by targeting the molecular machinery that controls cell fate decisions such as apoptosis, proliferation and differentiation. These host-virus interactions are likely to play a crucial role in the development of EBV-associated malignancies. We have previously shown that (i) EBV-infected Burkitt's lymphoma cell lines exhibit elevated levels of expression of the anti-apoptotic bfl-1 gene compared to their uninfected counterparts; (ii) ectopic expression of Bfl-1 can protect a Burkitt's lymphoma (BL) cell line from apoptosis induced by serum deprivation (D'Souza, B., Rowe, M. and Walls, D. 2000, J. Virol., 74, 6652) and (iii) the EBV Latent Membrane Protein 1 (LMP1) stimulates bfl-1 promoter activity through interactions with components of the cellular Tumour Necrosis Factor Receptor (TNFR/CD40)-signalling pathway by a mechanism which includes an essential role for the transcription factor NFkB (D'Souza et al., 2004, J. Virol, 78, 1800-1816). Bfl-1 is an anti-apoptotic protein of the Bcl-2 family, whose preferential expression in hematopoietic and endothelial cells is controlled by inflammatory stimuli. Here, we present evidence that EBNA2 upregulates bfl-1 mRNA levels in the absence of LMP1 in BL-derived cell lines. We show that EBNA2 trans-activates the bfl-1 promoter by a mechanism that is dependent upon its ability to bind to RBPJk/CBF1, and that trans-activation is inhibited by a non-DNA-binding mutant of this nuclear component of the Notch signalling pathway. We demonstrate an essential role for a novel RBPJk/CBF1-like binding site on the bfl-1 promoter and present evidence of a role for Ets-family transcription factors in the regulation of this gene by EBNA2. Upregulation of bfl-1 by EBNA2 does not involve activation of NFkB and is modulated by other EBV latent proteins that are known to co-operate with EBNA2 (EBNA-LP) or which have been shown to interact with the RBPJk/CBF1-co-repressor complex (EBNA3A, 3B, 3C and RPMS1). These virus-host interactions are relevant to our understanding of EBV persistence, its role in malignant disease, and the B cell developmental process.

The Epstein-Barr virus target gene RUNX3 regulates RUNX1 levels in human B cells
Whiteman HJ, Elgueta Karstegl C, Farrell PJ, Spender LC

Contact:
Hannah Whiteman
Ludwig Institute for Cancer Research (London, UK)
Email: hannah.whiteman@ic.ac.uk

Abstract:
Objective: RUNX3 is a direct target of EBNA2 and is induced upon EBV infection of primary B cells while RUNX1 is simultaneously downregulated. In Burkitt's lymphoma (BL) lines, Group I cells (EBNA2 negative) express RUNX1 and Group III cells (EBNA2 positive) express RUNX3. Our objective was to investigate the mechanism of this reciprocal expression and its role in EBV infection.

Methods: The regulation of RUNX expression was investigated using siRNA, transfection assays and EMSA. Cell proliferation assays, microarray and FACS analysis were used to investigate the function of RUNX proteins.

Results: Over-expression of RUNX3 in cells endogenously expressing RUNX1 decreased RUNX1 protein levels while siRNA knockdown of RUNX3 in lymphoblastoid cell lines (LCLs) increased RUNX1 protein expression. These results show that RUNX3 regulates RUNX1 expression at physiological levels. RUNX1 expression is regulated by two promoters, P1 and P2. P1 was active in BL cells, demonstrated by promoter specific RNase protection assays. The abundance of P1 transcripts was reduced upon RUNX3 expression and the activity of a P1 promoter-reporter construct also decreased when transiently co-expressed with RUNX3 expression plasmids. Nuclear extracts from BL cells containing conditionally expressed RUNX3 bound to a region of P1 containing two RUNX consensus sites. Mutation of these sites abolished both the interaction in EMSAs and the RUNX3 mediated repression in reporter assays. siRNA inhibition of RUNX3 also reduced the proliferation rate of LCLs suggesting that RUNX3 expression plays an important role in immortalisation. Potential target genes regulated by RUNX proteins, including the activation marker CD86 (B7-2), were identified by microarray analysis of inducible RUNX3 cells. Surface expression of CD86 and CD54 (ICAM1) was reduced in RUNX3 knockdown LCLs. RUNX proteins therefore contribute to phenotypic differences observed between the different BL cells.

Conclusions: RUNX3 regulates RUNX1 expression by repression of the RUNX1 P1 promoter, explaining the reciprocal expression observed in BL cell lines. RUNX proteins contribute to BL cell phenotypes through expression of the activation markers CD86 and CD54, and play a role in regulating proliferation of EBV infected B cells.

The EBV-encoded latent membrane proteins, LMP2A and LMP2B, promote epithelial cell adhesion and motility

Allen MD, Young LS, Dawson CW

Contact:
Dr. Christopher Dawson
University of Birmingham
CR UK Institute for Cancer Studies (Birmingham, UK)
Email: c.w.dawson@bham.ac.uk

Abstract:
Objective: The frequent expression of latent membrane proteins LMP2A and LMP2B in EBV-positive carcinomas implicates a role for these proteins in EBV-induced epithelial cell growth transformation. To examine the effects of LMP2A and LMP2B expression on epithelial cell growth and differentiation, epithelial cell lines were generated in which LMP2A and LMP2B were stably expressed. The effect of expression of these proteins on cell behaviour was then assayed by a variety of techniques.

Methods: Epithelial cell lines stably expressing LMP2A and LMP2B were generated by retroviral transduction. Stable drug-resistant lines were assayed for (i) their ability to terminally differentiate in organotypic raft culture, (ii) their ability to promote cell spreading and focal adhesion formation on extracellular matrix, and (iii) their ability to migrate in transwell migration assays.

Results: Expression of LMP2A and LMP2B in human epithelial cell lines was not associated with gross alterations in cell morphology and had little if any effect on the ability of cells to terminally differentiate in organotypic raft culture. However, closer inspection revealed that LMP2A and LMP2B not only promoted epithelial cell attachment and spreading, but also enhanced the motility of these cells on extracellular matrix. Although the mechanisms by which LMP2A and LMP2B influence the processes of cell spreading and motility are unknown, the use of selective pharmacological inhibitors established a role for tyrosine kinases in this phenotype, but ruled out contributions of PI3-kinase, ERK-MAP-kinase and PKC.

Conclusions: The ability of LMP2A and LMP2B to promote cell spreading and motility identifies a new phenotype for these proteins. That LMP2B is able to induce a phenotype very similar to LMP2A suggests that regions of the LMP2 protein in addition to the cytosolic amino terminus are capable of inducing phenotypic effects in epithelial cells. Our observations also suggest that LMP2A and LMP2B may provide a stimulus to facilitate tumour spread in the absence of LMP1 expression.

Three EBNA3A domains are critical for LCL growth

Maruo S, Johannsen E, Illanes D, Cooper A, Zhao B, Kieff E

Contact:
Dr. Elliott Kieff
Channing Laboratory, Harvard Medical School, and Brigham and Women's Hospital
Department of Medicine and Microbiology and Molecular Genetics (Boston, USA)
Email: ekieff@rics.bwh.harvard.edu

Abstract:
Epstein-Barr Virus nuclear antigen protein 3A (EBNA3A) is essential for the conversion of primary B lymphocytes to lymphoblastoid cell lines (LCLs) and is also critical for maintenance of LCL growth. Study of the mechanism(s) by which EBNA3A promotes LCL growth has been limited by the lack of genetic data defining the functional domains within this 944 residue protein. We recently reported LCLs transformed by a reverse genetic recombinant EBV genome in which the EBNA3A gene was fused to a mutant estrogen receptor hormone binding domain (Maruo et al., 2003. Journal of Virology 77: 10437-10447). LCLs transformed by this genome were dependent on 4-hydroxytamoxifen (4HT) for growth. Growth is rescued by transfection with ori-P based plasmids expressing EBNA3A, but not by plasmids expressing GFP, EBNA3B, or EBNA3C. Using this assay we have now tested the ability of sixteen EBNA3A deletion mutants to maintain LCL growth. Deletion of the EBNA3A RBP-Jk binding domain (aa170-240) prevented EBNA3A dependent LCL growth or inhibition of EBNA2 transactivation of a multimerized RBP-Jk reporter. A triple alanine substitution in the RBP-Jk binding domain also abrogated the ability of EBNA3A to support LCL growth. Deletion of the EBNA3A charged domain (aa 300-386) did not affect RBP-Jk binding, but did prevent EBNA3A dependent LCL growth and interfered with EBNA2 transactivation. EBNA3A deletions C-terminal to the charged domain also prevented EBNA3A maintenance of LCL growth, but did not prevent RBP-Jk binding and inhibited EBNA2 transactivation; these mutations did not globally impair EBNA3A function. Our results confirm a central role for EBNA3A binding to RBP-Jk in LCL growth and indicate that other mechanisms are also important for EBNA3A effects in LCL growth.

Epstein-Barr virus infection of naive and memory B cells in vitro

Begue Pastor N, Baldwin G, Rickinson AB, Delecluse HJ

Contact:
Dr. Noelia Begué Pastor
University of Birmingham
Cancer Research UK Institute for Cancer Studies (Birmingham, UK)
Email: beguep@cancer.bham.ac.uk

Abstract:
Objectives. EBV predominantly resides in resting memory B cells, and not naïve cells, in the blood of healthy individuals. It is not known how this preferential colonisation of the memory pool is achieved. We studied EBV infection of naïve and memory B cells in vitro to ask whether these B cell subsets are equally infectable and susceptible to immortalisation, and whether EBV immortalisation alters the immunoglobulin (Ig) genotype and/or surface Ig phenotype oftarget cells.

Methods. Peripheral blood B cells were FACS sorted into IgD+, CD27- naïve and IgD-, CD27+ memory subsets. Cells were exposed to EBV and virus binding assayed by quantitative PCR. Early events were monitored by EBNA2 staining and B cell outgrowth from limiting dilution cultures determined after 4 weeks. The surface Ig phenotype of LCLs was analysed by antibody staining. The rearranged VDJ region of the IgH gene was amplified by PCR from naïve and memory B cells before infection and from the resulting LCLs, the products cloned, sequenced and compared to the Ig germ line database

Results. Naïve and memory B cells bound the virus equally well and were equally susceptible to EBNA2 induction and subsequent immortalisation. The surface Ig phenotype of the resultant LCLs matched that of the starting population: naïve B cell-derived lines were consistently IgM+ IgD+, most memory B cell-derived lines were IgG+ or IgA+, though a small minority expressed IgM and IgD. Ig genotyping of cells ex vivo confirmed the predominance of naïve Ig sequences in IgD+ CD27- sorts and of mutated sequences in IgD-CD27+ sorts. Naïve B cell-derived lines generally retained a naive genotype (less than 1% divergence from germline) but occasionally showed slightly more mutations than the ex vivo population. Most memory B cell-derived lines had mutated Ig genotypes, including some lines with a IgM+ IgD+ phenotype.

Conclusions. EBV immortalises naïve and memory B cells in vitro with equal efficiency and does not substantially alter the Ig genotype or phenotype of infected cells; however the low level of divergence from germ line Ig sequences seen in some naïve B cell-derived lines needs further study.

Characterization of EBV gH residues important for B cell and epithelial cell fusion
Omerovic J, Longnecker R

Contact:
Jasmina Omerovic
Northwestern University
Microbiology and Immunology (Chicago, USA)
Email: j-omerovic@northwestern.edu

Abstract:
Epstein-Barr virus (EBV) infects both B lymphocytes and epithelial cells. The mechanism of EBV entry into target cells is still very poorly understood. Limited data suggest differences in the requirement for infection of B cells and epithelial cells. Viral glycoproteins required for entry into B cells are gp42, gH, gL and gB, while gp42 is not necessary for infection of epithelial cells. In EBV, gH and gL form two different complexes, a bipartite complex that contains only gH and gL, and a tripartite complex that also includes gp42. These two complexes have a mutually exclusive ability to mediate infection of epithelial cells and B cells, respectively. Previous reports have shown that EBV encoded gH and gL are essential for EBV induced membrane fusion and viral entry into both cell types (Haddad and Hutt-Fletcher, JV 63, 1989; Oda et al., Virol. 276, 2000). However, the exact role of gH/gL during EBV fusion process into either cell type and domains/residues required are not yet known. Recent data suggest a role of gH and gL in binding to epithelial cells and the existence of a possible receptor on these cells (Molesworth et al., JV 74, 2000; Borza et al., JV 78, 2004). In this study, mutagenesis of EBV gH was performed in order to identify residues important for fusion with B cells and epithelial cells with hopes of generating mutants defective in fusion with only one cell type.

Mutational Analysis of EBV gp42 Reveals New Binding Domain Essential for Membrane Fusion

Silva A, Omerovic J, Kirschner A, Jardetzky T, Longnecker R

Contact:
Amanda Lowrey Silva
Northwestern University
Microbiology/Immunology (Chicago, IL, USA)
Email: a-lowrey@northwestern.edu

Abstract:
Human herpesvirus-induced membrane fusion is essential for infection and pathogenesis, and although many key players have been identified, the mechanisms still remain poorly understood. In addition to the concerted action of gH, gL and gB for EBV-induced B-cell membrane fusion is the requirement of gp42. It was previously demonstrated that gp42 forms heterotrimeric complexes with gH and gL, and that gp42 inhibits infection of epithelial cells. The receptor of gp42 is the Class II human leukocyte antigen (HLA) and the crystal structure of soluble gp42 bound to the HLA-DR1 allele was recently solved. We have undertaken mutational analysis to further understand the spatial and temporal interactions of gp42 with other cellular and viral proteins. Our results confirm that three different domains of gp42 are involved in the Class II interface and are all required for binding and fusion. We also demonstrated that the hydrophobic pocket, which in other c-type lectin family members is critical for receptor binding, is not involved in Class II binding, but is essential for membrane fusion. Identification of the hydrophobic pocket ligand and how Class II binding induces a conformational change in gp42 which alters this and other interactions will allow further understanding of how gp42 triggers membrane fusion as well as generate a target for small molecule inhibitors.

Herpesvirus Reactivation in the JSC-1 Lymphoma Cell Line and the Effects on Cellular and Viral Gene Expression

Bagni RK, Marshall VA, Yuan C, Hines-Boykin R, Dittmer D, Vahrson W, Whitby D

Contact:
Rachel Bagni
SAIC-Frederick, NCI-Frederick
Viral Epidemiology Section (Frederick, USA)
Email: bagnir@mail.ncifcrf.gov

Abstract:
Objective: This study aims to identify cellular genes which are affected by gamma-herpesvirus reactivation (Kaposi's Sarcoma Associated Herpesvirus (KSHV) and Epstein Barr Virus (EBV)) and to further understand the kinetics of viral genes during viral reactivation.

Methods: KSHV and EBV were reactivated with 0.3mM sodium butyrate in a 72h time course experiment using the JSC-1 PEL cell line. DNA viral loads were determined using quantitative PCR assays. The Affymetrix microarray system was used to profile cellular genes. Whole genome quantitative PCRs for each KSHV and EBV were used to profile viral transcripts. Affymetrix data were analyzed using a conservative approach to identify cellular genes with changes >3 fold as compared to controls. k-means clustering and Principal Component Analysis (PCA) were used to identify genes and/or clusters of significance. Final gene lists include only those genes which pass the 1-Way ANOVA statistical test with a p-value cut-off 0.05. Viral array data was analyzed using Gaussian clustering based on normalized Pearson correlation of CT values.

Results: Visualization of the PCA as colored by defined k-means clusters identified groups of cellular genes with similar profiles over the time course. Inspection of the gene lists associated with each cluster revealed only one cluster to include cellular genes primarily associated with DNA replication and cell cycle progression. Of note, a detectable increase in viral load correlated with the first detectable decrease in cell viability during the time course. KSHV and EBV transcription commenced in an ordered cascade of early, delayed early and late classes and preceded increases in viral load. KSHV mRNAs clustered with other KSHV mRNAs, rather than with their homolog in EBV and KSHV latency I transcripts (LANA, vCYC) did not change upon lytic induction. Based upon these we were able to define a novel class of EBV latency transcripts. KSHV transcription increased prior to EBV transcription and declined as EBV transcription reached maximum levels.

Conclusions: This study reveals a tight cluster of DNA replication and cell division genes which follow a similar profile of lowered expression after gamma-herpesvirus reactivation. This analysis also provides insight into the kinetics of viral gene transcription in relation to changes in cellular gene expression.

Transfer Infection: a novel and efficient method of epithelial cell infection
Shannon-Lowe C, Rickinson AB, Delecluse HJ

Contact:
Dr. Claire Shannon-Lowe
University of Birmingham
Cancer Research UK Institute for Cancer Studies (Birmingham, UK)
Email: clairesl@cancer.bham.ac.uk

Abstract:
Objectives: EBV infection in healthy individuals is mainly confined to the B lymphocyte pool, but the association of the virus with oral hairy leukoplakia and with certain carcinomas indicates that epithelial infection can occur. Our understanding of the mechanism of gp350/CR2-independent entry into epithelial cells and of virus transcription post-infection would be helped if an efficient in vitro model of epithelial infection were available.

Methods: An in vitro infection system was developed using recombinant EBV strains (all with a GFP gene insert) bound to the surface of freshly-isolated, -irradiated, resting B cells as a means of virus delivery to epithelial cell cultures. Following a brief co-cultivation, B cells were washed off and the infection of epithelial cells was analysed by GFP expression, by quantitative RT/PCR assays for EBNA1, LMP1, LMP2A, BZLF2, Cp- and Wp-initiated transcripts, and by MAb staining for viral antigen expression. Finally, we used a panel of EBV glycoprotein gene knockouts to determine the contribution of individual glycoproteins to this transfer infection.

Results: Using transfer infection, virus entry into CR2-negative primary epithelial cell cultures (as monitored by GFP expression) was up to 10,000-fold more efficient than that achieved by virus alone. Similar levels of enhancement were observed for infection of CR2-negative epithelial cell lines. We demonstrate using a recombinant BZLF1 knockout virus that infection does not require de novo virus lytic replication in the B cell. Using a panel of EBV recombinants with individual glycoprotein knockouts, we show that BILF2 is dispensable for transfer infection, both gp110 and gp350 are required for optimal transfer (the latter reflecting its role in B cell binding), whereas the fusion protein gp85 is absolutely essential for transfer. Finally, assaying infection of primary human epithelial cultures by quantitative RT/PCR revealed transient expression of Qp-initiated EBNA1 and low level LMP1 transcripts, and an initial burst of BZLF1 transcription which then fell to a lower but sustained level. No Cp- or Wp-initiated transcripts were detected at any time point.

Conclusions: 'Transfer infection' is a novel and highly efficient method of introducing EBV (both recombinant and wild type strains) into primary epithelial cells and epithelial cell lines in vitro. We infer that this process also occurs in vivo; indeed the main route of entry of the virus into pharyngeal epithelium may be through interactions between epithelial cells and mucosal B cells that have recently bound infectious virions.

Novel Roles For The Basic Region Of Zta for both Transactivation And Viral Latency Reactivation Revealed by Mutation Of C189

Valencia S, Schelcher C, Delecluse HJ, Sinclair AJ

Contact:
Dr. Alison Sinclair
University of Sussex
School of Life Sciences (Brighton, UK)
Email: a.j.sinclair@Sussex.ac.uk

Abstract:
Objective: The bZIP domain of Zta is involved in nuclear localisation, dimer formation, cell cycle arrest and interaction with DNA and cellular proteins. This domain contains two highly conserved cysteine residues, one within the coiled coil region (C222) and one within the basic region (C189); this has been previously implicated in the redox control of DNA-binding. In this study we question the relevance of each of these cysteine residues for Zta function.

Methods: Mutations substituting a cysteine codon for a serine codon in Zta were generated. Proteins generated in vitro were used for EMSA, chemical cross-linking and biophysical investigations. ZRE-dependent reporter constructs were used for transactivation studies and the Zta knock-out 293-EBV cell line was employed for virus reactivation studies.

Results: Mutant forms of Zta that each substitute a Serine for a Cysteine residue at C189 and C222 were generated. Their ability to interact with three independent ZREs was assessed quantitatively in vitro. This revealed no differences in the ability to interact with DNA. The mutants also localised to the nucleus appropriately and were not compromised in their ability to form dimers. However, virus reactivation assays, measuring increases in the quantity of EBV genome using real-time PCR, revealed that the basic region mutant (C189S) was severely compromised in its ability to reactivate EBV from latency. Furthermore, the basic region mutant was also severely compromised in its ability to transactivate a ZRE-dependent reporter construct in vivo. In contrast, mutation of C222 in the coiled coil region of Zta had no effect on either of these functions.

Conclusions: We demonstrate that the basic region of Zta is required for both transactivation through ZREs and reactivation of the viral lytic cycle in vivo, independently of its ability to interact with DNA or to fold as a dimer. Residue C189 is critical for these functions. We have generated a working model proposing a structure for the basic region of Zta which incorporates this data and data from previously published mutants generated by Miller's and Flemington's groups and proposes the presence of two essential interaction faces on either side of the alpha helix.

EBV and multiple sclerosis

Ascherio A, Munger K

Contact:
Dr. Alberto Ascherio
Harvard School of Public Health
Nutrition and Epidemiology (Boston, USA)
Email: aascheri@hsph.harvard.edu

Abstract:
Objective: To determine the role of EBV in the etiology of multiple sclerosis (MS).

Methods: Review of several investigations on the role of EBV in the etiology of MS, including recent large prospective studies using blood samples collected before the onset of neurological symptoms.

Results: A critical analysis of the literature reveals the following facts:
(i) The risk of MS among individuals who are not infected with EBV is about 1/10 the risk of infected individuals. This result has been recently confirmed in a study of pediatric MS, and is thus unlikely to be due to atypical features of EBV negative adults;
(ii) Antibody titers to EBNA-1 measured among healthy young adults, are strongly predictive of their risk of developing MS. This result has been found consistently in two large prospective studies conducted by our group, and has been confirmed in blood samples collected several years before the first onset of MS symptoms. Preliminary results suggest that EBV viral load in plasma is also related to increased risk of developing MS.
(iii) The risk of MS among individuals with history of infectious mononucleosis is two to three times higher than among EBV positive individuals without history of infectious mononucleosis.
(iv) Several aspects of the epidemiology of MS could be explained if infection with EBV increased the risk of MS in genetic susceptible individuals, particularly when infection occurs at an age when IM is common.
(v) However, other aspects of the epidemiology of MS are not apparently explained by the distribution of EBV or age at infection with EBV. Among these, are the occurrence of an epidemics of MS in the Faroe islands and the gradient in MS prevalence observed in New Zealand.

Conclusions: Overall, there is strong evidence that EBV plays a role in the etiology of MS. Some aspects of MS epidemiology however could only be explained if either different strains of EBV have different propensity to cause MS, or if there are still unknown interactions between EBV and other infectious agents.

LMP1 strain distribution in Southeast Asia

Saechan V, Mitarnun W, Settheetham-Ishida W, Tiwawech D, Soemantri A, Isa MN, Ishida T

Contact:
Vannarat Saechan
The University of Tokyo
Biological Sciences (Tokyo, Japan)
Email: svannarat2002@yahoo.com

Abstract:
Objective: Epstein-Barr virus (EBV) is found in all human communities and associated with the development of certain lymphoid and epithelial neoplasms. It is well known that latent membrane protein 1 gene (LMP1) has variation that sometimes may lead to functional alteration or shows specific geographically distribution.

Methods: The EBV DNA was extracted from healthy individuals belonging to 10 Thai ethnic groups (Lahu, shan, Lisu, Red Karen, White Karen, Mlaburi, Hmong, Akha, Central Thais and Southern Thais), Kelantan Malays, and Indonesian (Sumbanese). By using a PCR-direct sequencing method, nucleotide sequences corresponding to the carboxyl terminus of the LMP1 were determined. Translated into amino acid sequences were aligned by using MEGA program and compared.

Results: Phylogenetic analysis of the carboxyl terminus of LMP1 amino acid sequence has identified five strains: B95-8 prototype, China 1, China 2, Mediterranean, and one new characteristic strain (SEA 2). The major strain in each population was the B95-8 prototype strain in Lahu; China 1 strain in Southern Thais and Kelantan Malays; China 2 strain in Akha, central Thais and Hmong; Mediterranean strain in Red Karen and SEA 2 in white Karen, Mlaburi, Lisu, Shan and Sumbanese. A new strain SEA1 that is specific to EBV associated T-cell proliferative disease in SEA was not found in these healthy individuals.

Conclusions: Distribution pattern of strains reflects in some part geographic location but the ethnicity or the history of population admixture may have contributed to the present pattern.

Analysis of sequence variations in EBNA3A and EBNA1 of Epstein-Barr virus isolates from Korea

Kang SH, Kim GN, Lee WK

Contact:
Dr. Won-Keun Lee
Myongji University
Bioscience and Bioinformatics (Yongin, Korea)
Email: wklee@mju.ac.kr

Abstract:
We have previously identified an intertypic recombinant of EBV with a genotype of type 1 EBNA2, -3A/type 2 EBNA3B, -3C from a Korean patient with an EBV-associated tumors. Recently, we also isolated a similar recombinant from a healthy carrier, corroborating previous reports that intertypic EBV recombinants are circulating in the general human polulations, along with two major types of EBV, type 1 and type 2. To reveal homologous recombination sites responsible for these recombinants sequences between the EBNA3A C-terminus- and the EBNA3B N-terminus-coding regions were amplified and determined from 23 EBV isolates including two recombinants. Similarly, to determine EBNA1 subtypes of Korean EBV isolates, nucleotide sequences for the EBNA1 N- and C-terminal regions were determined and analyzed. One of the recombinants showed uniformly type 1- and type 2-specific changes,separated by a stretch of 100-bp sequences in the EBNA3A C-terminal region, indicative of a single recombination event. However, the other recombinant showed no clear-cut boundary for variations in terms of type signatures but it had clusters of type 2 and type 1 signatures in order, between regions with unifomly type 1 and type 2-specific variations, suggesting that this recombinant might have been generated by multiple recombinations. Interstingly, the same pattern of alternating clustered type-specific variations were also found in three of 16 typical type 1 isolates analyzed, suggesting that these isolates might also be a recombinant. These results suggest that the EBNA3A C-terminal region and the intergenic region between EBNA3A and 3B might serve as a recombinational hotspot. The anlyses of EBNA1 N- and C-terminus-coding regions revealed the diversity of EBNA1 subtypes among Korean EBV isolates. The Q/V EBNA1 subtype (10/24) was most prevalent and strictly found among type 1 EBV. The second most common form was the Q/T subtype (6/24), which was preferentially associated with type 2 EBV.

Relation between malaria infection and increased EBV load

Falk K, Espmark E, Donati D, Kironde F, Wahlgren M, Linde A, Bejarano MT

Contact:
Dr. Kerstin Falk
Karolinska Institute and Swedish Institute for Infectious Disease Control
Microbiology and Tumorbiology Center (Stockholm, Sweden)
Email: Kerstin.Falk@mtc.ki.se

Abstract:
Objective: Children living in malaria-endemic regions have a high incidence of Burkitt's lymphoma (BL) whose aetiology involves Epstein-Barr virus (EBV) and P. falciparum malaria infections. The aim of this study was to analyse the correlation between EBV genome load and the course of malaria infection in Ugandan children.

Method: We quantified EBV DNA copies in plasma and saliva samples collected from 113 Ugandan children including: a) 45 children with acute malaria at the day of diagnosis (day 0) and 14 days after receiving anti malarial treatment (day 14), b) 40 malaria asymptomatic age matched controls, and c) 28 children with diagnosed BL.

Results: Viral DNA was detected in all saliva and serum samples, with higher levels in saliva than in serum. The viral load in serum was higher during acute malaria and decreased after malaria treatment was initiated. The mean copy levels of viral DNA/ml of sera were: 32100 at day 0 and 2.900 at day 14. In asymptomatic children the mean levels were lower, 100 copies/ml and contrasted with the extremely high levels detected in sera from BL patients in which the mean level was 998.000 copies /ml. The differences in saliva vs. serum were 2 logs during acute malaria, 3 logs after treatment and 4 logs in asymptomatic children. In saliva the values were 21.470.000 and 50.600.000 at days 0 and 14, respectively; 6.830.000 in asymptomatic and 35.850.000 in Burkitt's samples, respectively.

Conclusions: A significant correlation (p =0,01) between the levels of EBV DNA in serum at days 0 and 14 of malaria infection was demonstrated. Thus, acute malaria infection leads to EBV reactivation as shown by higher serum EBV viral load during and after a malaria episode.

Patients with Systemic Lupus Erythematosus have abnormally elevated EBV load in the blood

Moon UY, Park SJ, Oh ST, Kim WU, Park SH, Lee SH, Cho CS, Kim HY, Lee WK, Lee SK

Contact:
Prof. Dr. Suk Kyeong Lee
Catholic University of Korea
Catholic Reserch Institues of Medical Science (Seoul, Korea)
Email: sukklee@cmc.cuk.ac.kr

Abstract:
Objective: Various genetic and environmental factors seem to be involved in systemic lupus erythematosus (SLE). Epstein-Barr virus (EBV) is one of the environmental factors suspected to play a predisposing role in SLE based on the characteristics of EBV itself as well as sequence homologies between autoantigens and EBV antigens. In addition, elevated titers of anti-EBV antibodies and increased EBV seroconversion rates were observed in SLE patients than in healthy controls. This study was performed to clarify the precise status of the EBV infection in SLE patients. This would be valuable in understanding the role of EBV in SLE, as serological responses do not directly reflect the status of EBV in the body.

Methods: We tested EBV types infected in SLE patients (n=66) and normal controls (n=63) by a direct PCR analysis of mouthwash samples. We also compared EBV loads in the blood of SLE patients (n=24) with those of healthy controls (n=29) by a semiquantitative PCR assay.

Results: Infection rate (98.5% vs. 94%) and type distribution of EBV in the adult SLE patients were similar to those in the healthy controls. Interestingly, the EBV burden in the peripheral blood mononuclear cells was over 15 fold greater in the SLE patients compared with healthy individuals (mean +/- standard deviation, 463 +/- 570 vs. 30 +/- 29, p = 0.001), suggesting that EBV infection is abnormally regulated in SLE.

Conclusions: The abnormally increased EBV-infected B cells in the SLE patients may contribute to an enhanced autoantibody production in this disease.

Sexual Transmission of Epstein-Barr Virus

McAulay K, Thomas R, Macsween KF, Higgins C, Clutterbuck D, Anderson R, White J, Reid S, Harrison N, Swerdlow A, Crawford DH

Contact:
Karen McAulay
University of Edinburgh
Medical Microbiology (Edinburgh, UK)
Email: K.McAulay@ed.ac.uk

Abstract:
Objective: Epstein-Barr virus (EBV) is present in the saliva of most seropositive individuals and is spread by close contact. However, the virus has also been found in genital secretions, suggesting sexual transmission as a second route of spread (1,2,3). A cross-sectional study in Edinburgh University students provided strong epidemiological evidence to support this suggestion (4), but direct evidence of sexual spread is still lacking. This present study was undertaken to confirm the presence of EBV in genital secretions, and to determine if transmission of EBV occurs between sexual partners.

Methods: EBV strain analysis in blood and throat wash samples from patients with infectious mononucleosis (IM) and their close contacts was carried out by polymerase chain reaction (PCR) amplification of repeat sequences in EBNA 3C and LMP1 genes. Seventy five percent of sexual contacts carried strains identical to the IM case, whereas only 29% of close but non-sexual contacts carried matching strains.

Results: Cervical swabs, male urethral secretions and semen samples collected from healthy individuals were analysed for the presence of EBV DNA by PCR. Seven percent of cervical and 5% of urethral samples were EBV positive, whereas only 1 out of 24 semen samples contained detectable EBV DNA. Separation of cervical and urethral samples into cell associated and cell free fractions identified the majority of EBV DNA to be within the cell associated fraction.

Conclusions: Our results suggest that EBV can be transmitted sexually by transfer of virus infected cells.

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Infection of epithelial cells with Epstein-Barr virus

Kobayashi R, Ehara T, Sumida S, Igarashi M, Mizuno F

Contact:
Dr. Ryo Kobayashi
Tokyo Medical College
Department of Microbiology (Tokyo, Japan)
Email: ryo@tokyo-med.ac.jp

Abstract:
Objective: In a series of our studies to understand the natural transmission of EB virus to epithelial cells which did not bear the EBV receptor molecule CD21/CR2, we previously described that co-cultures of EBV-harboring lymphoblastoid cells as the virus donor and epithelial cells as its recipient, resulted in the promotion of the virus infection of the latter cells. In addition, this phenomenon obviously occurred when not only the virus-producer but also non-producer cells were used as the donors.

Methods: The EBV-nonproducer Raji cells which harbor fifty EBV genome equivalents per cell in entirely latent form with no evidence of virus replication, were used as relevant EBV-donor cells to these experimental conditions. Raji cells were labeled by transfection with episomes coding a proper marker protein, enhanced cyan fluorescent protein (ECFP) for easier analysis of these experiments. Epithelial MKN28 cells derived from human gastric cancer were employed as the expected targets of viral infection and were grown on coverslips. ECFP-labeled Raji cells in suspension were added to the monolayers of MKN28 cells.

Results: Within a few days of culture, co-cultures of two types of cells yielded adherent squamous cells positive for ECFP easily observed by optical microscope on coverslips, also positive for EBV-specific nuclear antigen (EBNA) observed by immunofluorescence, and positive for cytokeratin detected by mouse mAb, indicating that epithelial cells might become infectable with EBV by cell fusion.

Conclusions: In this study, we examined the mechanism of EBV "cell-to-cell" infection.

A quantitative evaluation of Epstein-Barr virus binding to B cells, nuclear delivery of the virus genome, latent antigen expression and immortalisation

Shannon-Lowe C, Baldwin G, Feederle R, Bell A, Rickinson AB, Delecluse HJ

Contact:
Dr. Andrew Bell
University of Birmingham
Cancer Research UK Institute for Cancer Studies (Birmingham, UK)
Email: A.I.Bell@cancer.bham.ac.uk

Abstract:
Objectives: B cell immortalisation to lymphoblastoid cell lines (LCLs) remains the standard biological assay for EBV but the process is poorly quantitated at each stage. Accurate quantitation is particularly important now, in the era of recombinant virus technology, where the effect of a virus gene knockout may be subtle

Methods: Recombinant EBV (B95- 8 strain with a GFP expression cassette and gp110 reconstituted) was exposed to primary B cells at varying multiplicities of infection (MOIs) and numbers of virions bound to the cell surface determined by quantitative PCR for the viral genome; anti-gp350 blocking and gp350-knockout virus confirmed specificity of the binding assay. Virus genome delivery to the nucleus was quantitated by fluorescence in-situ hybridisation (FISH) with an EBV cosmid probe, active viral infection by GFP and EBNA2 immunofluorescence staining prior to cell division, cell cycle entry by CFSE labelling, and LCL outgrowth by limiting dilution culture on feeder cells.

Results: At an MOI of 1, one virion is bound per cell and the genome reaches the nucleus in 10% cells; at an MOI of 100, a mean of 20 virions bind per cell and genomes (1-18 copies) reach the nucleus in 80% cells. Importantly, the number of nuclear genome-positive cells identifiable by FISH closely matches the number of EBNA2-expressing cells at 48 hours post infection and the majority of these cells went through at least one cell division. When equivalent numbers of EBNA2-positive cells from low and high MOI infections were seeded into limiting dilution culture, they showed equally efficient outgrowth to LCLs despite their different initial genome loads. In both cases, around 1 in 10 actively infected cells achieved successful outgrowth.

Conclusions: There are several points (virus binding, virus genome delivery, viral gene expression, cell cycle entry and LCL growth) at which EBV-induced B cell infection/immortalisation might stall. We find that EBV binding to B cells is very efficient whereas delivery of the viral genome to the nucleus is a critical rate limiting step. Interestingly, most cells in which the viral genome successfully reaches the nucleus then activate virus latent gene expression and enter cell cycle. The main rate-limiting step beyond this point is continued outgrowth to an LCL; to what extent this reflects in vitro constraints to which even established LCL cells are also subject is being determined.

Rheumatoid Factors Induce Signaling from B-cells, thus Leading to Epstein-Barr Virus and B-cell Activation

Yang L, Hakoda M, Iwabuchi K, Takeda T, Koike T, Kamatani N, Takada K

Contact:
Prof. Dr. Kenzo Takada
Institute for Genetic Medicine, Hokkaido University
Department of Tumor Virology (Sapporo, Japan)
Email: kentaka@igm.hokudai.ac.jp

Abstract:
B-cell antigen receptor signaling is initiated upon binding of the antigen to membrane-bound immunoblobulin (Ig), and the anti-Ig antibody (Ab) mimics this signaling. In B-cells latently infected with EBV, the same signals induce virus activation. This study examines whether rheumatoid factors (RFs), autoantibodies directed against the Fc portion of IgG, induce Epstein-Barr virus (EBV) and B-cell activation. As a source of RFs, RF-producing lymphoblastoid cell (LCL) clones were isolated from peripheral blood mononuclear cells (PBMC) and synovial cells from patients with rheumatoid arthritis (RA) by EBV transformation. Burkitt's lymphoma-derived Akata cells, which are highly responsive to EBV activation by anti-Ig Abs, were used for the assay of EBV activation. Akata cells expressed IgG3 as membrane-bound Ig. RFs from a synovium-derived LCL were directed to IgG3 and induced EBV activation in 16 to 18% of Akata cells, while RFs from another synovium-derived LCL were directed to IgG1 and did not induce EBV activation. Pretreatment of RFs with the purified Fc fragment of human IgG completely abolished EBV activation. Furthermore, B-cell activation was assessed by incorporation of 3H-thymidine. RFs from synovium-derived LCLs efficiently induced B-cell activation and addition of CD40 ligand had a synergistic effect. On the other hand, RFs from PBMC-derived LCLs were polyreactive, had a lower affinity to IgG, and did not induce EBV and B-cell activation. The present findings imply a possible role for RFs as EBV and B-cell activators.

Maintenance of serum IgG antibodies to Epstein-Barr virus nuclear antigen-2 in healthy individuals from different age groups in a Japanese population with a high childhood incidence of asymptomatic primary EBV infection.

Yanagi K, Harada S, Kamata Y, Kitamura R, Okabe N, Senpuku H, Kanegane H, Nakamura Y

Contact:
Dr. Kazuo Yanagi
National Institute of Infectious Diseases
Department of Virology I (Tokyo, Japan)
Email: kyanagi@nih.go.jp

Abstract:
Objective: Information about the long-term antibody responses to EBNA-2 and EBNA-1 following asymptomatic EBV infection would be provided by analyses of sera from different age groups of a population undergoing asymptomatic infection during infancy and young childhood. Thus, IgG antibodies to Epstein-Barr virus (EBV) nuclear antigens (EBNA)-2 and -1 were studied using sera from healthy individuals of a population with a high incidence of asymptomatic primary EBV infections during infancy or childhood in Japan.

Methods: Two CHO-K1 cell lines expressing EBNA-2 and EBNA-1 were used for anti-complement and indirect immunofluorescence assays.

Results: The positive rate for EBNA-2 IgG rose in the 1-2 year age group, increased and remained at a plateau (~45%) between 3 and 29 years of age (3-4, 5-9, 10-14, 15-29 groups), then reached 98% by age 40 (-40 age group). Both seropositivity for EBNA-1 and for EBNAs in Raji cells (EBNA/Raji) were detected in the 1-2 year age group, remained high, and finally reached 100% by age 40. The geometric mean titer (GMT) of EBNA-2 IgG reached a plateau in the 5-9 and 10-14 year old groups, and remained elevated in the older age groups (15-29 and -40 years). The GMT of EBNA-1 IgGs increased to a plateau in the 1-2 year-old group and remained unchanged in the older age groups. The GMT of EBNA/Raji IgGs also reached a plateau in the 1-2 year-old group, remained level throughout the 3-14 year age groups, and decreased in the15-29 year-olds. EBNA-2 IgGs emerged earlier than EBNA-1 IgGs in 8/10 patients with IM, who were between 1 and 27 years old, and declined with time in 3/8 cases.

Conclusions: These results suggest that EBNA-2 IgG antibodies evoked in young children by asymptomatic primary EBV infections remain elevated throughout life, probably because of reactivation of latent and/or exogenous EBV superinfection.

A human tonsillar tissue culture system can be used to study immune responses against Epstein-Barr virus infection

Ladell K, Berger C, Erbetta S, Nadal D

Contact:
Dr. Kristin Ladell
University Children's Hospital of Zurich
Experimental Infectious Diseases and Cancer Research (Zurich, Switzerland)
Email: Kristin.Ladell@kispi.unizh.ch

Abstract:
Objective: Epstein-Barr virus (EBV) which is associated with different malignancies is widely distributed among the adult population. The tonsils are a reservoir of persistent EBV infection. The reasons why some people develop symptomatic EBV infection or EBV-associated malignancies are not yet clear. We recently described a tonsillar tissue culture system which we characterized and compared to autologous tonsillar cell suspension cultures in respect of preservation of tissue structures, composition of the lymphocyte subsets, and constitutive cytokine gene expression levels. The aim was to infect these cultures with EBV to study EBV pathogenesis.

Methods: Expression of viral latent (EBNA1, EBNA2, LMP1, LMP2) and lytic (BZLF1, gp85) genes was quantified in parallel in human tissue and tonsillar cell suspension cultures +/- cyclosporin A (CSA) before and following EBV infection (B95-8 strain) by real-time polymerase-chain reaction in relation to the housekeeping gene hydroxymethylbilane synthase. Samples were taken at 0, 2 or 4, 24, 48, 72, 144 and 168 hours (h) of infection.

Results: A latent pattern of EBV gene expression was observed in both tissue and cell suspension cultures (n=4, respectively) within the first 4 days following EBV infection in vitro. The first EBV genes EBNA1 & 2 were expressed after 24 h, followed by LMP1 and 2 after 48 h. No immediate early lytic gene expression (BZLF1) was detected in tissue cultures, but was detected in one of the cell suspension cultures after 96 h of infection. The late lytic gene gp85 was expressed in cell suspension cultures as early as the first latent genes, whereas in tissue cultures gp85 was only expressed at 7 days of infection.

Conclusions: We show that tissue and cell suspension cultures can be infected with EBV in vitro. Similar patterns of EBV gene transcription are seen in these two types of cultures. The establishment of this ex vivo model can be exploited for the study of early events of cellular EBV infection within the preserved tonsillar organ structure.

Intranasal infection of newborn mice with MHV-68 - A model for gamma-herpesvirus induced neurological disease

Kleines M, Scheithauer S, Engler M, Ahlberg E, Sellhaus B, Ritter K, Hausler M

Contact:
Dr. Michael Kleines
UK Aachen
Division of Virology, Department of Medical Microbiology (Aachen, Germany)
Email: mkleines@ukaachen.de

Abstract:
Objective: The Epstein-Barr virus, a gamma-herpesvirus causes various neurological symptoms and complications ranging from facial palsy to encephalitis. The molecular pathogenesis of these complications has not been elucidated. Efficient therapeutic regimens are not available. For non-neurologic gamma-herpesvirus disease a model with the murine gamma-herpesvirus MHV-68 proved satisfactory. However, infection of the CNS could only be achieved by direct inoculation leading to lethal multisystem disease.

Methods, Results: In order to establish a satisfactory model for neurological disease caused by EBV BALB/c mice were intranasally inoculated with supernatant of MHV-68 infected BHK-21 cells one day after birth. Infected mice and controls were either killed one week or five weeks post infection or when symptoms occurred. All solid organs were checked for the presence of virus by PCR and for viral activity by RT-PCR. We detected viral DNA and viral transcripts in all organs including the brain. The organ-specific viral loads exceeded the viremia significantly. Viral loads declined with the interval post infection. Organ extracts were successfully used for re-infection of BHK-21 cells indicating productive infection with MHV-68. Presence of MHV-68 in the brain was shown by immunofluorescence. In the infected tissue accumulation of T-cells was demonstrated immunohistochemically. Inflammatory lesions gave the typical pictures of meningitis, focal encephalitis, diffuse cerebral vasculitis, or temporal lobe encephalitis, conditions which have all been diagnosed in human brain following EBV-infection.

Conclusions: Thus, we successfully established a natural model for neurological gamma-herpesvirus infections allowing characterization of the molecular pathogenesis as well as testing of medication regimens.

The use of RNAi silencing to study the function of LMP2A in carcinoma cells stably infected with rEBV

O'Neil JD, Stewart SE, Dawson CW, Takada K, Young LS

Contact:
Dr. John O'Neil
University of Birmingham
CR UK Institute for Cancer Studies (Birmingham, UK)
Email: johno@cancer.bham.ac.uk

Abstract:
In EBV-positive nasopharyngeal carcinoma (NPC), the virus-encoded latent membrane protein LMP2A is consistently expressed while the LMP1 protein appears to be restricted to only a proportion of tumours. In an attempt to understand the contribution of LMP2A to the pathogenesis of NPC, we have developed a model system in which carcinoma cell lines are stably infected in vitro with either a wild type recombinant EBV (rEBV) or a mutant rEBV in which LMP2A is deleted (rEBV-2A). An NPC like pattern of EBV gene expression including LMP2A but not LMP1 was consistently observed in carcinoma cells infected with rEBV. However, carcinoma cells infected with rEBV-2A expressed high levels of LMP1. The expression of LMP1 in the absence of LMP2A, precludes an assessment of the function of LMP2A on epithelial cell growth. To this end we have generated stable clones of rEBV-2A-infected cells in which LMP1 expression is eliminated through the use of small interfering RNAs (RNAi). In this way, the contribution of LMP2A to EBV-induced epithelial cell growth transformation can be assessed without the complication of LMP1 expression. Gene expression profiling, cytokine profiling and phenotypic studies are currently under way (and will be presented) in order to further understand the contributions made by LMP1 and LMP2 in NPC.

The Human Epigenome Project

Beck S

Contact:
Prof. Dr. Stephan Beck
Wellcome Trust Sanger Institute
Hinxton (Cambridge, UK)
Email: beck@sanger.ac.uk

Abstract:
The Human Epigenome Project (HEP) is a public/private collaboration that aims to identify, catalogue and interpret genome-wide DNA methylation phenomena. Occurring naturally on cytosine bases at CpG sequences, DNAmethylation is intimately involved in diverse biological processes and the aetiology of many diseases, particularly cancer. Differentially methylated cytosines give rise to distinct patterns and profiles thought to be specific for gene activity, tissue type and disease state. Such methylation variable positions (MVPs) are useful epigenetic markers that promise to significantly advance our ability to understand and diagnose human disease.
As a prelude to the HEP, the Human Epigenome Consortium has recently completed a pilot study of the methylation patterns within the human Major Histocompatibility Complex (MHC), a region on chromosome 6 that is associated with more diseases than any other region in the human genome. This involved the development of an integrated pipeline for high-throughput methylation analysis using bisulphite DNA sequencing, MVP discovery, epigenotyping by MALDI mass spectrometry and an integrated public database. The pilot study entailed the analysis of over 100,000 CpG sites at over 250 MHC loci, including regulatory, exonic and intronic regions in multiple (healthy) tissues and individuals. The generated data have been integrated with the human genome annotation using the ENSEMBL interface and are publicly available at http://www.epigenome.org.

TSLC1 involvement in nasopharyngeal carcinoma
Lung M, Lung HL, Xie D, Guan XY,Murakami Y

Contact:
Prof. Maria Lung
Hong Kong University of Science & Technology
Biology (Kowloon, Hong Kong (SAR), China)
Email: bomaria@ust.hk

Abstract:
Objective: The aim of these studies is to verify the importance of the TSLC1 gene in NPC and to evaluate its usefulness as a diagnostic and/or prognostic marker for this tumor.

Methods: The status of TSLC1 gene expression in four NPC cell lines was determined by using RT-PCR. Its inactivation by hypermethylation was studied by methylation-specific PCR (MSP) and the effect of a demethylating agent on its subsequent expression. The cloned TSLC1 cDNA was transfected into the HONE1 cell line to determine its effect on cell growth. Stable transfectants were injected into nude mice to evaluate their ability to suppress tumor formation. Immunohistochemical staining techniques were used on NPC tissue microarrays to determine the protein expression levels of this gene.

Results: The candidate tumor suppressor gene, TSLC1, mapping to 11q22-23, a critical region corresponding to tumor suppression in microcell hybrid analysis, is down-regulated in NPC cell lines. MSP analysis shows that the methylated allele was present for the four NPC cell lines tested. Treatment of the HONE1 NPC cell line with 5-aza-2'deoxycytidine reversed the down-regulation of this gene. TSLC1 transfected cells in culture exhibit decreased colony formation ability. In nude mice the tumorigenic potential of stably transfected cells was decreased as compared to the HONE1 NPC cell line. Using a TSLC1 antibody for immunohistochemical staining in tissue microarrays, we found in 67 informative NPC cases, 43% (29/67) cases showed TSLC1 down-regulation and 21% (14/67) showed loss of expression of this protein. The frequency of loss of expression of TSLC1 in lymph node metastatic NPC was 35%, which was significantly higher than the 12% observed in primary NPC ($P < 0.01$).

Conclusions: Results of these studies are consistent with the TSLC1 gene behaving as a tumor suppressor gene in NPC. Its loss of expression is significantly associated with metastatic NPC and a poor prognosis. TSLC1 is involved in cell adhesion and its loss is associated with tumor invasiveness. Thus, it may be a good prognostic biomarker for NPC.

Genome-wide screening for candidate tumor suppressor genes in nasopharyngeal carcinoma (NPC) using epigenetic/genetic approaches

Tao Q, Ying J, Qiu GH, Tan J, Liu D

Contact:
Prof. Qian Tao
Division of Johns Hopkins in Singapore (DJHS)
Cancer Epigenetics/Tumor Virology Laboratory (Singapore, Singapore)
and Department of Clinical Oncology, Chinese University of Hong Kong
Email: taoqia@hopkins.edu.sg

Abstract:
NPC is a strongly Epstein-Barr virus (EBV)-associated tumor, prevalent in Southern China and Southeast Asia. Its pathogenesis is a multi-step process involving a variety of genetic and epigenetic alterations including the disruption of certain tumor suppressor genes (TSGs) through promoter hypermethylation, in addition to EBV infection. Using DNA methyltransferase inhibitors as demethylation agents, epigenetic inactivation of TSGs can be reversed and exploited as a cancer therapeutic strategy. We tried to identify novel candidate TSGs, epigenetically or/and genetically inactivated in NPC, in a genome-wide way by employing various cancer epigenetic/genetic approaches such as subtraction, expression profiling, array-CGH, mutation mapping and functional analyses. More than 10 candidates, located at different chromosome regions, either ubiquitously expressed or tissue-specific, have been identified. All of these genes are hypermethylated and downregulated in NPC and in most of the common epithelial tumors. Functional analyses showed that some of these genes could induce apoptosis of tumor cells and suppress tumor cell growth. The silencing of these TSGs was mainly mediated through promoter hypermethylation which could be reversed by demethylation agents, indicating that these genes can be further explored as epigenetic therapeutic targets. Our studies demonstrated a promising, epigenetic way to identify candidate TSGs in tumors.

Hyperacetylation of histones H3 and H4 at Zp and Rp does not result in activation of the EBV lytic cycle

Countryman J, Gradoville L, Heston L, Miller G

Contact:
Dr. Jill Countryman
Yale University
Department of Molecular Biophysics and Biochemistry (New Haven, USA)
Email: jill.countryman@yale.edu

Abstract:
Activation of the Epstein-Barr virus lytic cycle is accomplished through the combined action of two virally encoded proteins, ZEBRA and Rta, the products of the BZLF1 and BRLF1 open-reading frames. Maintenance of the latent state, therefore, requires that these two genes be tightly repressed. A widely held hypothesis is that Zp and Rp, the promoters for BZLF1 and BRLF1, are repressed by chromatin. To investigate the mechanism by which these two genes are regulated, we used Chromatin Immunoprecipitation (ChIP) to examine the acetylation and phosphorylation state of histones H3 and H4 at Zp and Rp following treatment with sodium butyrate (NaB), Trichostatin A (TSA), tetradecanoyl phorbol acetate (TPA) or Azacytidine (AZC). In Raji cells, TPA and NaB act synergistically to activate the lytic cycle. Using antibodies directed against acetylated and phosphorylated histone H3, both markers for actively transcribed chromatin, we found an increase in histone modification at Zp, Rp, and EAp, the promoter for EA-D, an early lytic cycle protein. Surprisingly, however, when cells were treated with NaB or TSA alone, both of which fail to activate the lytic cycle in Raji cells, an increase of comparable magnitude of hyperacetylated and phosphorylated histone H3 at Zp, Rp and EAp was still observed. This finding indicates that following NaB and TSA treatment, the chromatin at these promoters was open, but open chromatin did not lead to the disruption of latency. We also tested the effect of AZC on activation of the lytic cycle and histone modification in HH514-16 cells. In these cells, AZC is a potent activator of immediate early mRNAs and lytic cycle proteins. At times when mRNA was expressed from Rp and Zp, we failed to observe any increase in hyperacetylation of histone H3, suggesting that the mechanism of lytic cycle activation by AZC does not require modification of histone tails at Rp or Zp. Taken together, our data suggests that open chromatin at EBV immediate early and early promoters is not sufficient to activate lytic cycle gene expression and is not an obligate step leading to activation of the lytic cycle.

The EBV lytic switch protein, Z, activates the methylated viral genome through a novel mechanism

Bhende PM, Seaman WT, Delecluse HJ, Kenney SC

Contact:
Dr. med. Shannon Kenney
University of North Carolina at Chapel Hill
Departments of Medicine and Microbiology, and Lineberger Comprehensive Cancer Center (Chapel Hill, USA)
Email: shann@med.unc.edu

Abstract:
Objective: Methylation promotes gene silencing. Nevertheless, the EBV IE protein, BZLF1 (Z), efficiently activates lytic viral genes even from a methylated viral genome. Here we have investigated the effect of viral genome methylation on the ability of Z versus BRLF1 (R) to disrupt viral latency.

Methods: The methylation status of the BRLF1 (Rp) promoter was determined in a variety of different latently infected lines. Z binding to the methylated versus unmethylated Rp was examined by EMSA. Z and R activation of the methylated versus unmethylated wild-type Rp-CAT construct, as well as Rp-CAT vectors missing specific ZRE sites, was examined in reporter gene assays. Z and R activation of methylated versus unmethylated (5-aza-2-deoxycytidine treated) genomes of the BZLF1-deleted and BRLF1-deleted viruses in 293 cells was examined.

Results: A Z-binding site (ZRE-2) in the BRLF1 IE promoter (TGAGCGA) that contains a CpG motif was methylated in all cell lines examined. Surprisingly, methylation of the CpG motif in this ZRE significantly enhanced Z binding. Another ZRE site, TTCGCGA (ZRE-3), was discovered in Rp and this site only bound Z in the methylated form. Z preferentially activated the methylated form of Rp-CAT, while R preferentially activated the unmethylated form. Z activation of the methylated Rp-CAT vector required the ZRE-2 and ZRE-3 sites, but not the ZRE-1 site (which cannot be methylated). Wild-type Z induced lytic genes more efficiently from the methylated genome in 293 cells latently infected with the BZLF1-deleted virus. In contrast, demethylation of the genome enhanced R-induced lytic gene expression in cells containing the BRLF1-deleted virus. A Z mutant containing a threonine (instead of serine) at residue 186 in the DNA binding domain bound only to the methylated form of the ZRE-2 site, and only activated lytic EBV gene expression from the methylated (but not unmethylated) viral genome.

Conclusions: Z is uniquely adapted for inducing lytic gene expression from a methylated viral genome. EBV has developed an intriguing and unexpected mechanism for circumventing the inhibitory effects of viral genome methylation.

Dynamic Chromatin Boundaries Delineate a Latency Control Region of Epstein-Barr Virus

Chau CM, Lieberman PM

Contact:
Dr. Charles Chau
Wistar Institute
Gene Expression and Regulation (Philadelphia, USA)
Email: chau@wistar.upenn.edu

Abstract:
The oncogenic potential of latent Epstein-Barr virus (EBV) can be regulated by epigenetic factors controlling LMP1 and EBNA2 gene transcription. The EBV latency control region (LCR) constitutes ~12 kb of viral sequence spanning the divergent promoters of LMP1 and EBNA2, and encompasses the EBV latent replication origin OriP and RNA polymerase III transcribed EBER genes. We have used the chromatin immunoprecipitation (ChIP) assay to examine the chromatin architecture of the LCR in different types of EBV latency programs. We have found that histone H3 K4 methylation (H3mK4) was enriched throughout a large domain that extended from the internal repeats 1 (IR1) to the terminal repeats (TR) in type III latency where EBNA2 and LMP1 genes are expressed. In type I latency where EBNA2 and LMP1 genes are transcriptionally silent, the H3mK4 domain contracts and does not enter the EBNA2 or LMP1 promoters. In contrast, histone H3 K9 methylation was enriched in the EBNA2 and LMP1 upstream control regions in type I, but not type III cells. MTA, a pharmacological inhibitor of protein methylation, globally reduced histone H3 K4 methylation and inhibited EBNA2 transcription in type III cells. 5'-azacytidine (AzaC), an inhibitor of DNA methylation that derepresses EBNA2 transcription in type I latency, caused H3mK4 expansion and a corresponding loss of H3mK9 at IR1. Chromatin boundary protein and transcription repressor CTCF was enriched at the EBNA2 transcription control region in type I, but not type III cells. We also present evidence that OriP binding factors EBNA1 and ORC2 can interact with sequences outside of OriP including a region within IR1 that may influence EBNA2 transcription status. These results indicate that type I and III latency programs have distinct histone methylation patterns in the LCR and suggest that chromatin organization mediated interactions between OriP and promoter elements of LMP1 and EBNA2.

High resolution methylation analysis and in vivo protein-DNA interactions at latent Epstein-Barr virus promoters Cp and Qp in the nasopharyngeal carcinoma cell line C666

Minarovits J, Bakos A, Banati F, Takacs M, Salamon D, Schwarzmann F, Wolf H, Niller HH

Contact:
Dr. Janos Minarovits
National Center of Epidemiology
Microbiological Research Group (Budapest, Hungary)
Email: minimicrobi@hotmail.com

Abstract:
C666 is a unique nasopharyngeal carcinoma cell line which maintains latent Epstein-Barr virus genomes during serial passages in vitro. We wished to characterize the epigenetic mechanisms silencing Cp and activating Qp in C666 cells.
We established, therefore, high resolution methylation maps of Cp and Qp regulatory regions using the method of automated fluorescent genomic sequencing of bisulfite modified DNA samples. We found a high level of CpG methylation 5 from Cp which is switched off in C666 cells. In contrast, Qp where EBNA 1 transcripts are initiated was completely unmethylated. We analysed protein-DNA interactions in the regulatoty region of Cp and Qp by genomic ('in vivo') footprinting. In control lymphoblastoid cell lines which actively use Cp we detected a typical footprint for the cellular regulatory protein CBF1. In contrast, in C666 cells the silent C promoter was not marked with a CBF1 footprint.
We established earlier that the activity of Qp, a promoter which is invariably unmethylated in lymphoid cells, is regulated by binding of cellular proteins. We detected strong footprints comparable to those of lymphoid cells in the regulatory region of Qp in the nasopharyngeal carcinoma cell line.
We concluded that DNA methylation contributes to silencing of Cp in C666 cells. The absence of a typical CBF1 footprint suggests that CBF1 is not bound to its recognition sequence 5' from Cp in C666 cells. Analysis of protein-DNA interactions at Qp showed that its regulation is similar in lymphoid and epithelial cells.

Genomic DNA methylation-subtraction identified functional tumor suppressor genes epigenetically silenced in nasopharyngeal carcinoma (NPC)

Ying J, Srivastava G, Hsieh WS, Gao Z, Murray PG, Tao Q

Contact:
Prof. Qian Tao
Division of Johns Hopkins in Singapore (DJHS)
Cancer Epigenetics/Tumor Virology Laboratory (Singapore, Singapore)
and Department of Clinical Oncology, Chinese University of Hong Kong
Email: taoqia@hopkins.edu.sg

Abstract:
Aberrant promoter hypermethylation inactivates tumor suppressor genes (TSGs) in cancers, including Epstein-Barr virus-associated tumors. We tried to identify hypermethylated TSGs in NPC, using a subtractive methylation-sensitive representational difference analysis. 29 DNA fragments with typical CpG islands were identified. Among them is a member of the DNA damage-inducible gene family (GADD45). Medium to high level expression of GADD45 was found in all normal fetal and adult tissues. However, its transcription silencing and promoter methylation was frequently detected in lymphoma and carcinoma cell lines including NPC, but not in normal epithelial cell lines, normal tissues and peripheral blood mononuclear cells. Treatment with DNA methyltransferase inhibitor led to GADD45 demethylation and re-expression. In primary tumors, aberrant methylation was frequently detected in lymphomas, and less frequently in carcinomas. Only one somatic mutation was detected in 1 of 25 cell lines, indicating that its genetic inactivation is very rare in tumors. Ectopic expression of GADD45 strongly suppressed tumor cell growth and colony formation in methylated cell lines. These results demonstrate that GADD45 is bona-fide new-age tumor suppressor gene, being frequently inactivated epigenetically in various tumors including NPC.

High-throughput genomic/epigenomic screening for silenced tumor suppressor genes on 16q in nasopharyngeal carcinoma

Tan J, Seng TJ, Ying J, Tao Q

Contact:
Prof. Qian Tao
Division of Johns Hopkins in Singapore (DJHS)
Cancer Epigenetics/Tumor Virology Laboratory (Singapore, Singapore)
and Department of Clinical Oncology, Chinese University of Hong Kong
Email: taoqia@hopkins.edu.sg

Abstract:
Loss of heterozygosity on the long arm of chromosome 16 is common in many tumors, including nasopharyngeal carcinoma (NPC). To identify candidate tumor suppressor genes (TSGs) located at the affected region, we have finely mapped the deleted regions in NPC by 1-Mb array-CGH. Furthermore, we used high-throughput array expression profiling to examine the differential expression of all the EST/genes located in the deleted regions, in a number of cell lines with or without demethylation treatment. Four genes and 9 ESTs were found to be downregulated in NPC, compared with normal controls. The 4 genes, designated as G2, G8, G39 and G54, were downregulated in NPC cell lines and the majority of a panel of other tumor cell lines, and were inducible with demethylation agents. Genes G8, G39, G54 were expressed in all normal tissues, while G2 was more tissue-specific. Further functional and regulatory studies showed that G8 and G54 were functional TSGs, but frequently inactivated epigenetically in NPC. Our findings suggest that there are likely a few candidate TSGs residing on the frequently deleted 16q22.3-23.1 region in NPC.

Epigenetic profiles of Epstein-Barr virus promoters and the therapeutic application to EBV-associated tumors

Tao Q, Goh HK, Huang H, Lim CY, Gong B, Chan ATC, Ambinder R

Contact:
Prof. Qian Tao
Division of Johns Hopkins in Singapore (DJHS)
Cancer Epigenetics/Tumor Virology Laboratory (Singapore, Singapore)
and Department of Clinical Oncology, Chinese University of Hong Kong
Email: taoqia@hopkins.edu.sg

Abstract:
Epigenetic regulation through CpG methylation is a fundamental transcriptional regulatory process, which is involved in embryonic development, tissue-specific gene expression, genetic imprinting and carcinogenesis. CpG methylation is also involved in the regulation of EBV promoters and viral latency. Hypermethylation silences the transcription of EBV promoters for immunodominant viral proteins, thus EBV lives as latency I or II and escapes from host immune surveillance in tumors. Using DNA methyltransferase inhibitors, epigenetic silencing of EBV promoters can be reversed and exploited as a therapeutic strategy towards EBV-associated tumors. Previously, we have shown the differential methylation profiles of Cp, Qp and Wp in tumors, and the in vivo demethylation of these promoters in patients with EBV+ tumors. We have furthered our study by systematically profiling the methylation status of other EBV latent (LMP1p) or lytic (Zp, Rp, Hp) promoters in various tumors, and searching for more efficient demethylating agents for EBV promoters. We developed both sensitive methylation-specific PCR (MSP) and high-resolution bisulfite genomic sequencing systems for this purpose, which can even be used for tiny paraffin tissue sections. It was found that all the EBV promoters studied could be demethylated by demethylating agents, although with different efficiencies, therefore can be used as therapeutic targets for EBV+ tumors.

Frequent epigenetic inactivation of the tumour suppressor lung cancer gene-1 (TSLC1) in EBV-positive and -negative Hodgkin's lymphoma

Murray PG, Waites ER, Flavell JR, Reynolds GM, Wei W, Young LS, Tao Q

Contact:
Dr. Paul Murray
University of Birmingham
CRUK Institute for Cancer Studies (Birmingham, UK)
Email: p.g.murray@bham.ac.uk

Abstract:
Objectives: We recently identified frequent hypermethylation of the RASSF1A tumour suppressor gene in the tumor cells of Hodgkin's lymphoma (HL) pointing to a potential role for this anti-apoptotic gene in the survival of HL progenitor cells. In the present study we have investigated the nature and extent of tumour suppressor gene hypermethylation in HL cells and also whether this is influenced by EBV status.

Methods: Hypermethylated tumour suppressor genes were initially identified by comparison of gene expression profiles obtained from KM-H2 HL cells in the presence or absence of the demethylating agent, 5-azacytidine. Methylation-specific PCR and bisulfite genomic sequencing (BGS) were used to confirm the methylation status of tumour suppressor genes in cell lines and also in primary HL tissues.

Results: Methylation of a number of tumour suppressor genes was identified in HL cells, none of which were previously shown to be methylated in HL. Of these TSLC1 emerged as a candidate for further study. TSLC1 (IGSF4) has already been reported as a tumour suppressor gene in several human cancers including non-small cell lung cancer and nasopharyngeal carcinoma and encodes a member of the immunoglobulin superfamily of proteins. TSLC1 is an adhesion molecule involved in cell-cell interactions. Methylation of the TSLC1 promoter and loss or reduced expression of TSLC1 mRNA was demonstrated in the majority of HL cell lines and also in non-Hodgkin lymphoma-derived cell lines. This methylation was partially reversible in cell lines by demethylating treatments. Furthermore, TSLC1 methylation was observed in 69% of primary HL tissues. Importantly, aberrant methylation was localised to the malignant HRS cells of HL tumors by analysis of microdissected cell populations. No association between TSLC1 methylation and EBV status was found.

Conclusions: TSLC1 expression is frequently lost or reduced in Hodgkin's lymphoma cells as a result of aberrant promoter methylation. We are currently determining the functional consequences of this loss and its role in HL pathogenesis

Multiple EBNA1-binding sites within oriPI are required for EBNA1-dependent transactivation of the Epstein-Barr virus C promoter

Zetterberg H, Borestrom C, Nilsson T, Rymo L

Contact:
Cecilia Boreström
Institute of Laboratory Medicine, Göteborg University
Department of Clinical Chemistry and Transfusion Medicine (Göteborg, Sweden)
Email: cecilia.borestrom@clinchem.gu.se

Abstract:
Objective: The transactivating function of the oriPI-EBNA1 complex is essential for activation of the Epstein-Barr virus (EBV) C promoter (Cp) in lymphoblastoid cell lines expressing the viral growth programme. Furthermore, the oriPI-EBNA1 complex is believed to play an important role during promoter switching upon primary infection of B-lymphocytes and establishment of latent infection in vivo. Previously, it was shown that at least six EBNA1-binding sites within oriPI are required for transactivation of the heterologous thymidine kinase promoter. This study was conducted to reveal the biological importance of the previous results using the EBV C promoter.

Methods: Deletion fragments of oriPI were made as restriction fragments containing 0, 1 and 4-20 EBNA1-binding sites by partial digestion of an oriPI-containing plasmid with NdeI that cuts at multiple sites within oriPI. Fragments containing 2-3 EBNA1-binding sites were made by synthesis of overlapping doublestranded oligonucleotides. The oriPI-fragments were cloned into the previously described pgCp(-248)CAT plasmid (Nilsson et al., J. Vir. 2001). Co-transfections were performed in the EBV-negative BL cell line DG75 with 10 µg reporter plasmid and 10 µg expression plasmid for EBNA1 (pCI-EBNA1) or an equal molar amount of the corresponding empty vector.

Results: We show that 20 EBNA1-binding sites within oriPI, i.e. the full length oriPI, result in a 280-fold activation of Cp by EBNA1. This activation decreased to below 100-fold, when the number of EBNA1-binding sites was reduced to eight. Four to six EBNA1-binding sites within oriPI could still respond to EBNA1 with an approximately 10-20-fold up-regulation of promoter activity. Constructs containing three or fewer EBNA1-binding sites were not significantly activated by EBNA1.

Conclusions: Here, we define the number of EBNA1-binding sites within oriPI necessary for its biological function as EBNA1-dependent Cp enhancer. We show that four EBNA1-binding sites within oriPI lead to significant up-regulation of Cp in response to EBNA1 and eight or more to full activation. Thus, multiple EBNA1 homodimers at oriPI are required for formation of a transcriptionally active Cp structure, a process that involves EBNA1-induced changes in chromatin structure including DNA looping and nucleosome destabilization.

The Epstein-Barr virus oncogene product, latent membrane protein 1, activates DNA methyltransferase 1 via JNK signaling

Tsai CL, Tsai CN, Tse KP, Yu JS, Chang YS

Contact:
Dr. Chi-Neu Tsai
Graduate Institute of Basic Sciences
Chang-Gung University (Taoyuan, Taiwan)
Email: pink7@mail.cgu.edu.tw

Abstract:
Epstein-Barr virus latent membrane protein 1 (LMP1) induces cell migration via a mechanism that involves activation of DNA methyltransferases (DNMT) 1, 3a and 3b, resulting in hypermethylation of a cellular E-cadherin gene. Although it is established that LMP1 activates the expression of dnmt genes, the underlying mechanism remains to be elucidated. In this study, we demonstrate that LMP1 directly activates both the major promoter (P1) and the minor promoter (P2-4) of the dnmt1 gene. Experiments with LMP1 mutants lacking the YYD domain, dominant-negative JNK1, and the JNK-specific inhibitor, SP600125, reveal that LMP1-mediated P1 activation involves JNK signaling. Data from the ChIP assay disclose that LMP1 induces a transcriptional repression complex comprising DNMT1, DNMT3a, DNMT3b, MeCP2, and HDAC1, associated with the hypermethylated E-cadherin gene promoter. This interaction is inhibited by SP600125 in LMP1-inducible cells. The results imply a mechanistic link between JNK signaling and DNA methylation induced by the EBV oncogene product, LMP1.

Improving survival, expansion and persistence of adoptively-transferred tumor-specific CTLS

Rooney CM, Huls MH, Bollard C, Straathof KC, Okamura T, Gottschalk S, Brenner MK, Heslop HE

Contact:
Prof. Dr. Cliona Rooney
Center for Cell and Gene Therapy, Baylor College of Medicine
and Texas Children's Hospital and The Methodist Hospital (Houston, USA)
Email: CMRooney@txccc.org

Abstract:
After infusion into recipients of T cell-depleted stem cell transplants (SCT), EBV-specific CTL expand by up to four logs, reconstitute immunity to EBV, reduce high virus load, home to tumor sites and produce complete remission of EBV-associated lymphomas. Gene-marking studies further demonstrated the persistence of the infused cells for up to seven years. By contrast tumor-specific T cell lines or clones infused into immunocompetent tumor bearing individuals have been disappointing, rarely producing anti-tumor responses and persisting only days or weeks. The factors influencing CTL survival and efficacy include the composition of the CTLs, with polyclonal T cell lines recognizing multiple tumor epitopes and containing both CD4+ T helper cells and CD8+ CTL being ideal. Both patient and tumor environment also influence the fate of the infused CTL. In a lymphopenic environment, mature T cells should proliferate by homeostatic lymphoproiliferation, until they fill the T cell compartment, but if the T cell compartment is full, little expansion or even contraction may result. After SCT, the presence of both an antigenic tumor and the presence of normal EBV-infected B cells likely also provide a proliferative boost. Immunogenic tumors that arise in immunocompetent hosts express a range of T cell inhibitory molecules and recruit and induce T regulatory cells. Therefore, for optimal CTL function, either the host or tumor environment must be altered, or the CTL must be made resistant to tumor-mediated inhibition.We will discuss the results of using EBV-specific CTL lines in patients with NPC and Hodgkin, as well as new protocols that use specific antibodies to deplete the T cell compartment before infusion and planned projects for genetic enhancement of CTLs.

Non-invasive diagnosis of nasopharyngeal carcinoma by detection of EBV DNA load plus BARF1 and EBNA1 mRNA in Nasopharyngeal brushings

Stevens SJC, Verkuijlen SAWM, Hariwiyanto B, Harijadi H, Fachiroh J, Paramita DK, Tan IB, Haryana SM, Middeldorp JM

Contact:
Dr. Servi Stevens
Vrije Universiteit Medical Center
Department of Pathology (Amsterdam, The Netherlands)
Email: s.stevens@vumc.nl

Abstract:
Nasopharyngeal carcinoma (NPC) is a prevalent malignancy in South-East Asia, virtually 100% associated with EBV. Currently, an invasive nasopharyngeal biopsy is required for diagnosis of the tumour and demonstration of EBV involvement. We investigated the diagnostic value of EBV DNA load quantification and EBV-encoded BARF1 and EBNA1 mRNA detection and tumour cell detection in cytological smears using nasopharyngeal (NP) brushings in a cohort of Indonesian NPC patients (N=87) and controls (N=14). Patients originated from the Yogyakarta region, a geographical NPC hotspot. EBV DNA loads in NP brushings of NPC patients were extremely elevated (up to 4*108 EBV DNA copies/swab) compared to non-NPC controls (<104 copies/swab, P<0.001). High EBV DNA copy numbers per NPC cell, as determined by combining EBV load and cytological data indicated possible lytic replication in approx. 20% of patients (>50 viral DNA copies/NPC tumour cell in the NP swab). To confirm the presence of tumour cells, EBV RNA was determined as well. BARF1 RNA was assessed because it represents a viral oncogene exclusively expressed in EBV-carrying carcinomas, whereas EBNA1 is universally expressed in all EBV-linked malignancies. EBNA1 and BARF1 transcription was detected in respectively 83.8% and 72.5% of all NP swab samples and was even higher in fresh samples (84.8% for both mRNAs). EBV RNA-negative NP swabs had the lowest EBV DNA loads but normal cellular DNA levels indicating inaccurate sampling. No EBV RNA was detected in control NP swabs from healthy donors and non-NPC cancer patients. In conclusion, EBNA1 and BARF1 mRNA detection, combined with EBV DNA load quantification in NP swabs are non-invasive tools for NPC diagnosis, directly showing carcinoma-specific EBV activity at the anatomical site of tumour development. This approach reduces the number of invasive nasopharyngeal biopsies, can be more easily repeated and may thus be suited for prognostic monitoring during and after therapy.

The role of Epstein-Barr virus DNA measurement in plasma in the clinical management of Dutch nasopharyngeal carcinoma (NPC) patients

Kroes ACM, Kalpoe JS, Douwes Dekker PB, Claas ECJ, van Krieken JHJM, Baatenburg de Jong RJ

Contact:
Prof. Dr. Aloys C.M. Kroes
Leiden University Medical Center
Department of Medical Microbiology (Leiden, The Netherlands)
Email: kroes@lumc.nl

Abstract:
Objective: To evaluate the role of quantitative measurement of Epstein-Barr virus (EBV) DNA in the clinical management of NPC patients, in an area where this tumor is non-endemic, with regard to the establishment of EBV-association and the correlation with tumor progression and response to treatment.

Methods: EBV DNA loads in plasma samples from 14 consecutive caucasian Dutch NPC patients were determined by an internally controlled real-time quantitative PCR based on the non-glycosylated membrane protein BNRF1. An EM counted standard, containing 6.68×10^9 EBV particles/ml was used for quantification. Efficiency of DNA extraction and PCR inhibition were monitored by spiking all clinical samples with a fixed amount of phocine herpes virus (PhHV) prior to DNA extraction. For each patient pre-treatment as well as post-treatment samples were included, in 2 cases patients were also regularly sampled during treatment. RNA in situ hybridization for the detection of EBV-encoded RNAs (EBERs) was performed on nasopharyngeal tumor biopsies. Treatment consisted of radiotherapy or combined chemotherapy and radiotherapy.

Results: Twelve out of fourteen NPCs were EBER positive as determined by in situ hybridisation. All patients with EBER positive NPCs (12/14) showed a positive EBV DNA load in plasma at time of diagnosis with a median EBV DNA level of 16,000 (or 4.2 log) copies/ml (range: 2.6 log - 5.1 log), whereas the patients with EBER negative NPCs (2/14) had no detectable EBV DNA load in plasma. After treatment, none of the 14 NPC patients had detectable EBV DNA load in plasma and all patients showed sustained complete remission of the tumor as confirmed by clinical and fiber-optic nasopharyngoscopic examinations. During treatment, in two cases, a gradual decline in the EBV DNA levels was observed towards the limit of detection within four weeks after start of therapy.

Conclusions: The presence of EBV DNA in plasma correlated exactly with the EBER-based diagnosis of EBV-associated NPC. The disappearance of EBV-DNA after treatment confirmed the complete regression of the tumors on treatment. Plasma EBV DNA load measurement appears to have broad clinical utility in the management of NPC patients.

EBV serology: stochastics and basic patterns
Bauer G

Contact:
Prof. Dr. rer. nat. Georg Bauer
Universität Freiburg
Abteilung Virologie (Freiburg, Germany)
Email: georg.bauer@uniklinik-freiburg.de

Abstract:
EBV serology has a great impact for differential diagnosis, as the symptoms of clinically overt EBV infections may be overlapping with the symptoms of many other diseases. Classical EBV serology, based on the determination of VCA-IgG, VCA-IgM and Anti-EBNA-1 has allowed to establish a basic scheme for the interpretation of EBV serology, but may be misleading in a substantial number of cases. For example, VCA-IgM may be missing in acute infections or the VCA-IgM response may persist after acute infection. Positive Anti-EBNA-1 excludes acute infection, but negative Anti-EBNA-1 may be found in acute EBV infections and in 6 % of healthy persons with past infections. In addition, immunosuppression may lead to secondary loss of Anti-EBNA-1. The use of immunoblots or lineassays with purified recombinant antigens allows to resolve the problems inherent to classical EBV serology. As immunoblots and lineassay represent strictly quantitative systems, they allow precise measurements of antibody concentrations and thus allow avidity determination as additional way for a significant serological diagnosis. The use of the antigens p23, p54, p138, p72 (EBNA-1) and a specific form of p18 allows the precise determination of the serostatus in each individual case. Modified p18 is recognized late in infection, similar to p72. However, there is no secondary loss of p18-IgG during immunosuppression and persons with missing p72-IgG can usually be recognized as past infection by their positive p18-IgG. The study of 500 defined cases of past EBV infections and 1500 nonselected cases demonstrated that the general pattern of the serological response in past infections is modified by stochastics, leading to the appearance of numerous, mathematically predictable aberrant serological constellations. The application of avidity determination allows a secure serological determination also in these puzzling cases.

Induction of MHC class-I and -II restricted epitope presentation by urea-adjuvated soluble proteins: A novel technology for the simultaneous restimulation of various populations of antigen-specific T-cells

Barabas S, Bauer T, Weinberger B, Pullmann K, Lindner J, Wagner R, Jilg W, Wolf H, Deml L

Contact:
Dr. Ludwig Deml
University of Regensburg
Institute for Medical Microbiology and Hygiene (Regensburg, Germany)
Email: Ludwig.Deml@klinik.uni-regensburg.de

Abstract:
Rationale: The present study was designed to develop a novel and attractive technology for the simultaneous activation of various populations of protein-specific T-cells for diagnostic and therapeutic applications based on the urea-treatment of soluble proteins.

Methods: Peripheral blood lymphocytes (PBL) as well as pure populations of immature and mature dendritic cells (DC) have been stimulated with either (i) synthetic peptides, soluble Epstein-Barr virus (EBV) BZLF1 or urea-treated BZLF1 (uBZLF1) and analysed for the capacity to specifically restimulate various T cell populations. The number and identity of antigen-specific T cells was determined by measuring IFN-γ production by the ELISPOT and FACS technology. Detailed evaluation of the molecular mechanisms underlying uBZLF1 uptake and processing for MHC-I and -II mediated epitope presentation was performed by determining protein aggregation (chemical cross-linking), cell-surface adherence (immunostaining), as well as antigen processing and presentation using specific inhibitors of the MHC-I and II pathway.

Results: Stimulation of PBL from EBV-positive individuals with synthetic CTL peptides resulted in an exclusive stimulation of CD8+ CTL. In contrast, an activation of various populations of antigen-specific T-cells including CD8+ CTL, CD4+CD8 + T cells, CD4+ T cells was observed upon incubation of PBL with uBZLF1, indicating that uBZLF1 was efficiently introduced into the MHC-I and II presentation pathway. Herein, both immature and mature DC were able to incorporate und process uBZLF1 for MHC-I restricted epitope presentation. Removal of urea from the stimulator antigen resulted in an almost complete loss in the activation of CD8 + CTL, strongly supporting the importance of urea for the antigen delivery to MHC-I molecules. Our cell-biological analysis revealed a strong surface adherence of uBZLF1 resulting in endocytosis and epitope presentation on MHC-I by a cross-presentation pathway.

Conclusions: These results strongly suggest urea-treatment of proteins to be a suitable technology to deliver polypetides towards the MHC-I and MHC-II pathway, thus providing a useful tool for activating various populations of protein-specific T-cells for diagnostic and therapeutic purposes.

Cancer Cell's Secreted Proteome as a Basis for Searching Potential Tumor Markers - Nasopharyngeal Carcinoma as a Model

Wu CC, Chien KY, Tsang NM, Chang KP, Hao SP, Tsao CH, Huang WC, Chang YS, Yu JS

Contact:
Chih-Ching Wu
College of Medicine, Chang-Gung University
Department of Cell and Molecular Biology (Tao-Yuan, Taiwan)
Email: chihchingwu@yahoo.com.tw

Abstract:
Objective: Nasopharyngeal carcinoma (NPC) is endemic in Southern China and Taiwan. NPC is commonly diagnosed late because of its deep location and vague symptoms. Therefore, it is important to identify biomarkers for early diagnosis. For this purpose, we analyzed the secreted proteomes of two NPC cell lines (NPC-TW02 and 04), and three of those proteins identified in both cell lines (fibronectin, Mac-2 BP, and PAI-1) were selected for further characterization for their potentials as NPC biomarkers.

Methods: Secreted proteins in the cultured medium of NPC-TW02 and 04 cells were identified by SDS-PAGE combined with MALDI-TOF mass spectrometry, followed by three Western blot analysis. Immunohistochemistry and fluorimetric sandwich ELISA were established to measure the levels of fibronectin, Mac-2 BP, and PAI-1 in nasopharyngeal tissue and sera from NPC patients and normal individuals, as well as from nude mice tumor model.

Results: Twenty three secreted proteins were identified in cultured medium of both NPC-TW02 and 04 cells. Among them, fibronectin, Mac-2 BP, and PAI-1 were further confirmed by Western blot analysis. The three proteins were highly expressed in NPC biopsies, but weakly or not expressed in normal nasopharyngeal tissues. The serum level of all the three proteins were significantly higher in NPC patients (n=46) than in normal controls (n=47)($p<0.01$). Nude mice bearing NPC tumors (derived from NPC-TW02 cells)(n=9) also show an elevated serum level of Mac-2 BP and PAI-1 when compared with tumor-free mice (n=9)($p<0.01$).

Conclusions: Systematic analysis of cancer cell's secreted proteome in conjunction with animal tumor model can be a feasible and convenient strategy for searching multiple potential tumor markers. Fibronectin, Mac-2 BP, and PAI-1 may be potential markers for diagnosis of NPC.

Benefits of ELISA and Western-blot for EBV serology in diagnosis and monitoring of nasopharyngeal carcinoma

Karray H, Ayadi W, Fki L, Drira M, Daoued J, Frikha M, Hammami A, Middeldorp JM

Contact:
Prof. Dr. Jaap M. Middeldorp
VU Medical Center
Pathology (Amsterdam, The Netherlands)
Email: j.middeldorp@vumc.nl

Abstract:
Nasopharyngeal carcinoma (NPC) in Tunisia shows a bimodal age distribution with adult patients of 40-60 years and a second peak in young people of 10-24 years. The usefulness of EBV serology for clinical management of NPC is well established. Our study aims: (1) to determine the EBV serological profile at primary diagnosis of 117 NPC patients in two age groups, using immunofluorescence assay (IFA) and ELISA, and (2) to evaluate the diagnostic and prognostic value of three EBV serological methods in 21 NPC patients followed for a mean of 4.2 years after treatment, for which we used Immunoblot next to IFA and ELISA.

Methods: Standard IFA serology testing was used to titrate IgG/VCA, IgG/EA, IgA/VCA and IgA/EA. ELISA was used for measuring IgG and IgA against EA-specific native protein extracts prepared from EA-induced HH514. c16 cells and for detecting IgA against VCA-p18 and EBNA1 peptides, respectively. Immunoblot analysis was used to study the diversity of IgG and IgA responses in NPC patients against EBV antigens from VCA-induced HH514. c16 cells expressing EBNA, EA, VCA and MA.

Results: Comparing the 2 age groups of the 117 NPC patients tested before treatment, a statistically significant difference was found between them: 78% of young people had elevated titers of IgG VCA/EA versus 91.7% for the group of elder patients ($p=0.08$) and IgA VCA/EA were detected in only 50% of young versus 89.4% for elder patients ($p<0.0001$). A good concordance was found between reactivities in IFA and ELISA. Concerning the evolution of antibody responses during treatment of 21 patients, a good concordance was seen between the three serological methods except in one patient who showed an increase of bands in immunoblot corresponding to IgG VCA-p18, VCA-p40 and EA-D-p47, while EBV antibody levels detected by IFA and ELISA decreased and remained stable. This patient was free of disease at this moment but 16 months later, he developed a recurrent NPC in the same site. For the remaining patients, declining IgG and IgA responses correlated with good treatment result, whereas patients with bad outcome showed increases in serological responses by all methods.

Conclusion: Overall, ELISA for IgA anti-VCA-p18 and immunoblot proved most sensitive for predicting tumour relapse. VCA-p18 IgA ELISA seems suitable for routine diagnosis and early detection of NPC complication.

Epstein-Barr virus-associated infectious mononucleosis in Chinese children
Chiang AKS, Chan CW, Lee PPW, Chan KH

Contact:
Dr. Alan Kwok Shing Chiang
The University of Hong Kong
Paediatrics and Adolescent Medicine (Hong Kong, HK)
Email: chiangak@hkucc.hku.hk

Abstract:
Objective: Seroprevalence studies showed that the majority of children in Hong Kong are infected by Epstein-Barr virus (EBV) before 10 years of age. This study aims at defining the clinical presentation and complications of infectious mononucleosis (IM) in Chinese children.

Methods: A retrospective study was performed on >100 consecutive Chinese childhood IM patients who fulfilled the serologic criteria for the diagnosis of primary EBV infection (VCAIgM+/-VCAIgG+EBNA-) over a 6-year period. The clinical, hematologic and biochemical findings were evaluated among four age groups of less than 2 years, 2-4 years, 5-9 years and 10-15 years.

Results: EBV-associated IM occurred at all age groups with a peak incidence at 2-4 years, corresponding to the rapid rise in the seroprevalence of EBV in early childhood in the Hong Kong Chinese. Although a significant number of children presented with classical features of fever, tonsillopharyngitis, lymphadenopathy and hepatosplenomegaly, a spectrum of milder presentation that could not be easily discerned from other common viral infections was also found. The majority recovered without major complications. Marked lymphocytosis with the presence of atypical lymphocytes was a consistent haematologic finding in all age groups and the occurrence of hepatitis showed an association with advancing age (p=0.003).

Conclusions: EBV-associated infectious mononucleosis presents frequently in Hong Kong Chinese children with a spectrum of clinical features ranging from mild to classical IM and corresponds to seroepidemiology of EBV infection in our population.

Epstein-Barr Virus-associated diseases: study of antibody patterns and circulating DNA

Banha L, Martins L, Cunha M, Ornelas C

Contact:
Luis Banha
Instituto Português Oncologia Francisco Gentil, Centro Regional de Oncologia de Lisboa, SA
Laboratório Virologia (Lisbon, Portugal)
Email: lbanha@ipolisboa.min-saude.pt

Abstract:
Objective: Study of serological markers detected by ELISA and circulating DNA in serum of patients with EBV associated diseases.

Methods: Ninety one patients, 46 male and 45 females (mean age of 43 years old, range from 3 months to 81 years old), with EBV associated diseases (Nasopharyngeal Carcinoma (NPC), Non-Hodgkin Lymphoma (NHL), Hodgkin Lymphoma (HL) and other diseases that could be associated with EBV), and fiftyfive healthy donors (included as control and reference group for EBV PCR and serology). Serum of all patients and controls were analyzed by Nested PCR (amplification of 54 bp from the DNA polimerase gene, with β-globin as internal control, and detection in agarose gel, 4%) and serological markers, by indirect ELISA: anti-EBV IgM (EA+VCA+EBNA), anti-EBV IgG (EA+VCA+EBNA), anti-EBV IgA (EA+VCA+EBNA), anti-EBV EA-D IgG and anti-EBV EBNA-1 IgG. Statistical analysis of data was performed using t-student.

Results: All patients were EBV IgG positive. In NPC, all patients (14 cases) were EBV IgA positive, 71.4% were positive for EBV EA-D IgG, 92.9%were positive for EBV EBNA-1 IgG and 71.4% had DNA detected. In NHL (21 cases), 90% of the patients were positive for EBV IgA, 80% for EBV EBNA-1 IgG and 25% had DNA detected. For HL (7 cases), 57.1% were positive for EBV IgA, 71.4% for EBV EBNA-1 and 28,6% had DNA detected. In the remaining EBV associated tumors (49 cases), 68% were positive for EBV IgA, 80% for EBV EBNA-1 and 4% had DNA detected. In NHL, HL and other EBV associated tumors, 45% of the patients were positive for EBV EA-D IgG. In the control group, 87.3% were positive for EBV IgG, 41.8% for EBV IgA and 88.2% for EBV EBNA-1 IgG. All the donors were negative for EBV EA-D IgG and circulating DNA.

Conclusions: Traditionally, the best way for evaluate recent versus past infection in healthy individuals are the serological markers. Comparing the control group and the EBV associated tumors, the serological markers are not enough to define disease, but when we associate this with DNA detection they seem to have prognostic and diagnostic value. In EBV associated tumors, the activity of antibody for EBV IgG is 8.4 times higher than in control group (1249.5 UI/ml versus 148 UI/ml). The EBV IgA is marker that has high specificity for NPC (100%) and NHL (90%). For HL and others EBV associated tumors, there aren't significative differences.

Nasopharyngeal Carcinoma: the use of synthetic peptides and native Epstein Barr-Virus (EBV) proteins for diagnostic screening

Fachiroh J, Paramita DK, Harijadi A, Hariwiyanto B, Haryana SM, Middeldorp JM

Contact:
Prof. Dr. Jaap M Middeldorp
VU Medical Center
Pathology (Amsterdam, The Netherlands)
Email: j.middeldorp@vumc.nl

Abstract:
Nasopharyngeal carcinoma (NPC) is one of the most prevalent tumours in SE-Asia and Northern Africa. NPC is 100% linked to EBV infection, with active viral gene-expression in all tumour cells. EBV-IgG and -IgA serology using cell-based immunofluorescence assays is still widely used for diagnosis and monitoring of NPC, and progress to more simple testing is required.
Our extensive immunoblot studies revealed that IgG and IgA antibodies in NPC patients recognize a variety of EBV proteins, including previously defined EBNA1, Major Early Antigen (EA-Dp47/p138), ZEBRA, DNAse, TK and VCA-p18. However antigen recognition patterns may significantly differ between individuals. Furthermore, analysis of the antigen fine-specificity revealed that IgG and IgA in the same individual frequently recognize different sets of EBV proteins and epitopes, suggesting independent B-cell triggering (J. Fachiroh et al.,JID 189 (2004) in press).
For some EBV proteins the IgG and IgA reactive epitopes were further defined by PEPSCAN analysis and the reactivity of individual epitope domains was analysed in ELISA by using single or combined peptide coatings. Based on this information, a synthetic peptide-based EBV-IgA ELISA was developed for primary NPC screening using a defined mixture of EBNA1 and VCA-p18 epitopes. This assay showed a sensitivity and specificity of 85.3% and 90.1% for diagnosing NPC in a panel of 154 NPC sera and 254 healthy regional controls. Initially EBV-IgA ELISA positive sera were confirmed by immunoblot IgG diversity analysis, which gives a distinct reactive pattern with NPC sera compared to controls. This increased sens./spec. values to >95%, but remains a laborious method. To replace immunoblot, a defined set of non-VCA, non-EBNA1 antigen(s) consisting of native EA proteins was used to design a confirmation ELISA (see Paramita et al., abstract 06.35). EA-D-specific peptide epitopes gave inferior results when compared to native EA-D proteins, indicating the importance of conformational epitopes for EBV-IgA interaction. ELISA using purified native EA-D proteins showed sensitivity and specificity of 92.86% and 97.61% for IgG and 86.26% and 97.62% for IgA detection, respectively. This combination of IgA-ELISAs may be suited for field-screening, (early) diagnosis and monitoring of NPC in high risk populations.

Immune Responses of Patients with Nasopharyngeal Carcinoma to Epstein-Barr Virus Lytic Proteins

Liu MY, Huang YT, Sheen TS, Chen JY, Tsai CH

Contact:
Dr. Mei-Ying Liu
National Health Research Institutes (Taipei, Taiwan)
Email: myl@nhri.org.tw

Abstract:
Objective: To elucidate immune responses of patients with NPC to three EBV lytic proteins, i. e. DNase, thymidine kinase (TK) and BMRF-1 gene products (50/52 kD diffused early antigen, EA-D complex).

Methods: To avoid differences among various assays, immunofluorescence assay (IFA) was used to determine humoral immune response of NPC patients by using recombinant EBV proteins as the antigens produced from the same baculovirus-expression system. Cell proliferation assay was performed to evaluate cellular immune response by monitoring incorporation of 3H-thymidine into the antigen-treated peripheral blood mononuclear cells. In total, 70 NPC patients and 32 non-cancer controls were analyzed.

Results: 1. IgG antibodies against EA-D complex, DNase and TK were detected in 94%, 90% and 80% of the 70 NPC patients, respectively, but only 25%, 19% and 9% of the controls were positive for the antibodies at low titers.
2. IgA to EA-D complex, DNase and TK (69%, 49% and 39%) were lower than those of IgG. However, none of the 32 control subjects was positive for the IgA antibodies. Thus, the IgA class appeared to be more specific for the detection of NPC ($p<0.05$).
3. Metastases of cervical lymph nodes were strongly associated with elevated antibody titers to VCA, EA, EA-D, DNase and TK, particularly the isotype IgG antibody (all of $p< 0.01$).
4. Peripheral blood mononuclear cells from 54 NPC patients were not noticeably induced to divide when treated by EA-D complex or DNase, except few individuals. No good correlation was observed between the humoral immunity and cellular immunity to the EBV lytic proteins.

Conclusions: 1. Both positivity rates and antibody titers to DNase, TK and EA-D complex were higher for NPC patients, especially for the IgA class.
2. The antibody titers to the three EBV lytic proteins were significantly associated with metastases of cervical lymph nodes.
3. In general, except few cases, NPC patients showed weak cellular immune responses to the EA-D complex and DNase.

Primary diagnostic value of circulating EBV DNA and carcinoma-specific viral RNA in Indonesian nasopharyngeal carcinoma patients

Stevens SJC, Verkuijlen SAWM, Hariwiyanto B, Harijadi H, Fachiroh J, Paramita DK, Tan IB, Haryana SM, Middeldorp JM

Contact:
Dr. Servi Stevens
Vrije Universiteit Medical Center
Department of Pathology (Amsterdam, The Netherlands)
Email: s.stevens@vumc.nl

Abstract:
Nasopharyngeal carcinoma (NPC) is a prevalent malignancy in South East Asia, strongly associated with EBV. We investigated the primary diagnostic value of circulating EBV DNA load in a large cohort of Indonesian NPC patients (N=149), from a high incidence region. In a 213 bp LightCycler-based real-time PCR, 72.5% of patients was positive for EBV DNA in whole blood, with only 29.5% above a previously determined clinical cut-off value (COV) of 2,000 EBV DNA copies/ml blood, the upper level in healthy carriers. In a novel 99 bp LightCycler PCR, 85.9% of patients was positive and 60.4% was above the COV. The 99 bp assay gave a significant higher EBV DNA load than the 213 bp assay ($P<0.0001$), indicating the EBV DNA in the circulation to be highly fragmented. Using data from 11 different previous studies, this finding was further substantiated by showing a significant inverse correlation between PCR amplicon size and percentage of primary diagnosed NPC patients positive for circulating EBV DNA (Spearman's rho = -0.91, $p<0.0001$). Presence of BARF1 RNA, a viral oncogene exclusively expressed in EBV-carrying carcinomas, was assessed in 19 whole blood samples using NASBA. Despite high EBV DNA loads (range 2,600-75,200 copies/ml in 213 bp PCR) and positivity for EBV-encoded EBNA1 (in 36.8%) and human U1A snRNP mRNA (in 100%) of the same samples, BARF1 mRNA was never detected in whole blood of NPC patients, indicating absence of circulating tumour cells or transcriptional silencing of this gene. We conclude that the primary diagnostic value of EBV DNA load determination is limited, due to low or negative EBV DNA loads in a considerable number of patients, but may be increased by using small sized amplicon in real-time PCR. It may however be useful for prognostic monitoring in NPC patients with initial elevated loads. BARF1 RNA detection in blood has no diagnostic value for primary NPC.

Quality control and standardization of EBV DNA

Niesters HGM, Fries E, van Loon AM, Forde C, Wallace P, Puchhammer-Stockl E

Contact:
Dr. Hubert Niesters
Erasmus University Hospital Rotterdam
Virology (Rotterdam, The Netherlands)
Email: h.g.m.niesters@erasmusmc.nl

Abstract:
Objective: An international quality control program for the detection and quantification of EBV DNA was organized to support efforts for standardization.

Methods: Serial dilutions of EM quantified EBV particles were made in citrate plasma in the range between 100 and 10.000 copies per ml. This range was chosen based on relative EBV DNA ranges of clinical significance for pre-emptive treatment of bone-marrow transplant patients at risk for the development of PTLD. A total of 8 samples were distributed both in 2002 and 2003 among respectively 75 and 84 participants in 19 and 21 countries respectively. Both commercially available as well as home-brew assays were used for the qualitative or quantitative detection of EBV DNA.

Results: The majority of laboratories (60.2%) detected EBV DNA qualitative, using mostly home-brew assays (73.6%). In both years, over 41% of the assays were based on real time detection. False positivity was a limited problem (2.3%). Samples containing more than 1000 copies per ml were detected in 93%; samples below 1000 copies were detected infrequently likely due to the small sample input. Five hundred copies was detected in 80%, while 250 and 100 copies per ml was detected in respectively 65% and 33%. No significant difference was seen between commercial and home-brew assays, or between real time and conventional amplification systems. The principal discrepancies observed appear to be due to the lack of method standardization, and the lack of suitable EBV DNA QC material for assay quantification. This presentation reports the first results on the implementation of a better characterized standard method.

Conclusions: The first two international quality control programs on the detection of EBV DNA showed that the majority of laboratories are able to detect clinically relevant levels of EBV DNA in plasma. However, efforts on the standardization of quantification need to be made. The multi-centre studies co-ordinated through QCMD help support the standardisation process through the continued appraisal of current and developing molecular technologies. Furthermore, as with most current QC programs on the detection of herpes viruses, real-time technologies are now becoming the most abundant technology used.

Performance and technical validation of the first commercially available and CE marked Real-Time PCR assay for the detection and quantitation of Epstein-Barr virus

Laue T, Raith S, Hess M

Contact:
Thomas Laue
artus
R&D (Hamburg, Germany)
Email: laue@artus-biotech.de

Abstract:
Objective: For lack of a standardized, commercially available assay, determination of Epstein-Barr virus (EBV) viral loads in cell free (e.g. plasma) or cell associated (e.g. blood) specimen is still based on home brew methods, set up and performed by a limited number of highly specialised laboratories or hospitals. Therefore, artus GmbH developed a real-time PCR based assay for the detection and quantification of EBV.

Methods: Different variants of the assay were developed and optimised so that they can be used on different real-time PCR platforms: LightCycler (ROCHE), TaqMan Series (Applied Biosystems), Rotorgene (Corbett Research). A heterologous amplification and detection system (Internal Control) was incorporated in order to be able to monitor the sample preparation procedure (DNA extraction) and the amplification efficiency of the system. The specificity of the assay and the ability to detect all relevant EBV genotypes was ensured by sequence comparison analysis.

Results: Sensitivity: The detection limit of the assay, determined by Probit analysis, is 5.78 copies/µl (p_ 0.05), 5.2 copies/µl (p_ 0.05), and 3.8 copies/µl (p_ 0.05) on the LightCycler, TaqMan 7000, and Rotorgene, respectively. Specificity: No false positive results were detected testing EBV negative blood, serum or plasma samples nor testing closely related viruses. Precision: The total variance of the specific detection system was determined to be 14.54%, 21.2% and 16.9% on the LightCycler, TaqMan 7000, Rotorgene, respectively.

Conclusion: As shown, artus GmbH was able to develop a very sensitive and specific real-time PCR assay for the detection and quantification of EBV in plasma and serum.As this assay was developed and validated in accordance with the requirements of the ISO 9001, EU In vitro Diagnostic Device Directive (98/79/EG), and FDA (21 CFR) and received its CE certification in 2004, it can easily be used by in vitro diagnostic laboratories on a routine base. But there is still one problem remaining with the quantification of EBV. Because most laboratories worldwide are using their own quantification standard material there is no way to compare quantification results between different laboratories. Therefore, there is an urgent need for a standardized quantification material preparation, comparable to the international standards supplied by the WHO.

The Novel In Vitro Diagnostic REALQUANT EBV TEST is a Robust, Specific and Highly Sensitive Assay for the EBV Viral Load Determination

Galdenzi G, Louis Y, Menghi M

Contact:
Dr. Gabriella Galdenzi
DiaTech Italy
Research and Development (Jesi, Italy)
Email: diatech@diatech-meet.it

Abstract:
Objective: REALQUANT EBV (DiaTech Italy) is a technology that offers a real standardization for the EBV quantitation. The aim of our study was to examine the performances of REALQUANT. The assay is based on the real-time amplification of the EBV Nuclear Antigen 1 gene (EBNA-1) and the human ß-actin gene (Internal Control, IC) on the Rotor-Gene system (Corbett Research). The amplicons produced are detected by two specific dual labelled probes in FAM (EBV) and in JOE (ß-actin) channels. The kit contains all the necessary reagents for 96 amplifications. The 8 standard curve (EBV 95-8) is pre-dispensed and lyophilized in tubes with concentrations ranging from 10 to 10^{-6} copies/reaction.

Methods: The analytical sensitivity was determined testing 8 times, in triplicate, a set of 8 EBV DNA dilutions from 100 to 0.0316 copies/µl. The specificity was examined testing 18 DNA samples: 12 from certified negative plasma and 6 from whole blood of EBV negative subjects. The cross-reactivity was evaluated by testing twice a list of pathogens: Chlamydia t., HBV, HCMV, HPV(18,44), HSV1/2 and Parvo B19. The whole system failure rate was measured on 3 experiments including 39 EBV DNA samples with 3.3 copies/µl (three folds LOD). The cross-contamination was evaluated by performing 2 independent experiments, each run with alternating 10 high-positive and 10 negative samples.

Results: The 95% limit of analytical sensitivity (LOD), calculated by a probit analysis, is 1.15 copies/µl. EBV DNA wasn't detected in any negative sample (18/18) as well as in the cross-reactivity panel. The whole system failure rate is 0% (39/39 samples detected and correctly quantified). The amplification of the IC was monitored in all experiments. The IC is robust and effective for detecting the presence of inhibitors: it was amplified in all samples except for few extracts, containing PCR inhibitors. No negative sample, in any experiment, was EBV false-positive because of cross-contamination.

Conclusions: The results suggest that REALQUANT EBV kit is highly sensitive, specific and robust. The use of the IC appears essential to prevent false negative results due to PCR inhibitors and/or extraction issues.

Quantification of lytic mRNAs for monitoring of Epstein-Barr virus (EBV) reactivation in patients after stem cell or renal transplantation

Weinberger B, Plentz A, Jilg W

Contact:
Birgit Weinberger
University of Regensburg
Institute for Medical Microbiology and Hygiene (Regensburg, Germany)
Email: birgit.weinberger@klinik.uni-regensburg.de

Abstract:
Objective: Despite being inapparent in healthy carriers reactivation of Epstein-Barr virus in latently infected individuals during immunosuppression may be associated with severe complications. We established real-time RT-PCR to sensitively quantitate viral transcripts expressed at different times of the lytic cycle. Currently we are prospectively monitoring patients after stem cell or renal transplantation.

Methods: RNA-isolation was optimized to eliminate contaminating DNA and our RT-PCR systems have been shown to detect 100 copies of RNA per well by using serial dilutions of in vitro transcribed RNA which corresponds to a detection limit of 800 copies/10^6 PBMC.

Results: First results show detectable levels of lytic mRNAs in patients with acute Infectious Mononucleosis as well as in some patients under immunosuppression. Within our prospective study 115 blood samples of 19 patients after stem cell transplantation and 83 blood samples of 18 patients after renal transplantion were analyzed. Viral DNA was detectable in isolated PBMC in 10,1% of the samples (100-1000 copies / 10^6 PBMC) but not in serum. Borderline levels of viral mRNA of the late transcript BLLF1 (gp220/350) could be detected in 8,6 % of the samples. This result is comparable to the situation in healthy carriers.

Conclusions: Preliminary results show that despite viral DNA in isolated PBMC of patients under immunosuppression is detected more often than in healthy carriers, expression of lytic viral genes is comparable in both groups. The slight increase of viral load may be due to proliferation of latently infected B-cells. Quantification of latent viral transcripts will be carried out to adress this question.

Quantification of gp350/220 Epstein-Barr Virus (EBV) mRNAs by Real-Time RT-PCR in EBV-associated diseases

Germi R, Morand P, Brengel-Pesce K, Fafi-Kremer S, Genoulaz O, Ginvera C, Ballout M, Bargues G, Seigneurin JM

Contact:
Dr. Raphaele Germi
Université Joseph Fourier - Faculté de Medecine
Laboratoire de Virologie Moléculaire et Structurale (Grenoble, France)
Email: raphaele.germi@ujf-grenoble.fr

Abstract:
Objective: Quantification of Epstein-Barr Virus (EBV) DNA is useful to monitor EBV-linked diseases but gives no information on EBV late gene expression which could reflect a productive infection in these diseases. This study presents the quantification by real-time RT-PCR of the EBV envelope glycoprotein (gp350/220) late mRNAs, in cell cultures and clinical samples.

Methods: Taqman-technology and serial dilutions of in vitro transcripts were used to quantify gp350/220 mRNAs in B95-8, P3HR1 and Akata EBV-positive cell lines. EBV mRNA and DNA loads were then assessed in peripheral blood mononuclear cells (PBMCs) and biopsies from clinical samples.

Results: The real time RT-PCR sensitivity was 50 copies and coefficients of variation were inferior to 5% attesting a good reproducibility. This method allowed the monitoring of gp350/220 mRNAs in EBV-infected cell lines after induction or inhibition of mRNA production. gp350/220 mRNAs were not detectable in PBMCs from healthy individuals whereas they were expressed in 7/8 PBMCs from transplant recipients. mRNA load was higher in PBMCs from transplanted patients with asymptomatic primary infection (median=4.38 log copies/µg) than in PBMCs from patients with post-transplantation lymphoproliferative disorders (PTLD) (median=2.32 log copies/µg), even though these samples presented similar DNA load. gp350/220 mRNAs were detected in 4/4 of PTLD, 3/5 of nasopharyngeal carcinoma and 0/8 of Hodgkin's disease biopsies.

Conclusions: Real-time RT-PCR is a simple and accurate tool to assess late stage of EBV productive infection in vitro or ex vivo. This method was able to demonstrate various patterns of gp350/220 mRNA expression in EBV-associated diseases.

Dynamics of Epstein-Barr virus (EBV)-specific T cell responses in immunocompromized patients after renal transplantation

Barabas S, Jilg W, Wolf H, Deml L

Contact:
Dr. Ludwig Deml
University of Regensburg
Institute for Medical Microbiology and Hygiene (Regensburg, Germany)
Email: Ludwig.Deml@klinik.uni-regensburg.de

Abstract:
Introduction: In immunocompetent individuals, Epstein-Barr virus (EBV) causes mild, self-limiting illness followed by a live-long period of latency in which virus-replication is efficiently controlled by EBV-specific cytotoxic T cells. By contrast, in hosts with impaired T-cell activity e. g. because of pharmacologic immunosuppression after renal transplantation (RT) EBV has been clearly associated with the occurrence of post-transplant lymphoproliferative disorder (PTLD). Thus information on the number and functionality of EBV-specific T-cells after RT might be helpful to assess risk for EBV reactivation and EBV associated disorders.

Method: We initiated a three year prospective study to examine peripheral blood lymphocytes (PBL) of presently 35 patients kidney recipients for the presence of functional EBV-specific T-lymphocytes. Therefore, blood samples were taken one day before and at weeks 1, 2, 4, 12, 24, 36, 48 after transplantation and analysed for the presence of specific T-cell responses by determining the number of IFN-γ secreting T-cells upon stimulation with selected synthetic peptides representing CTL epitopes with well-known HLA-restriction and/or urea-adjuvated BZLF1 (uBZLF1) by ELISPOT. Monitoring of EBV reactivation was performed weekly by serological assays and detection of viral DNA.

Results: The preliminary report of this study after one year includes results of 35 RT patients: all of them with positive EBV serology. All patients showed a strong decline of EBV-specific T-cells within the first week after the onset of immunosuppressive therapy. In the majority of patients numbers of EBV-specific T-cells only slowly arose over time concurrent with the reduction of the doses of immunosuppressive drugs. However, despite the substantially reduced numbers of EBV-specific T cells, only 2 of 35 analysed patients showed slight reactivation of EBV, indicated by an slightly increased numbers of circulating EBV (102 -103 viruses/ml serum) and the occurrence of anti-EBV IgM isotypes. Both patients were characterized by notably low numbers of EBV-specific T cells before the onset of immunosuppressive therapy.

Conclusion: The preliminary results of this ongoing study indicate, that even low numbers of functional EBV-specific T cells may confer protection against uncontrolled EBV reactivation and the development of EBV-associated disorders. The frequency and functionality of EBV-specific T cells prior to immunosuppressive therapy may be a prognostic marker for an increased probability of EBV reactivation.

Relationship between the expression of (active) caspase 3 and clinical outcome in patients with nasopharyngeal carcinoma (NPC)

Harijadi A, Hariwiyanto B, Astuti I, Haryana SM, Oudejans JJ, Middeldorp JM

Contact:
Dr. Ahmad Harijadi
Faculty of Medicine Gadjah Mada University
Pathology (Yogyakarta, Indonesia)
Email: hariyadi_a@hotmail.com

Abstract:
Objective: To investigate the relationship between the expression of (active) caspase 3 and clinical outcome of NPC patients. To clarify effects of radiotherapy on caspase 9 pathway, we also examined the expression of p53, p21, bcl-2, pro-caspase 3 and X-linked inhibitory apoptosis protein (XIAP)

Methods: 41 Indonesian NPC biopsies from patients without evidence of distant metastasis who were treated with radiotherapy only. Standard immunohistochemistry staining with monoclonal anti bodies against pro-caspase 3, active caspase 3, XIAP, p53, p21 and bcl-2. Levels of caspase 3 activation were determined by quantifying positive ly staining tumor cells. Expression of pro-caspase 3 wasscored relative to expression levels in surrounding lymphocytes. Tumour-infiltrating activated cytotoxic T-lymphocytes were detected via Granzyme-B staining as described before (Oudejans et al., J. Pathol. (2002) 98; 468-475).

Results: Low levels of caspase 3 activation were associated with high levels of granzyme-B expressing tumour-infiltrating cytotoxic lymphocytes ($p=0.001$). High levels of active caspase 3 showed a strong association with good clinical outcome (CR; $P<0.0001$). Positive active caspase-3 was associated with younger age ($p=0.04$), but not with gender. A positive correlation was found between detectable caspase 3 activation and expression levels of pro-caspase 3 ($p=0.02$). High levels of active caspase 3 were associated with absence of XIAP expression ($p=0.05$). Bcl-2 was detected more frequently in cases with active caspase-3 ($p=0.07$), but P53 and p21 were not significantly different among cases positive or negative for active caspase 3.

Conclusions: The expression of active caspase 3 in NPC tumor cells predicts clinical outcome in NPC patients. Presence of abundant activated T-cell infiltrate correlates with absent caspase-3 and supports the hypothesis on their role in selecting apoptosis resistant tumour cells. Our data support the notion that an intact apoptosis cascade in tumor cells is essential for effective tumour cell killing by radiotherapy.

Detection of HHV-8 specific humoral and cellular immune response as a more accurate measure to assign the individual HHV-8 status.

Gartner BC, Lonard B, Sester U, Pees H, Kohler H, Muller-Lantzsch N, Sester M

Contact:
Dr. Barbara C. Gärtner
University of the Saarland
Institute of Virology (Homburg/Saar, Germany)
Email: vibgae@uniklinik-saarland.de

Abstract:
Objective: Prevalence of human herpes virus (HHV)-8-IgG is higher among transplant recipients (15-30%) than in healthy controls (<5%). These figures could not exclusively be explained by an increased rate of primary infection by graft or blood transfusion, indicating that the humoral immune response is underestimating the prevalence of HHV-8. Thus, we investigated if the analysis of HHV-8 specific T-cells may represent a more accurate tool to assign HHV-8 status.

Methods: Specific cellular and humoral immune response was analysed in 129 controls, 70 hemodialysis patients, 68 transplant recipients and 72 HIV-infected patients by the flow cytometric analysis of HHV-8 specific T-cells and immunofluorescence assay (against latent and lytic antigens), respectively.

Results: A higher seroprevalence was confirmed in the immunocompromised individuals (13-43%) than in controls (4%). HHV-8 reactive cells were CD8 T-cells producing interferon-γ and TNFα. Among controls, 12% had HHV-8 reactive T-cells. However, T-cell reactivity increased to 36% in hemodialysis patients, and decrease when analysing the immunocompromised patients (transplant recipients 18%, HIV-infected patients 20%). Interestingly, there was no strict correlation between cellular and humoral immunity within the study groups.

Conclusions: Data on the immune response against HHV-8 are still puzzling. Serological assays alone may underestimate HHV-8 prevalence. Thus, apart from primary infections, the high prevalence of HHV-8 reactivity in immunocompromised individuals may be explained by an immunosuppression-related decrease in T-cells that may be followed by increasing HHV-8 replication and concomitant booster effect on humoral immunity. Thus, the combined analysis of specific humoral and cellular immunity may represent a more accurate measure to assign the individual HHV-8 status.

Vaccination against Epstein-Barr virus (EBV): report of phase ii studies using recombinant viral glycoprotein gp350 in healthy adults

Denis M, Haumont H, Bollen A

Contact:
Dr. Martine Denis
GlaxoSmithKlineBiologicals
R&D (Rixensart, Belgium)
Email: martine.denis@gskbio.com

Abstract:
The development of an EBV vaccine was based on the rationale that pre-existing antibodies against EBV could reduce the viral load upon infection and thereby prevent occurrence of infectious mononucleosis (IM). The vaccine candidate includes recombinant gp350, a viral envelope antigen produced in mammalian cells.
A first study (80 subjects) evaluated three different vaccine formulations: antigen alone, antigen formulated with Alum, or antigen in combination with the AS04 adjuvant (Alum/MPL based, GlaxoSmithKline Biologicals). All three formulations proved generally safe and were well tolerated in the population of initially EBV seronegative healthy adults. While the non-adjuvanted vaccine appeared significantly less immunogenic than the adjuvanted preparations, the AS04-based formulation showed a trend towards increased immunogenicity compared with the Alum-based vaccine. The second study, a phase II randomised, double-blind and placebo-controlled clinical trial started in October 2001 and enrolled 180 subjects. It was designed to evaluate the efficacy of a vaccine formulation containing 50 µg of gp350 combined with AS04. Two groups of 90 healthy adolescents and young adults received the vaccine or placebo according to a 0-1-5-month schedule.
The primary and secondary efficacy analyses will respectively determine the attack rate of IM and compare the incidence of EBV infection in vaccine and control groups over 18 months after the second injection.

Pre-clinical studies to assess the efficacy of a polyepitope vaccine for nasopharyngeal carcinoma

Duraiswamy J, Tellam J, Connolly G, Cooper L, Davis J, Tscharke D, Corban M, Smith M, Busson P, Khanna R, Moss DM

Contact:
Dr. Jai Duraiswamy
Queensland Institute of Medical Research
EBV Biology (Brisbane, Australia)
Email: jaiD@qimr.edu.au

Abstract:
Background: Nasopharyngeal carcinoma (NPC) cells are characterised by the expression of weakly immunogenic Epstein-Barr virus (EBV) proteins, latent membrane protein (LMP)1 and LMP2 which are considered to be the suitable targets for T-cell based therapy.

Objective: To determine whether adoptive transfer of LMP-specific T cells from HLA-A2/Kb mice can protect or regress LMP-expressing tumours in vivo.

Methods: A polyepitope vaccine encoding multiple epitopes from LMP1 and LMP2 was developed using replication-incompetant adenovirus vector. HLA A2/Kb mice were immunised with this vaccine and T-cell response was tested in splenocytes using ELISPOT and in vitro cytotoxicity assays. These LMP-specific T cells were expanded in vitro, and the efficacy of these expanded T cells was tested in vivo in both prophylactic and therapeutic models.

Results: Immunisation of HLA-A2/Kb mice with this polyepitope vaccine and subsequent ex vivo stimulation of splenocytes resulted in activation of a strong LMP-specific T cell response, which was readily detected by ex vivo and in vitro functional assays. Preliminary studies suggest that adoptive transfer of these expanded T cells resulted in killing LMP-expressing tumour in vivo in both prophylactic and therapeutic models.

Conclusions: These studies suggest that a T-cell based therapy using adenoviral polyepitope vaccine may be efficient in treating NPC in mice.

An Epstein-Barr virus (EBV) BDLF2 knock-out mutant lacking expression of LMP1, EBNA1, and EBNA2 and failing in immortalization of B-Lymphocytes as a potential EBV life vaccine aiming at sterile immunity

Kleines M, Schellenberg K, Ritter K, Scheithauer S

Contact:
Dr. Michael Kleines
UK Aachen
Division of Virology, Department of Medical Microbiology (Aachen, Germany)
Email: mkleines@ukaachen.de

Abstract:
Objective: The molecular details of EBV infection and immortalization of B-lymphocytes are not understood completely, so far. Detailed investigations on poorly characterized EBV-encoded genes are necessary to elucidate their function in EBV biology.

Methods: We constructed a genomic EBV-library and a transposon-based mutagenesis vector allowing in vitro generation of random mutant clones covering the entire EBV genome. For generation of mutant-EBV from the mutant clones, B95-8 cells were transfected with the mutated EBV-clones allowing homologous recombination with wildtype EBV. Raji cells infected with mutant virions from the supernatant of these cultures were selected using the transposon-encoded resistance genes. To characterize EBV-mutants, B-lymphocytes were infected with the supernatant of these Raji cell cultures.

Results: A mutant containing a single insertion of the transposable element in the BDLF2 gene was shown to be able to infect B-lymphocytes of EBV-negative donors. Compared to wildtype EBV the mutant lacked the capacity of triggering aggregation of infected B-lymphocytes. Several cell surface antigens induced by wildtype EBV remain unaffected by the BDLF2 mutant including the adhesion molecules CD11a, CD54, and CD58. Expression of genes indicative of the viral lytic cycle as BZLF1, BMRF1, BHRF1, BRLF1 is unchanged in mutant-infected B-lymphocytes compared to wildtype infections. The latent genes LMP2, EBNA3A, EBNA3B, EBNA3C, and EBER1 are expressed, while expression of LMP1, EBNA1, and EBNA2 is not detectable. The BDLF2-negative mutant lacks the capacity to immortalize B-lymphocytes. Infected cells show a self-limiting viral activity and die within 2 month post infection.

Conclusions: Thus, the EBV-BDLF2-mutant which combines self-limiting replicative activity, lack of expression of important latency genes and failure in immortalization of B-lymphocytes is a potential EBV life vaccine aiming at sterile immunity.

Emerging Therapeutic Strategies for Nasopharyngeal Carcinoma and Hodgkin's Lymhoma

Khanna R, Cooper L, Connolly G, Duraiswamy J, Tellam J

Contact:
Dr. Rajiv Khanna
Queensland Institute of Medical Research
Division of Infectious Diseases and Immunology (Herston, Australia)
Email: rajivK@qimr.edu.au

Abstract:
Advances in our understanding of the role of cytotoxic T lymphocytes (CTLs) in the control of Epstein-Barr virus (EBV)-associated malignancies and the overall biology of these diseases have led to the development of novel therapeutic strategies designed to specifically target viral antigens expressed in these malignancies. Long-term success of many of these strategies are constrained by the latency phenotypes adopted by different diseases. Adoptive transfer of polyclonal virus-specific CTLs has been successfully used to reverse the outgrowth of malignancies such as post-transplant lymphoproliferative disease (PTLD). On the other hand, limited viral gene expression in other EBV-associated malignancies such as Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma limits the efficacy of immunotherapeutic strategies used for PTLD. Preclinical studies based on specific targeting of viral antigens expressed in these malignancies have provided very encouraging results and thus are likely to serve as an important platform for the treatment of human patients.

Retargeting EBV-specific CTL to ErbB2 positive tumors for the adoptive immunotherapy of breast cancer

Gottschalk S, Ratnayake M, Brenner MK, Rooney CM

Contact:
Dr. Stephen Gottschalk
Baylor College of Medicine
Pediatrics (Houston, USA)
Email: smg@bcm.tmc.edu

Abstract:
The long-term objective of this project is to develop an innovative, immunotherapeutic approach for ErbB2 positive breast cancer. One approach to generate antigen-specific T cells for adoptive immunotherapies is to genetically modify T cells with retroviral vectors expressing a tumor antigen-specific chimeric T-cell receptor, which is compromised of the antigen-binding domain of a monoclonal antibody, linked to the transmembrane and cytoplasmic signaling domain of the T-cell receptor. T-cell receptor expressing T cells (chimeric T cells) lyse and secrete cytokines upon exposure to tumor cells expressing the target antigen. However, translating these preclinical findings into clinical trials revealed a disappointing lack of correlation between ex vivo cytotoxicity and therapeutic efficacy. The most pertinent issue being that chimeric T cells fail to expand and rapidly lose their function in vivo. We hypothesize that the current functional limitation of chimeric T cells can be overcome by expressing chimeric T-cell receptors on EBV-specific CTL, which 1) can be readily prepared from EBV-positive individuals and 2) persist long-term in vivo after infusion. Our group has recently documented the feasibility of this approach using a chimeric T-cell receptor recognizing the GD2 antigen commonly expressed by malignant neuroblastoma cells. Here we demonstrate that EBV-specific CTL can be successfully retargeted to ErbB2, a tumor antigen expressed in 20 to 30% of invasive breast cancers.
EBV-specific T cells were transduced with a RD114 pseudotyped retroviral vector containing the ErbB2 chimeric T-cell receptor gene with a zeta-signaling domain (provided by Dr. Wels, Frankfurt, Germany). EBV-specific T cells expressed the chimeric T-cell receptor as judged by FACS analysis and lysed autologous ErbB2 positive breast cancer cell lines (MCF-7, SK-BR-3) in addition to autologous EBV-transformed B cells (LCL). In contrast, ErbB2 negative tumor cell lines were not lysed. Moreover, ErbB2/EBV-specific CTL could be expanded with autologous LCL stimulations similar to naïve EBV-specific CTL.
In summary, these results indicate that EBV-specific T cells expressing ErbB2 chimeric T-cell receptors may represent a new source of antigen-specific T cells that would persist and function long-term after their adoptive transfer to breast cancer patients.

Fas down-modulation in EBV-specific Cytotoxic T-Lymphocytes (CTLs) reduces their sensitivity to Fas/FasL-induced apoptosis

Savoldo B, Pule M, Straathof KC, Biagi E,Yvon E,Vigouroux S, Brenner MK, Rooney CM, Dotti G

Contact:
Dr. Barbara Savoldo
Baylor College of Medicine
Center for Cell and Gene Therapy (Houston, USA)
Email: bsavoldo@bcm.tmc.edu

Abstract:
Activated T cells expressing the Fas receptor (Apo-1/CD95) are switched to an apoptotic program by cross-linking with Fas-ligand (FasL). Consequently, the many tumors that express FasL, including Hodgkin's lymphoma (HD) and Nasopharyngeal carcinoma (NPC), can induce apoptosis of Fas positive T-lymphocytes and subdue any anti-tumor host immune response. Thus we determined whether EBV-specific CTLs could be genetically modified to resist this evasion strategy.
We show that transduction of CTLs with siRNA targeting the Fas mRNA encoded in a retrovirus significantly down-modulates the expression of Fas compared to control cells (Fas MFI 107 ± 47 vs. 402 ± 47, $p<0.001$). This effect paralleled with a significant reduction of apoptosis induced by the Fas agonistic antibody (clone CH-11) in modified CTLs compared to control cells (23% ± 3% vs. 44% ± 7% $p=0.006$). Fas down-modulation was stable overtime in modified CTLs and the addition of CH-11 antibody to the culture selected a uniformly Fas-low, CH11-resistant population (Fas MFI 79 ± 29). The modified CTLs remained polyclonal and their survival and proliferation retained dependence on antigen specific stimulation and the presence of other physiological growth signals. In order to mimic in vitro a situation in which tumor cells express the natural hFasL, CTLs transduced with siRNA were co-cultured (1: 1 ratio) with COS-7 cells expressing hFasL or control cells. Even in this situation modified CTLs were more resistant to Fas/FasL induced cell death compared to control cells (23% ± 8% vs. 43% ± 13% $p=0.008$).

These results suggest that responsiveness to this single death signal may be ablated in an effector-memory population of CTLs without adversely affecting their in vitro function and characteristics. EBV-specific CTLs with lower expression of Fas receptor should have a selective functional and survival advantage over unmodified CTLs in the presence of tumors expressing FasL, such as HD and NPC, and may be of value for adoptive cellular therapy.

Identification of LMP2-epitopes in cytotoxic T cell lines (CTL) from patients with nasopharyngeal carcinoma (NPC) and Hodgkin's disease (HD).

Straathof KC, Bollard C, Leen AM, Heslop HE, Rooney CM

Contact:
Dr. Karin Straathof
Baylor College of Medicine
Pediatrics (Houston, USA)
Email: kcstraat@texaschildrenshospital.org

Abstract:
Objective: The significant failure rate and treatment associated morbidity of current treatment regimens for nasopharyngeal carcinoma and Hodgkin's disease indicate the need for additional treatment modalities. Immunotherapy either by infusion of ex-vivo expanded EBV-specific T cells or vaccination with EBV-antigens is an attractive therapeutic strategy for these type II latency malignancies. So far epitopes for the tumor associated antigens LMP1 and LMP2 have been identified for only a limited number of HLA alleles. Broadening this panel would provide useful tools for vaccination strategies as well as for monitoring of tumor-specific T cell populations after immunotherapeutic interventions.

Methods: A peptide library consisting of 122 pentadecamers overlapping by 11 amino acids covering the entire LMP2 sequence, was divided into 23 pools in such a way that each peptide was present in 2 pools as previously described by Kern et al, J Virol 1999. In an IFN-gamma ELISPOT assay LCL-reactivated cytotoxic T cell lines (CTL) from 12 NPC patients and 12 HD patients were screened for the presence of LMP2-specific T cells using these peptide pools. After the recognized pentadecamers were identified the minimal epitopes as well as their HLA-restriction were mapped.

Results: In 8 of 12 CTL lines from NPC patients and in 9 of 12 CTL lines from HD patients T cell responses against 1-4 LMP2 epitopes were identified. These included responses to previously described epitopes as well as to newly identified HLA-A2, A29, A68, B27, B35 and B53 restricted epitopes. The latter epitopes were located both in the transmembrane and in the cytoplasmatic region of the LMP2 molecule. The T cell responses against these LMP2 epitopes ranged from 7 to 1990 (median 154) SFC/1x10e5 CTL and, as expected, were overall weaker than responses to epitopes derived from lytic antigens and EBNA2/3. Nevertheless, responses to LMP2 were present in the majority of LCL-reactivated CTL lines from patients.

Conclusions: Screening for IFN-gamma secreting cells in an ELISPOT assay upon stimulation with a peptide library is a rapid and sensitive method to identify the presence of T cells specific for the antigen of interest within a polyclonal CTL line. Using this method we identified 8 new LMP2-epitopes with a diverse HLA restriction that provide useful tools for immunotherapy and immune monitoring.

Production of T cell populations for immuonotherapy of EBV malignancy by the transfer of LMP2-specific T cell receptor sequences

Jurgens L, Khanna R, Weber J, Orentas R

Contact:
Dr. Lisa Jurgens
Medical College of Wisconsin
Pediatrics (Milwaukee, USA)
Email: lbircher@mcw.edu

Abstract:
Objective: Adoptive immunotherapy with expanded cytotoxic T cell (CTL) populations for the treatment of malignancies expressing the EBV latency membrane protein 2 (LMP2) is an attractive alternative to current therapy. We have proposed creating CTL populations in vitro by direct transfer of recombinant T cell receptors(TCR)that recognize LMP2 in an HLA-restricted manner.

Methods: TCR-encoding DNA was transferred into activated PBMC by retroviral transduction and nucleofection with plasmid-based vectors. TCR sequences were derived from two T cell clones (CS1C7; HLA-A23-restricted and NB20; HLA-A2 restricted) that have the ability to lyse unmodified B-LCL.

Results: PBMC transduced with the retroviral vectors A7-LMP2. A2 or A7-LMP2. A23 were able to specifically recognize and lyse B-LCL in an HLA-restricted manner. In order to accelerate the process of identifying TCR sequences suitable for immunotherapy, we explored non-viral gene-transfer. Nucleofection (Amaxa, Inc.) transfers plasmid DNA directly into the cell nucleus by electroporation in a proprietary transfection solution. OKT3 and IL-2 activated PBMC, depleted of CD56+ cells, were nucleofected with pBudA7-LMP2.2, a plasmid expressing the alpha-2 and beta-21 chains of the NB20 clone. Expression of recombinant TCR by nucleofection was demonstrated by increased cell surface expression of the TCR beta-21 subunit and sequence-specific tetramer binding. Functional activity was demonstrated by lysis of peptide-loaded T2 target cells, giving greater than 45% lysis at a 1: 30 target to effector cell ratio. Nucleofection in the absence of plasmid gave only background levels of lysis against K562 or peptide-loaded T2. Cell death following nucleofection is considerable, 50-80%, but can likely be overcome by nucleofecting a sufficient number of PBMC.

Conclusions: Retroviral transduction of patient-derived PBMC produces an effector cell population suitable for the adoptive immunotherapy of EBV+ malignancy. The ability of nucleofected PBMC to express a functional TCR was demonstrated by antibody and tetramer-binding, as well as by the peptide specific lysis of T2 cells. Data generated thus far indicate that nucleofection provides a method by which the lytic potential of TCR sequences being considered for gene therapy can be rapidly evaluated.

Peptides encoding conformational epitopes and generating high affinity anti-LMPs antibodies for immunotherapy of EBV-associated diseases

Tranchand-Bunel D

Contact:
Prof. Denis Tranchand-Bunel
IBL, UMR CNRS 8527 (Lille, France)
Email: denis.bunel@ibl.fr

Abstract:
Objective: The presentation herein relies on novel peptides comprising conformational epitopes designed to mimic the structure of extracellular domains of Epstein-Barr virus (EBV) encoded latency-and tumour-associated type III membrane proteins (LMPs) for the preparation of therapeutic antibodies (Abs).

Results: The designed peptides induced high affinity mouse and rat anti-LMP1 and LMP2 antibodies (Ka = 7 to 17 nM-1 and 0.16 to 0.007 nM-1 for mouse and rat). These Abs did not cross-react with peptide coding for a single extracellular domain or with SDS-denaturated full length proteins. In contrast, anti-LMP1 Abs immunoprecipitated a protein showing a clear band at the appropriate position in immunoblot analysis, and thereby confirm their binding to native protein and their conformational peptidic structure recognition. EBV-transformed human lymphoblastoid B and T cell line (LCLs and E1 cells) cultures in the presence of anti-LMP Abs show total LCL and E1 culture growth arrest and induce agglutination and apoptosis of LCLs and E1 cells. Serotherapy with two injections of rat anti-LMP2 Abs three days after in vivo inoculation of high tumorigenic human E1 cells to immunodeficient mice resulted in delayed development of E1 tumours and increased by 30% survival of treated animals. To test preventive and therapeutic anti-LMP1 vaccination efficiency in immunocompetent mice, we have created a mouse tumorigenic cell line expressing LMP1 or LMP2. So, we have cloned LMP1 or LMP2 in the stable and episomal pREP4 plasmid and transfected malignant Sp2o cells. Sp2o-pREP4-LMP1 cells (6 $x10^6$) were injected to anti-LMP1 immunized or sensitized Balb/c mice. 90% of the control mice immunized with an irrelevant peptide did not survive more than 20 days. At day 23 the low anti-LMP1 responders (30%) started to develop aggressive tumours which totally regress in 50% of them or were stabilized for the others after two new anti-LMP1 immunizations. These results suggest that this new anti-EBV approach may have promise for treatment or prevention of EBV-associated lymphomas and carcinomas in which LMP1 and/or LMP2 proteins are expressed at the surface of the cells

Conclusions:To our knowledge, this is the first report in which peptides with conformational epitopes can generate high affinity Abs recognizing non-denaturated extracellular domains of type III membrane proteins.

Methotrexate induces reactivation of latent Epstein-Barr virus (EBV): a potential contributor to methotrexate-associated lymphomas

Feng W, Cohen J, Fischer S, Li L, Sneller M, Goldbach-Mansky R, Raab-Traub N, Delecluse HJ, Kenney SC

Contact:
Dr. Wenhai Feng
University of North Carolina at Chapel Hill
Lineberger Comprehensive Cancer Center (Chapel Hill, USA)
Email: fengw@med.unc.edu

Abstract:
Objective: Patients with rheumatoid arthritis (RA) or polymyositis (PM) treated with methotrexate (MTX) develop EBV-positive lymphomas more commonly than RA and PM patients treated with other regimens, whereas equally immunosuppressive regimens are not associated with the development of EBV-related lymphomas in these patients. We have determined if MTX, in contrast to other commonly used medications for RA and PM, is unique in its ability to induce the release of infectious Epstein-Barr virus from latently infected cells.

Methods: The effect of MTX on viral replication in vitro was assessed using latently infected, EBV-positive cell lines, and its effect on the transcription of the two EBV immediate-early promoters was examined using reporter-gene assays. EBV viral load in vivo was quantitated using real-time PCR in 29 patients with RA, and 12 patients with polymyositis, receiving various immunosuppressive medications.

Results: MTX activated the release of infectious EBV from latently infected cell lines in vitro, and MTX treatment was associated with activation of the two viral immediate-early (IE) promoters in reporter gene assays. Induction of lytic EBV infection by MTX required the p38 MAP kinase, PI3 kinase, and MEK pathways, as well as specific cis-acting motifs in the two viral IE promoters. In accordance with our in vitro results, we found that patients with RA and PM treated with MTX-containing regimens were significantly more likely to have an abnormally high EBV load in their blood than patients treated with other immunosuppressing regimens that did not include MTX ($p=0.011$).

Conclusions: The results suggest that MTX may promote EBV-positive lymphomas in RA and polymyositis patients both by its immunosuppressive properties as well as by reactivating EBV.

NF-kB is essential for progression of KSHV- and EBV-infected lymphomas in vivo
Keller SA, Hernandez-Hopkins D, Schattner E, Cesarman E

Contact:
Dr. Ethel Cesarman
Weill Medical College of Cornell University
Pathology and Laboratory Medicine (New York, USA)
Email: ecesarm@med.cornell.edu

Abstract:
Objective: Constitutively activated NF-kB is thought to be a vital mechanism by which EBV and KSHV infected lymphoma cells are protected from apoptotic stimuli. The goal of this study was to determine whether pharmacologic inhibition of NF-kB is a valid approach for the treatment of EBV and KSHV-associated lymphoma cells by using a murine xenograft model.

Methods: As a model for EBV-associated immunodeficiency-associated lymphoproliferative disorders, we injected NOD/SCID mice subcutaneously with the lymphoblastoid cell line LCL 9001 followed by intraperitoneal (IP) injection with vehicle, or with the NF-kB inhibitor, Bay 11-7082 at 20 mg/kg on days 1, 3, and 5 and once weekly, and measured the tumors each week. After 4 weeks animals were euthanized, and tumors excised and weighed. For evaluation of KSHV-associated lymphoma responses to NF-kB inhibition, we challenged male NOD/SCID mice with BC-3 primary effusion lymphoma (PEL) cell line intraperitoneally (IP) and then injected them IP with Bay 11 at 5 or 20 mg/kg, or with vehicle alone, on days 1, 3, and 5 after injection with tumor cells. We sacrificed the mice that developed PEL, and evaluated disease-free survival by the Kaplan-Meier method. We collected tumors and ascites and examined them by immunohistochemistry and in situ hybridization to confirm that the malignancies were of correct histology and contained viral RNA and proteins, and to evaluate potential induction of lytic gene expression.

Results: In the EBV tumor model, the mean tumor area was greater in untreated mice than in Bay 11-treated mice at all time points ($P<0.0352$), and the mean weight of excised tumors was greater in untreated mice than in the mice that received Bay 11 ($P<0.027$). In the KSHV lymphoma model, all mice injected with BC-3 cells developed PEL while approximately one third of the mice treated with Bay 11-7082 never developed lymphoma. Mice injected with Bay 11 had delays in tumor development as compared to mice injected with vehicle alone ($P<0.001$). The tumors showed expression of EBV and KSHV latent genes, and no viral lytic reactivation.

Conclusion: Our data provide proof of concept that a selective NF-kB inhibitor can prevent, delay the appearance, or slow the growth of EBV and KSHV associated lymphomas in vivo.

EBV infected NPC in Indonesia: Improvement of clinical management

Hariwiyanto B, Dahlia HL, Harijadi A, Astuti I, Paramita DK, Fachiroh J, Dhamiyati W, Haryana SM, Tan IB, Middeldorp JM

Contact:
Dr. Bambang Hariwiyanto
Faculty of Medicine Gadjah Mada University
ENT (Yogyakarta, Indonesia)
Email: asialinknpc@med.ugm.ac.id, sari_wuland@yahoo.com

Abstract:
Objective: To improve clinical management by developing standardized treatment and improvement of diagnostic & follow up based on pathology and serology.

Methods: To improve clinical management, internal coordination between departments namely ENT, Internal medicine, Radiodiagnostic, Radiotherapy, Pathology and serology was performed. Diagnostic done based on clinical examination (ENT), serology (VCA, EA, EBNA - IgA) and pathology. Treatment based on the degree of staying of NPC cases. Stage I & II will be treated by Radiotherapy, while stage III & IV by neo adjuvant therapy (Cisplatin and 5FU). Follow up were done 6-8 week after treatment by serology and swab.

Results: It was shown by this new clinical management, the number of dropout (DO) patients decreased significantly (20%). By this kind of weekly coordination, the communication and clinical atmosphere among departmentsis very conducive, and obstacles faced before could be overcome.

Conclusions: According to the new clinical management the number of DO was decreased significantly.

Epstein-Barr virus nuclear antigen 1 (EBNA1) evades direct immune recognition by CD4+ T helper cells

Mautner J, Adhikary D, Bornkamm GW, Hammerschmidt W, Behrends U

Contact:
Dr. Josef Mautner
Technical University Munich
Children's Hospital (Munich, Germany)
Email: mautner@gsf.de

Abstract:
The Epstein-Barr virus (EBV) nuclear antigen-1 (EBNA1) is the only viral protein regularly expressed in EBV-associated malignancies. An internal glycine-alanine repeat (GAr) has been reported to block proteasome-dependent degradation, preventing immune recognition of EBNA1 by CD8+ cytotoxic T cells. To test whether EBV-infected cells could be recognized by T helper cells, CD4+ T cell clones specific for EBNA1 were isolated from latently EBV-infected individuals. These T cells neither recognized autologous nor HLA-matched LCL, nor Burkitt's lymphoma cell lines. To investigate whether endogenous presentation of EBNA1 epitopes on MHC class II was prevented by the GAr domain, a mutant EBV strain with an EBNA1 lacking the GAr (EBNA1Δ GA) was generated and used to establish LCLs. These EBNA1Δ GA LCLs were not recognized by the EBNA1-specific T cell clones, indicating that the GAr domain does not mediate this effect. Immune recognition could be restored by overexpression of EBNA1 in LCL, for which at least 60-fold higher levels of both EBNA1 or EBNA1Δ GAr protein were required. These results demonstrate that EBNA1 evades direct recognition by CD4+ T helper cells since its steady state level is below the threshold required for efficient presentation on MHC class II. These results have important implications for the design of immunotherapeutic approaches to target EBV-positive malignancies.

The CD4+ T helper cell response against Epstein-Barr virus latent antigens is directed against several antigens

Adhikary D, Behrends U, Mautner J

Contact:
Dr. Dinesh Adhikary
GSF
Institute for Clinical Molecular Biology (Munich, Germany)
Email: adhikary@gsf.de

Abstract:
The CD4+ T helper cell response against Epstein-Barr virus (EBV) is poorly defined. T helper cells specific for several EBV latent cycle antigens have been detected in the peripheral blood of healthy virus carriers, but the low frequencies of such T cells has hampered assessment of breadth and magnitude of the EBV-specific T helper response. Here, recombinantly expressed and purified EBV latent proteins were used to reactivate and clone latent cycle protein-specific T cells from peripheral blood of healthy virus carriers. T helper cells specific for at least five latent EBV proteins were consistently detected in all donors, namely EBNA1, EBNA2, EBNA-3A, -B, and -C. Notably, no responses against EBNA-LP and the latent membrane proteins LMP1 and LMP2A were observed. All latent antigen-specific T cells were of Th1 type, mostly HLA-DR-restricted, and comprised less than 0.01% of the peripheral CD4+ T cell pool. These results demonstrate that in comparison to the EBV-specific CD8+ CTL response, a significantly lower number of EBV-specific CD4+ T helper cells is directed against a broader set of latent EBV antigens.

Both CD4+ and CD8+ T cells are required to generate EBV-specific CTL in vitro

Vanhoutte VJ, Turner ML, Crawford DH, Haque T

Contact:
Victoria Vanhoutte
The University of Edinburgh
The Laboratory for Clinical and Molecular Virology (Edinburgh, UK)
Email: Victoria.Vanhoutte@ed.ac.uk

Abstract:
CD8+ T cells and more recently CD4+ T cells have been shown to be cytotoxic to EBV-infected cells and have been used as immunotherapy of EBV-associated diseases such as PTLD. Here we aimed to immunophenotype these T cells and examine their cytotoxic mechanisms and in vitro growth trends.
CTLs were generated by weekly stimulations with X-irradiated autologous LCL using unseparated PBMCs and PBMCs separated into CD4+ T cells alone, CD8+ T cells alone and CD4+ and CD8+ T cells in combination (2: 1 ratio). Chromium release assays, using inhibitors such as BFA, CMA and EGTA, were used to determine the cytotoxic pathways. Immunophenotyping was done by FACS analysis and granulysin was detected by Western blotting.
Of six CTL lines, generated using unseparated PBMCs, two comprised of >79% CD4+ T cells and four of >90% CD8+ T cells. All CTL lines displayed specific autologous LCL killing. CMA and EGTA reduced cell-specific lysis by 42% and 63% respectively, whilst BFA reduced this by 8%, indicating that the CTL used the perforin/granzyme pathway. Separated PBMCs from two donors were stimulated weekly and showed no growth impairment compared to CTLs grown from unseparated PBMCs. All 3 separated T cell lines from donor 1 evolved into CD8+ cells, with >60% of cells expressing CD56 at stimulation 3. These cells expressed granulysin and were cytotoxic to autologous LCL and K562 cells. From donor 2, only the CD4: CD8 combination line expressed CD56, whilst CD4+ alone and CD8+ alone lines retained their original phenotype. The 3 T cell lines from donor 2 exhibited low killing of autologous LCL with <20% cell lysis. Cytotoxicity to K562 cells was restricted to CD56-expressing CD4: CD8 combination cell line (50.2%), in conjunction with granulysin expression. After 5 stimulations all cell lines from both donors displayed a mature phenotype of CD45RO+, CD27-, CD28-, CD62L- and CD69+. Low level expression of IL-2, IL-4, TNF-α, IFN-γ, FasL, granzyme B and perforin was also detected in these cells.
Although there was no difference in the growth of T cell lines from separated and unseparated PBMCs, the former did not exhibit EBV-specific killing, highlighting the importance of CD4+ and CD8+ T cell interaction in generating CTL lines. Separated T cells developed into a natural killer cell-like phenotype in vitro, the significance of which will be investigated.

Improved gene expression in HIG-82 synoviocytes using EBV-based plasmid
Kim JY, Oh ST, Youn J, Lee SK

Contact:
Prof. Dr. Suk Kyeong Lee
Catholic University of Korea
Catholic Reserch Institues of Medical Science (Seoul, Korea)
Email: sukklee@cmc.cuk.ac.kr

Abstract:
Objective: In rheumatoid arthritis (RA), over-expression of inflammatory cytokine and hyperplasia of synovial tissue cause joint destruction. Gene therapy has been developed to treat RA. However, viral vectors have safety problems and side effects, while non-viral vectors suffer from inefficient gene transfer and fast loss of gene expression. To overcome the limits of non-viral vectors, an EBV-based plasmid which is known to exert prolonged high level gene expression can be used. The aim of this study is to investigate whether enhanced anti-inflammatory cytokine production could be achieved by transfecting genes cloned in EBV-based plasmid.

Methods: pEBVGFP, pEBVIL-10, and pEBVvIL-10 were constructed by cloning GFP, IL-10, and vIL-10 genes into an EBV-based plasmid (pECP4), respectively. For comparison, pGFP was derived from pEBVGFP by deleting out the oriP and EBNA-1 sequences. All gene constructs were lipofected into HIG-82 rabbit synoviocyte cell line. The expression of GFP was monitored by FACS and confocal microscopy. IL-10 and vIL-10 expressions were measured by ELISA.

Results: Around 30% of synoviocytes transfected with pEBVGFP or pGFP showed GFP expression 2 days after transfection. The percent of GFP-expressing cells transfected with pGFP decreased rapidly from 5 days after transfection and completely disappeared by 11 days. On the contrary, the percent of GFP expressing cells transfected with pEBVGFP decreased slowly and maintained above the initial expression level up to 7 days. For these cells, GFP expression was detected for 20 days at least. In addition, HIG-82 cells transfected with pEBVIL-10 (12.4 +/-2.8 ng/μl) or pEBVvIL-10 (7.6 +/- 6.1 ng/μl) secreted these cytokines at quite high levels. This high level cytokine production was maintained for up to several days after transfection.

Conclusions: These results suggest that the EBV-based plasmid has a potential to improve non-viral gene transfer system and may be applicable to treat RA without the drawbacks of viral vectors.

Human CD40 ligand gene transfer into chronic lymphocytic leukemia (CLL) B-cells via EBV-based vectors

Hettich E, Janz A, Weissflog B, Neuhierl B, Hellebrand E, Hammerschmidt W, Zeidler R

Contact:
Dr. Eva Hettich
GSF - Department of Gene Vectors (München, Germany)
Email: Hettich@gsf.de

Abstract:
Background/Aim: B-CLL is the most frequent adult leukemia in the western world and is characterized by the of CD5+ monoclonal B cells in the blood, bone marrow, and lymphoid tissues. CLL B-cells are inable to properly act as antigen-presenting cells (APC) due to the lack of expression of costimulatory molecules which are necessary for induction of a T cell response. In addition, activated T cells of patients with CLL fail to express detectable amounts of CD40 ligand (CD40L). It has been demonstrated before by Wierda et al. that adenoviral transfer of murine CD40L into B-CLL cells induces the expression of a variety of immune coreceptors, including CD80 (B7-1) and CD86 (B7-2), on both transduced and bystander leukemia cells. This restores the capacity of CLL cells to stimulate autologous T cells (1). Instead of using the murine CD40L as a therapeutic gene we chose the human homolog (hCD40L) and used a gene therapy system based on Epstein-Barr virus (EBV), a gamma-herpesvirus which naturally infects human B-cells.

Methods/Results: Gene transfer of hCD40L into B-CLL cells requires the production of modified EBV particles carrying the gene encoding for hCD40L. For this purpose we constructed a packaging cell line (HEK293-V) harboring a recombinant EBV helper genome. This EBV mutant lacks its packaging sequences (TR), but supports the packaging of EBV-based gene vectors in trans (2). For safety reasons, we further knocked out BZLF1, which induces the lytic cycle of EBV, as well as the genes encoding for proteins that are important for immortalization of primary B-cells in vitro. These include EBNA2, LMP1, and the EBNA3 family. Our therapeutic gene vector, carrying the human CD40L, encompasses only a few cis-acting elements of wild-type EBV that are necessary for the amplification of episomal vector DNA during the lytic cycle of EBV (oriLyt) and for packaging of this DNA into viral particles (TR). Instead of cloning an antibiotic resistance gene onto the vector, we took advantage of the E. coli supF gene system. This also includes the construction of a modified E. coli strain that carries the amber amp and amber tet resistance genes for propagation of the gene vector DNA. Infection experiments with Raji cells showed that unwanted recombination between the EBV helper virus genome and our therapeutic vector is minimized. In addition, incubation of CLL B-cells with low MOIs (0.5 to 1) of the recombinant EBV particles resulted in efficient transduction of the cells and hCD40L expression. As expected, upregulation of costimulatory molecules such as CD80, CD86, and CD54 was observed.

Summary: We could establish a safe and helper-free EBV packaging cell line that enables us to package EBV-based therapeutic gene vectors for the efficient infection and genetic modification of B-cells as e. g. B-CLL cells.

(1) Wierda et al. (2000) Blood 96, 2917-2924
(2) Delecluse et al. (1999) Proc. Natl. Acad. Sci. USA 96, 5188-5193

Ad5F35 adenoviral vector modified LCL enable the expansion of polyclonal antigen-specific cytotoxic T cells for adoptive immunotherapies

Gottschalk S

Contact:
Dr. Stephen Gottschalk
Baylor College of Medicine
Pediatrics (Houston, USA)
Email: smg@bcm.tmc.edu

Abstract:
Cytotoxic T cells (CTL) have been used successfully for the adoptive immunotherapy of viral infections and malignancies. Infused T cells 1) were safe, 2) produced anti-viral and anti-tumor effects and 3) did not persist without the co-infusion of CD4 T helper cells. Moreover T-cell escape mutants were observed when clonal or oligoclonal T-cell lines were infused. These results imply that therapeutic T-cell products should target multiple antigens and contain CD4 as well as CD8 positive T cells. Dendritic cells (DC) have been used to reactivate antigen-specific CTL, however the expansion of polyclonal, antigen-specific CTL for therapeutic use has proved challenging, since 1) patient's DC are frequently limiting and 2) non-specific T-cell stimulation does not support the long-term growth of antigen-specific, polyclonal CTL. To overcome these limitations, we have combined initial DC stimulations to reactivate antigen-specific CTL with subsequent LCL stimulations for CTL expansion.
Since T-cell stimulation with LCL results in the generation of EBV-specific CTL lines dominated by T-cell clones specific for immunodominant EBV proteins we reasoned that overexpression of antigens in LCL would compete out immunodominant peptides for MHC class I presentation and replace them with naturally - processed peptides of the antigen of interest. For antigen expression in LCL we utilized Ad5F35 adenoviral vectors, which have a serotype 5 capsid containing the short-shafted fiber protein of serotype 35. As model antigens we either used the CMV protein pp65 or the subdominant EBV associated LMP antigens. Recombinant Ad5F35 vectors encoding either pp65 or LMP antigens transduced LCL efficiently. CMV and LMP-specific CTL were initially activated with DC and subsequently expanded with antigen overexpressing LCL. Depending on the donor, the resultant CTL lines contained an increased frequency of antigen-specific CTL (4-11%) when compared to CTL lines generated with unmodified LCL (<0.1%). Moreover, we were able to generate sufficient numbers of poylconal, antigen-specific CTL for clinical trials.
In summary, the developed 'DC-LCL technology' 1) does not require prior knowledge of a particular HLA type or epitope and 2) serves as the core technology for our current clinical trials with LMP2-specific CTL for EBV-positive Hodgkin's lymphoma and CMV-specific CTL post transplant.

Inhibition of NF-κ B activity induces cytotoxicity in Epstein-Barr virus positive tumor cells

Wang H, Liu S, Tang M, Yi W, Cao Y

Contact:
Dr. Hai Wang
Cancer Research Institute
Central South University (Changsha, China)
Email: wanghai97@hotmail.com

Abstract:
Objective: Nasopharyngeal carcinoma (NPC), Kaposi's sarcoma, and B-cell lymphomas are human malignancies associated with Epstein-Barr virus infections. EBV is characterized by its ability to establish latent infections almost exclusively in tumor cells. In these EBV positive tumor cells, NF-κB is highly activated. Inhibition of NF-κB activity leads to lytic replication of EBV. Switching from latent to Lytic replication of EBV in EBV positive carcinoma cells can cause tumor cell death. So, we try to prove whether NF-κB inhibitor can cause significant cytotoxicity in EBV positive carcinoma cells by lytic replication of EBV. This will provide a new strategy for EBV associated carcinoma therapy.

Methods: Two NPC cell lines: HNE3 (EBV negative), 5-8F (EBV Positive) were used in this experiment. Two chemicals: Bay 11-7082 and Z-LLF-CHO were NF-κB inhibitors. NF-κB activity were estimated by indirect immune fluorescent staining and western blot. EBV lytic replication was verified by detection of BZLF1, EA-D and gp125 protein which expressed only in lytic EBV. Gardella gel, which could distinguish linear (implying lytic replication of EBV) and circular EBV (latent EBV) DNA was also used to tell lytic replication from latent EBV. Cell toxicity was assessed by MTT.

Results: Bay 11-7082 and Z-LLF-CHO inhibited p65 (subunit of NF-κB) nuclear translocation (barometer of activity of NF-kB) through indirect immune fluorescent staining and western blot assay of nuclear and cytoplasm protein. The two NF-κB inhibitors induced EBV lytic replication in EBV positive 5-8F cells through detection of EBV lytic replication associated protein and gardella gel assay. Furthermore, the two inhibitors induce about 30% cells death in EBV positive 5-8F at the concentration of 30mmol/L and 5mmol/L individually. Most important of all, in EBV negative HNE3 cell, the two inhibitors have no significant cell killing effects. So this strategy is a specific therapy for EBV positive carcinoma. Lytic replication of EBV can express EBV-TK gene (suicide gene). Adding of GCV (100mg/ml) to cells treated by Bay 11-7082 or Z-LLF-CHO killed over 70% in EBV positive 5-8F cells.

Conclusions: Inhibition of NF-κB activity can disrupt EBV latency, resulted in potential, specific EBV positive tumor cell killing.

Anti-viral activity of statins - a novel strategy for blockading lytic EBV replication
Jones RJ, Kenney SC

Contact:
Dr. Richard Jones
University of North Carolina
Lineberger Comprehensive Cancer Center (Chapel Hill, USA)
Email: richardj@email.unc.edu

Abstract:
Therapeutic strategies for inhibiting lytic EBV replication are limited to drugs such as acyclovir (ACV) and ganciclovir Therapeutic strategies for inhibiting lytic EBV replication are limited to drugs such as acyclovir (ACV) and ganciclovir (GCV), which inhibit viral replication but do not prevent expression of immediate-early (IE) and early lytic genes. We recently demonstrated that fatty acid synthase inhibitors prevent the lytic form of EBV infection at the level of IE gene expression. Here we have investigated whether the 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, Lovastatin and Simvastatin, also inhibit the lytic form of EBV infection.

EBV-positive AGS gastric carcinoma cells were generated using either a recombinant Akata or B95-8 EBV. EBV-positive AGS cells were transfected with BZLF1 or BRLF1 expression vectors, or treated with the HDAC inhibitor, valproic acid, to induce lytic EBV infection. The effect of lovastatin and simvastatin on constitutive lytic EBV gene expression, as well as lytic gene expression induced by valproic acid, BZLF1 transfection, or BRLF1 transfectioin, was examined by immunoblot analysis.

Initial studies have clearly demonstrated that doses between 4-8uM of Lovastatin or Simvastatin are able to inhibit constitute expression of the BZLF1 IE gene, as well as the BRMF1 early gene, in EBV-positive gastric carcinoma cell lines. This dose of statins is not toxic to AGS-EBV cells. The statins also inhibit lytic EBV gene induction by valproic acid. Interestingly, preliminary data suggest that the statins reduce the ability of transfected BRLF1 to induce lytic gene expression, but that transfected BZLF1 (driven by a strong heterologous promoter) activates lytic EBV expression even in the presence of the statins.

These findings indicate that the statins may be a novel way of inhibiting lytic EBV infection. This effect, similar to the effect of fatty acid synthase inhibitors, appears to be mediated at the level of IE gene expression. The finding that both fatty acid synthase inhibitors, as well as statins, inhibit lytic EBV gene expression suggests that one or more cellular proteins located within lipid rafts may promote lytic EBV gene expression.

hTERT/re-Caspase-3 system induce apoptosis in hTERT-positive tumor cells

Yang LF, Zhao Y, Zeng L, Gong JP, Cao Y

Contact:
Dr. Lifang Yang
Cancer Research Institute
Xiangya School of Medicine,Central South University (Changsha, China)
Email: yanglifang99@hotmail.com

Abstract:
Objective: To restore the ability to undergo apoptosis is promising for the treatment of tumor. However, we need to explore a tumor-specific expression system of inducing apoptosis genes. Telomerase is an attractive target because approximately 90% of tumors have telomerase activity whereas most normal cells do not have. Activation of telomerase is tightly regulated at the transcriptional level of the human telomerase reverse transcriptase (hTERT). In the present study, we developed the telomerase-specific transfer system of apoptosis-inducible genes re-Caspase-3, through utilizing the promoter of the hTERT gene, and then investigated its antitumor effect on tumor cells and tissues. The reason we used the re-Caspase-3 gene is that it is capable of autocatalytic processing and inducing apoptosis independent of the initiator Caspases.

Methods: Based on the expression of hTERT mRNA in tumor and control cells, determined by real-time quantitive RT-PCR,we confirmed that the activation of hTERT transcription was significantly up-regulated only in the hTERT-positive cells by reporter plasmids. We constructed hTERT promoter plasmids carrying re-Caspase-3. First, we determined whether the hTERT/re-Caspase-3 system induces Caspase-3 expression only in hTERT-positive cells by the Caspase-3 enzyme activity analysis; Second, we observed that the hTERT/re-Caspase-3 system induced apoptosis in hTERT-positive tumor cells by the LSCM and Flow Cytometry; Third, the in vivo antitumor effect of the hTERT/re-Caspase-3 system was demonstrated by animal experiments; Last, detection ofthe changes of Caspase-3 expression and apoptosis was used to determine the in vivo antitumor effect of the hTERT/re-Caspase-3 system, the sections from the treated tumors were analyzed by immunohistochemistry for Caspase-3 and TUNEL technique.

Results: We demonstrated here that the hTERT/re-Caspase-3 system induced apoptosis in hTERT-positive tumor cells: CNE1 (nasopharyngeal carcinoma), HRT-18 (colonic carcinoma), MGC (stomach carcinoma), but not in hTERT-negative Hacat (human normal keratinized epithelium) cell. In addition, the growth of s.c. tumors in nude mice was significantly suppressed by the treatment with hTERT/re-Caspase-3 system.

Conclusions: The telomerase-specific transfer of the re-Caspase-3 gene may be a novel and promising targeting approach for the treatment of tumor.

Eradication of Epstein-Barr Virus Episome and Associated Inhibition of Infected Tumor Cell Growth by Adenovirus Vector-Mediated Transduction of Dominant-Negative EBNA1

Kuroda M, Nasimuzzaman MD, Dohno S, Yamamoto T, Iwatsuki K, Matsuzaki S, Kumita W, Mizuguchi H, Hayakawa T, Taguchi T, Wakiguchi H, Imai S

Contact:
Dr. Masayuki Kuroda
Kochi Medical School, National University Corporation Kochi University
Molecular Microbiology and Infections
Program of Bio-signaling and Infection Control (Nankoku, Japan)
Email: kurodam@med.kochi-u.ac.jp

Abstract:
Objective: The Epstein-Barr virus (EBV) is causally associated with certain human cancers of diverse tissue origin. Many studies so far have presented evidence that EBV genome itself or the specific gene(s) is actually implicated in the cellular acquisition of malignant phenotypes, providing an exploitable opportunity of virus-specific cancer therapy to eradicate EBV episomes from tumor cells. According to this concept, this study corroborated the significance of a novel therapeutic strategy to target EBV nuclear antigen 1 (EBNA1), a latent viral protein which is consistently expressed and essentially required to maintain EBV episomes in infected proliferating cells.

Methods: We constructed a mutant (mt) EBNA1 lacking the N-terminal-half, relative to wild-type (wt) EBNA1, and examined whether it exerted dominant-negative effects on maintenance of the viral episome thereby leading to abrogation of EBV-infected tumor cell growth.

Results: Using lymphocyte and epithelial cell lines converted with neomycin-resistant recombinant EBV (rEBV) as models, adenovirus vector-mediated transduction of mtEBNA1, but not lacZ, brought about rapid and striking reductions of rEBV-derived wtEBNA1 expression levels and viral genomic loads in converted cell lines of three major viral latencies. This outcome was further validated at the single cell level by cellular loss of G418-resistance and viral genomic signals or viral transcripts (EBER1) in situ. The mtEBNA1 transduction significantly impaired growth of Burkitt's lymphoma cells, which naturally harbor EBV, largely in association with the eradication of viral episomes. Expression of mtEBNA1 per se caused no detectable cytotoxicity in EBV-uninfected cells including normal human fibroblasts.

Conclusions: These results indicate that mtEBNA1 can act as a dominant-negative effector that efficiently impedes the EBV-dependent malignant phenotypes in cells regardless of viral latency, tissue origin or resident viral strain. Therefore, the mutant will afford an additional therapeutic strategy specifically targeting EBV-associated malignancies.

Targeted suppression of EBV-LMP1 mRNA by deoxyribozymes as a potential therapeutic strategy for EBV associated carcinoma

Lu ZX, Ye M, Yan GR, Li Q, Tang M, Lee LM, Sun LQ, Cao Y

Contact:
Dr. Zhong-Xin Lu
Cancer Research Institute, Xiangya School of Medicine
Central South University (Chang Sha, China)
Email: lzx71@yahoo.com

Abstract:
Objective: The latent membrane protein (LMP1) encoded by Epstein-Barr virus (EBV) has been suggested to be one of the major oncogenic factors in EBV mediated carcinogenesis. RNA-cleaving DNA enzymes are catalytic nucleic acids that bind and cleave a target RNA in a highly sequence-specific manner. In this study, we explore the potential of using DNAzymes as a therapeutic approach to EBV associated carcinomas by targeting the LMP1 gene.

Methods: Fourteen different phosphorothioate-modified '10-23' deoxyribozymes (DNAzymes) were designed and synthesized against the LMP1 mRNA and transfected into B95-8 cells, which constitutively express the LMP1. Fluorescence microscopy was used to determine the intracellular uptake and distribution in B95-8 cells. Western blotting was used to determine the expression of LMP1, Bcl-2 and the release of Cytochrome c. alamarBlue Assay was used to evaluate cell growth activity and flow cytometry was used to evaluate the progression of the cell cycle.

Results: As demonstrated in Western blotting, three out of fourteen deoxyribozymes significantly down-regulated the expression of LMP1 in B95-8 cells. These DNAzymes were shown to markedly inhibit B95-8 cell growth compared with a disabled DNAzyme and untreated controls, as determined by an alamarBlue Assay. It was further demonstrated that these DNAzymes arrested the B95-8 cells in G0/G1 using flow cytometry. Interestingly, the active DNAzymes could also down-regulate the expression of Bcl-2 gene in treated cells, suggesting a close association between the LMP1 and Bcl-2 and their involvement in apoptosis. This was further confirmed with the result that the DNAzymes could induce the release of Cytochrome c from mitochondria, which is the hallmark of apoptosis.

Conclusions: The present results suggest that the LMP1 may present a potential target for DNAzymes towards the EBV associated carcinoma through cell proliferation and apoptosis pathways.

Tumor regression by Epstein-Barr virus latent membrane protein 1

Hsieh YH, Liu HP, Lo SJ, Chang YS

Contact:
Prof. Dr. Yu-Sun Chang
Graduate Institute of Basic Medical Science
Chang-Gung University (Kwei-Shan, Taiwan)
Email: ysc@mail.cgu.edu.tw

Abstract:
Latent membrane protein 1 (LMP-1) is an Epstein-Barr virus (EBV)-encoded integral membrane oncoprotein that is cytotoxic when over-expressed. NLMP-1 is an LMP1 gene isolated from EBV-positive nasopharyngeal carcinoma tissues. In this study, we present data showing that expression of NLMP-1 from a strong promoter (CMV-IE) activates caspases and induces apoptosis. Mapping studies indicate that NLMP-1 transmembrane segments 1-3 are required for induction of cytotoxicity. Intratumoral delivery of vectors expressing NLMP-1 or its membrane domain via in vivo electroporation induces tumor tissue damage, suppresses tumor growth in mice and prolongs the life span of treated animals. In addition, the membrane domain of NLMP-1 alone induces effects similar to those induced by co-treatment with NLMP-1 and IL-12. These data suggest that high-level expression of NLMP-1 or its membrane region leads to significant inhibition of tumor growth. Mice surviving 120 days after the initial treatments were further challenged with CT-26 tumor cells. No tumor growth was observed, suggesting that treatment with NLMP-1 may induce immune memory. Thus, NLMP-1, and more specifically the transmembrane domain of NLMP-1, may be promising new therapeutic agents for inhibition of tumor growth

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