# EBV CONFERENCE 2006

**Boston/Cambridge**
Massachusetts · USA
July 8–12, 2006

The 12th Biennial Conference of the International Association for Research on Epstein-Barr Virus and Associated Diseases

## Final Program and Abstracts

# Welcome

Welcome to Boston/Cambridge and the 12th Biennial Conference of the International Association for Research on Epstein-Barr Virus and Associated Diseases! After enjoying the splendors of Australia and the warm hospitality in the heart of Bavaria, we are proud to host the EBV Association in the hub of New England. Boston is one of the oldest cities in America, and Cambridge is…, well, it is Cambridge, as you will see.

The area is rich in history, education, culture, and sports. The shot "heard round the world" was fired in nearby Lexington/Concord (apologies to our British conferees), and the old North Church, where lanterns were hung to signal Paul Revere, can be found in Boston's North End. Walking all—or part—of the Freedom Trail would be a great way to experience the historical sights and tradition. The area is also the home of Harvard University, Tufts University, the Massachusetts Institute of Technology (MIT), and a bevy of other higher educational institutions. The oldest public school in the USA, Boston Latin School (founded in 1630), can be found adjacent to the Longwood Medical area, the home of Harvard Medical School. You can go for a jog along the banks of the Charles River, or run across the Boston Marathon finish line on Boylston Street in Copley Square. For those who find swiping a credit card the exercise of choice, you can try shopping at historic Faneuil Hall Market Place or for the more upscale, the posh stores along Newbury Street. For the culturally minded, you might try visiting the Museum of Fine Arts, the Isabella Gardner Museum, or some of the museums at Harvard University (Fogg Museum: European and North American painting, prints, and photography; Sackler Museum: ancient, Asian, Islamic, and Indian arts). Boston is also the home of the Boston Pops, who finished their season with the Fourth of July celebration along the Charles River, and the Boston Symphony, who spend the summers at Tanglewood in the Berkshire Mountains of western Massachusetts. And lest we forget, Boston is also the home of the Red Sox, who finally broke Babe Ruth's curse by winning the World Series in 2004 (correlating with a dip in research productivity during the fall of 2004 in Boston), the Boston Celtics, winner of 16 NBA championships, and the New England Patriots, Super Bowl winners in 2002, 2004, and 2005.

Thanks to all of you, we have a wealth of science to be presented in the talks and in the posters. We have tried to supplement that knowledge base with invited talks from a number of investigators in the EBV field, as well as two plenary sessions from exceptional investigators outside the field to give us new perspectives on NF-kB signaling and antigen presentation. We will recognize another of our outstanding EBV investigators with the Henle Lectureship, and we will pay special tribute to two distinguished investigators that we recently lost from our community.

The meeting format this year is slightly different from previous years. Rather than extend the meeting an extra day for a single, group-wide event, we chose to compact the meeting schedule and provide conferees with time before or after the meeting to explore and enjoy the area. We hope this provides for an intense and enriching scientific experience. By holding all events within the same hotel, we also hope this provides the most important ingredient—an opportunity for scientific interaction. As always, posters will be up during the whole meeting, and there will be two designated poster sessions. The Sunday poster session will be a typical evening event with beer and wine, but the Monday poster session is moved up earlier and will be accompanied with hors d'oeuvres. This will enable you to get out and try out some of the wonderful restaurants in Cambridge and Boston after a full day of science. We will conclude the meeting with a banquet at the nearby Museum of Science.

We hope you have a wonderful time in Boston/Cambridge and find the meeting to be an enriching experience. Thank you for your science and support to make this a successful meeting!

Fred Wang
on behalf of the Organizing Committee

# Committees

## EBV Association Officers

Fred Wang, President
Yi-Xin Zeng, Vice-President
Tom Sculley, Secretary
Nancy Raab-Traub, Treasurer

## EBV Association Governing Board

Friedrich Grasser, Germany
Shannon Kenney, USA
Richard Longnecker, USA
Evelyne Manet, France
Andrew Morgan, UK
Gerald Niedobitek, Germany
Takeshi Sairenji, Japan
Clare Sample, USA
Ih Jen Su, Taiwan
Sankar Swaminathan, USA (adjunct member)
Joanna Wilson, UK

## Local Organizing Committee

Elliott Kieff
Nancy Raab-Traub
Sankar Swaminathan
Fred Wang

## Scientific Program Committee

Alfieri, Carolyn
Allday, Martin
Bornkamm, Georg
Cohen, Jeffrey
Delecluse, Henri
Ernberg, Ingemar
Farrell, Paul
Flemington, Erik
Frappier, Lori
Grässer, Friedrich
Hayward, Diane
Hutt-Fletcher, Lindsey
Khanna, Rajiv
Lieberman, Paul
Longnecker, Richard
Masucci, Maria
Miller, George
Morgan, Andrew
Moss, Denis
Niedobitek, Gerald
Ooka, Tadamasa
Rickinson, Alan
Robertson, Erle
Rooney, Cliona
Rowe, Martin
Sairenji, Takeshi
Sample, Jeff
Sample, Clare
Sergeant, Alain
Sixbey, John
Takada, Kenzo
Wilson, Joanna
Wolf, Hans
Yanagi, Kazuo
Zeng, Yi-Xin

# General Information

## Opening Hours of the Registration & Information Desk

| | |
|---|---|
| Saturday, July 8, 2006 | 13:30–19:00hrs |
| Sunday, July 9, 2006 | 08:00–17:00hrs |
| Monday, July 10, 2006 | 08:00–17:00hrs |
| Tuesday, July 11, 2006 | 08:30–17:00hrs |

## Conference Office

Meeting Services International
1330 Beacon Street, Suite 228
Brookline, MA 02446, USA
Phone: (617) 738-9951
Fax: (617) 278-9113
info@ebv2006.com

## Congress Venue

Boston Marriott Cambridge
2 Cambridge Center (Broadway and 3rd Street)
Cambridge, MA 02142 • USA
Phone: (617) 494-6600

## Registration Fees

| | before/on May 31, 2006 | after May 31, 2006 |
|---|---|---|
| EBV Association-members | $ 600.00 | $ 750.00 |
| Non-Member | $ 725.00 | $ 850.00 |
| Accompanying Persons/Before | $ 475.00 | $ 475.00 |

## Conference Banquet

The conference banquet on Tuesday, July 11, 2006 will take place at the Museum of Science (MoS) from 18:30 to 22:00hrs. Please make sure that you obtain a ticket from the registration desk. A bus transfer to the Museum of Science will be provided leaving the hotel at 18:00hrs. If you prefer to walk to the Museum it will take you approximately 15 to 20 minutes from the Marriott Cambridge hotel.

## Badges

Please wear your name badge at all times during the conference in order to gain access to the scientific program and all conference functions (breakfast, lunches, dinner, banquet).

## Poster Sessions

**Poster Session I**
**Sunday, July 9, 2006 20:00–22:00hrs**

**Poster Area/Level 2:**
Session 5: Latent Infection
Session 6: Oncogenesis & Cell Cycle

**Poster Area/Level 3:**
Session 7: Signal Transduction
Session 8: Viral Replication

**Poster Session II**
**Monday, July 10, 2006, 17.30–19.00hrs**

**Poster Area/Level 2:**
Session 13: PTLD & Other Malignancies
Session 14: Diagnostics, Vaccines, and Therapy
Session 15: Gene Regulation

**Poster Area/Level 3:**
Session 16: Immune Mechanisms
Session 17: Comparative Systems
Session 18: Human Infection
Session 19: Burkitt & Hodgkin Lymphoma
Session 20: Nasopharyngeal Carcinoma

# Program-at-a-Glance

| | SATURDAY July 8, 2006 | SUNDAY July 9, 2006 | MONDAY July 10, 2006 | TUESDAY July 11, 2006 |
|---|---|---|---|---|
| **6:30** | | Breakfast until 8:30 | Breakfast until 8:30 | Breakfast until 8:30 |
| **8:15** | | **WELCOME** | | |
| **8:30** | | **Session 1:** Latent Infection I | R. Longnecker | |
| **8:45** | | | | **Session 21:** Signal Transduction |
| 9:00 | | | **Session 9:** Replication II | |
| 9:15 | | | | |
| 9:30 | | | | |
| 9:45 | | | | |
| **10:00** | | Coffee Break | Coffee Break | Coffee Break |
| **10:30** | | H. Delecluse | L. Young | S. Harrison **Structures and Interactions of Rel-Family Proteins** |
| 10:45 | | | | |
| **11:00** | | **Session 2:** Latent Infection II | **Session 10:** Latent Infection III | |
| 11:15 | | | | |
| **11:30** | | | | EBV Association Meeting |
| 11:45 | | | | |
| **12:00** | | Lunch | Lunch EBV Board Meeting | Lunch |
| 12:30 | | | | |
| **13:30** | **REGISTRATION** | **TRIBUTE:** J. Ho & D. Huang | **HENLE LECTURE** D. Moss **Are We Reaching a Clinical Application Era After 40 Years of Basic EBV Research?** | R. O'Reilly |
| 13:45 | | Y.-X. Zeng | | |
| **14:00** | | | | **Session 22:** Immunology |
| 14:15 | | **Session 3:** Malignancy | | |
| **14:30** | | | **Session 11:** Therapy | |
| 14:45 | | | | |
| 15:00 | | | | |
| 15:15 | | | | |
| **15:30** | | Coffee Break | Coffee Break | Coffee Break |
| **16:00** | | S. Kenney | R. Kuppers | J. Yewdell **Gained in Translation: Generation of MHC Class I Peptide Ligands from Biosynthesized Viral Proteins** |
| 16:15 | | | | |
| **16:30** | | **Session 4:** Replication I | **Session 12:** Human Infection | |
| 16:45 | | | | |
| **17:00** | | | | **CLOSING** |
| 17:15 | | | | Buses for Banquet will leave from the Marriott Cambridge Hotel at 18:00hrs |
| **17:30** | Welcome Cocktail Reception | | **Poster Session II** Sessions 13–20 | |
| 17:45 | | | | |
| **18:00** | | Dinner | | |
| 18:15 | | | | |
| **18:30** | | | | Banquet Museum of Science |
| 19:00 | | | Free Time On Your Own | |
| **20:00** | | **Poster Session I** Sessions 5–8 | | |
| **22:00** | | | | |


# Final Program

**Saturday, July 9, 2006**
**Foyer Grand Ballroom (Level 2)**
**Arrival and Registration**
**13:30–17:30**

**Saturday, July 9, 2006**
**Room: Grand Ballroom (Level 2)**
**Welcome Cocktail Reception**
**17:30–19:00**

| Welcome | Sunday, July 9, 2006<br>08:15–08:30 | Grand Ballroom (Level 2) |
|---|---|---|

**Fred Wang**
Boston, MA (US)

| Session 1 | Sunday, July 9, 2006<br>08:30–10:00 | Grand Ballroom (Level 2) |
|---|---|---|

## Session 1: Latent Infection I

**Co-Chairs:** Lori Frappier
Jeff Sample

1.001 Negative Autoregulation by EBV EBNA-1 Protein is Mediated through a Posttranscriptional Mechanism
**M. Yoshioka**, M.M. Crum, J.T. Sample
Memphis, TN (USA)

1.002 Regulation of Epstein-Barr Virus Latency Type by the Chromatin Boundary Factor CTCF
**C.M. Chau**, X. Zhang, S.B. McMahon, P.M. Lieberman
Philadelphia, PA (USA)

1.003 Conceptual Promoter Analysis and Subsequent Validation Reveals a Role for EBNA1 in Enhancing the Expression of Angiogenic Factors in an NPC Cell Model
**J.D. O'Neil**, V.H.J. Wood, K.L. Date, C.W. Dawson, L.S. Young
Birmingham (United Kingdom)

1.004 EBNA-1 Binds the Promoters of Cellular Genes Whose Transcript Levels Are Altered in the Presence of Dominant Negative EBNA-1
**D. Vereide**, B. Sugden
Madison, WI (USA)

1.005 Functional Characterization of the EBNA1-USP7 Interaction
**F. Sarkari**, T.S. Alcaraz, Y. Sheng, V. Saridakis, C. Arrowsmith, L. Frappier
Toronto (Canada)

1.006 Identification of a Specific, Non-random Mechanism for the Partitioning of Epstein-Barr Virus Plasmids
**A. Nanbo**, B. Sugden
Madison, WI (USA)

10:00–10:30 Coffee Break

| Invited Presentation | Sunday, July 9, 2006<br>10:30–11:00 | Grand Ballroom (Level 2) |
|---|---|---|

**Chair:** Georg Bornkamm

Genetic Analysis of Epstein-Barr Virus Infection
**Henri J. Delecluse**
Heidelberg (Germany)

| Session 2 | Sunday, July 9, 2006<br>11:00–12:00 | Grand Ballroom (Level 2) |
|---|---|---|

## Session 2: Latent Infection II

**Co-Chairs** Paul Ling
Clare Sample

2.001 Epstein-Barr Virus Nuclear Protein EBNA3C is Required for Continuous Cell Cycle Progression and Growth Maintenance of Lymphoblastoid Cells
**S. Maruo**, Y. Wu, S. Ishikawa, T. Kanda, K. Takada
Sapporo (Japan)

2.002 EBNA3C and the Regulation of Mitosis
G.A. Parker, M. Leao, E. Anderton, P. Young, R.E. White, **M.J. Allday**
London (United Kingdom)

2.003 EBNA-3B and EBNA-3C Regulated Cellular Genes in Epstein-Barr Virus Immortalized Lymphoblastoid Cell Lines
**A. Chen**, B. Zhao, E. Kieff, J.C. Aster, F. Wang
Boston, MA (USA)

2.004 Epstein-Barr Virus EBNA-3C is Targeted to and Regulates Expression from the Bidirectional LMP-1/2B Promoter
C. Jimenez-Ramirez, A. Brooks, L. Plym Forshell, K. Iakimtchouk, B. Zhao, T. Fulgham, **C. Sample**
Memphis, TN (USA)

12:00–13:30 Lunch in Grand Ballroom/Level 2

| Tribute | Sunday, July 9, 2006<br>13:30–13:45 | Grand Ballroom (Level 2) |
|---|---|---|

**Tribute to John Ho and Dolly Huang**
Elliott Kieff & Alan Rickinson

| Invited Presentation | Sunday, July 9, 2006<br>13:45–14:15 | Grand Ballroom (Level 2) |
|---|---|---|

**Chair:** Nancy Raab-Traub

Sequence Variations and Functional Significance
**Yi-Xin Zeng**
Guangzhou (China)

| Session 3 | Sunday, July 9, 2006<br>14:15–15:30 | Grand Ballroom (Level 2) |
|---|---|---|

## Session 3: Malignancy

**Co-Chairs** Pierre Busson
Georg Tsao

3.001 Regulation of Tumor Suppressor-related Genes in Nasopharyngeal Carcinoma Cells
**C.T. Lin**
Taipei (Taiwan)

3.002 Bmi-1 is a Novel Molecular Marker of Nasopharyngeal Carcinoma Progression and Immortalizes Primary Human Nasopharyngeal Epithelial Cells
L.B. Song, **M.S. Zeng**, W.T. Liao, L. Zhang, G.S.W. Tsao, G. Dimri, V. Band, Y.X. Zeng
Guangzhou, Hong Kong (China); Evanston, IL (USA)

3.003 Dynamics of EBV-DNA Load in Nasopharyngeal (NP) Brushings and Changes in IgA VCA/EBNA1 Serology during Treatment of Nasopharyngeal Carcinoma
M. Adham, A. Harahap, G. Suhartati, R. Averdi, A.N. Kurniawan, A. Djumhana, S.A.M.W. Verkuijlen, I.B. Tan, S.J.C. Stevens, **J.M. Middeldorp**
Jakarta (Indonesia); Amsterdam (Netherlands)

3.004 High Serum Levels of Soluble CD40 Ligand in Undifferentiated Nasopharyngeal Carcinoma Patients are Associated with Distant Metastases and Lack of LMP-1 Expression at Presentation
L. Caggiari, M. Guidoboni, E. Vaccher, L. Barzan, G. Franchin, A. Gloghini, D. Martorelli, P. Zancai, M.T. Bortolin, M. Mazzucato, D. Serraino, A. Carbone, P. De Paoli, **R. Dolcetti**
Aviano, Milano, Pordenone (Italy)

| Session 3 continued | Sunday, July 9, 2006 14:15–15:30 | Grand Ballroom (Level 2) |
|---|---|---|

3.005 Survival of Epstein-Barr Virus-infected T Cells from TNFa-induced Apoptosis through Down-regulation of TNFR1 and Recruitment of TRADD by LMP1
**H.C. Chuang**, J.D. Lay, I.J. Su
Tainan, Taipei (Taiwan)

15:30–16:00 Coffee Break

| Invited Presentation | Sunday, July 9, 2006 16:00–16:30 | Grand Ballroom (Level 2) |
|---|---|---|

**Chair:** Sankar Swaminathan

Contribution of Lytic Viral Proteins to Epstein-Barr Virus Associated Malignancies
**Shannon Kenney**
Chapel Hill, NC (USA)

| Session 4 | Sunday, July 9, 2006 16:30–17:45 | Grand Ballroom (Level 2) |
|---|---|---|

## Session 4: Replication I

**Co-Chairs** Evelyne Manet
George Miller

4.001 Role of the Viral Protein Kinase BGLF4 in Epstein-Barr Virus Nuclear Egress
**E. Gershburg**, D. Scheswohl, S. Raffa, M.R. Torrisi, J.S. Pagano
Rome (Italy); Chapel Hill, NC (USA)

4.002 Systematic Identification of Cellular Signals Reactivating Kaposi's Sarcoma-Associated Herpesvirus
**F. Yu**, J.N. Harada, H.J. Brown, S. Lee, H. Deng, M.J. Song, T.T. Wu, J. Kato-Stankiewicz, C.G. Nelson, J. Vieira, F. Tamanoi, S.K. Chanda, R. Sun
Los Angeles, CA, San Diego, CA, SAn Diego, CA, Seattle, WA (USA)

4.003 The Plasma Cell Differentiation Factor, X-Box-binding Protein-1 (XBP-1), Activates Lytic Epstein-Barr Virus Gene Expression
P. Bhende, E. Holley-Guthrie, **S. Kenney**
Chapel Hill, NC (USA)

**Session 4** continued | **Sunday, July 9, 2006 16:30–17:45** | **Grand Ballroom (Level 2)**

4.004 Viral Trans-factors Essential for Expression of Late Genes in a Gammaherpesvirus
**T.T. Wu**, V. Arumugaswami, Q. Jia, D. Martinez-Guzman, H. Hana, T. Tran, L. Tong, N. Reyes, H. Deng, R. Sun
Los Angeles, CA (USA)

4.005 Characteristics of EBV Gene Dependence on SM Expression
Z. Han, E. Marendy, J. Yuan, M. Wang, J. Sample, **S. Swaminathan**
Gainesville, FL, Boston, MA, Memphis, TN (USA)

18:00–19:30 Dinner in Grand Ballroom/Level 2

**Poster Session I** | **Sunday, July 9, 2006 20:00–22:00**

**Poster Session I**

**Poster Area/Level 2:**
Session 5: Latent Infection
Session 6: Oncogenesis & Cell Cycle

**Poster Area/Level 3:**
Session 7: Signal Transduction
Session 8: Viral Replication

## Session 5: Latent Infection

5.001 B-Cells Infected with EBNA-3C Negative EBV Mutants Have a Limited Life Span
C. Popp, A. Altmann, B. Neuhierl, W. Hammerschmidt, **B. Kempkes**
Munich (Germany)

5.002 Origin Recognition Complex Binding to Telomere Repeat Factor 2 Stimulates OriP Replication
**Z. Deng**, C. Atanasiu, J. Norseen, P.M. Lieberman
Philadelphia, PA (USA)

5.003 Phosphorylation of the Nuclear Localization Signal of EBNA-1 Up- and Down Regulates Its Nuclear Import Mediated by Importin Alpha5
R. Kitamura, T. Sekimoto, S. Ito, S. Harada, Y. Yoneda, **K. Yanagi**
Osaka, Tokyo (Japan)

5.004 Silencing of the Epstein-Barr Virus (EBV) Gene *LMP2B* Decreases Induction of Lytic EBV in Burkitt's Lymphoma Cells
**M.P. Rechsteiner**, D. Nadal, C. Berger, M. Bernasconi
Zurich (Switzerland)

5.005 Identification of Proteins Associated with the EBV-encoded Nuclear Antigen-5 in the Cell
**A. Stromberg**, U. Rüetschi, L. Rymo
Gothenburg (Sweden)

5.006 Gene Expression Profiling of LMP1-expressing Epithelial Cells Reveals Alterations Characteristic of Hyperkeratotic or Inflamed Epithelium
**J.R. Arrand**, M. Morris, W.B. Wei, C.W. Dawson, L.S. Young
Birmingham, UK (United Kingdom)

5.007 The Expression Profile of Epstein-Barr Virus-Encoded MicroRNAs in Akata Cells
K.H. Lee, D.N. Kim, J.M. Lee, S.K. Lee, **W.K. Lee**
Seoul, Yongin (Republic of Korea)

5.008 Increased B-cellular Infection with EBV Using a Novel Protocol: Improvement for Studies on Host Gene Expression and Cell Transformation
**M. Dorner**, F. Zucol Fröhlich, R.F. Speck, **D. Nadal**
Zurich (Switzerland)

5.009 Variable Methylation and Efficiencies in Splicing of Intron 1 Modulate LMP2A Expression Levels
**R.S. Scott**, C.A. Moody, E.T. Anye, J.W. Sixbey
Shreveport, LA (USA)

5.010 EBNA2-independent Induction of EBV-encoded LMP1 by CD40-ligand, IL-4 and IL-13: Involvement of Cytokines in the Expression of Type II Latency
**L.L. Kis**, N. Nagy, M. Takahara, K. Ferev, G. Klein, E. Klein
Asahikawa (Japan); Stockholm (Sweden)

5.011 EBNA2 Interferes with Germinal Center Phenotype by Downregulating Bcl6 and Tcl1
F. Boccellato, P. Rosato, E. Anastasiadou, B. Kempkes, L. Frati, **A. Faggioni**, P. Trivedi
Munich (Germany); Rome (Italy)

5.012 Genetic Analysis of EBNA3C Using an EBV Bacterial Artificial Chromosome
**C.Y. Lai**, E. Johannsen, M. Calderwood, S. Sakakibara, A. Holthaus, E. Kieff
Boston, MA (USA)

5.013 The BZLF1 Gene is Expressed as an Immediate Early Gene Following Primary Infection of B-lymphocytes
**K. Takada**, W. Wen, D. Iwakiri, K. Yamamoto, S. Maruo, T. Kanda
Sapporo (Japan)

5.014 Expression of Viral miRNAs during Primary Infection of Epstein-Barr Virus in B Cells
J.H. Lee, K.H. Lee, D.N. Kim, S.K. Lee, W.K. Lee, **J.M. Lee**
Seoul, Yongin (Republic of Korea)

5.015 EBNA1 Can Distribute Replicated 'Sister Viral Molecules' Evenly to Sister Chromatids
**T. Kanda**, M. Kamiya, S. Maruo, K. Takada
Sapporo (Japan)

5.016 OCTs and Grg/TLEs Compete with EBNA 1 in Regulation of FR - Implications for Switching between Cp and Qp
J. Zou, J. Almqvist, M. Werner, C. Boreström, S. Pettersson, E. Aurell, L. Rymo, **I. Ernberg**
Gothenburg, Stockholm (Sweden)

5.017 A Regulatory Loop Between Kaposi's Sarcoma-Associated Herpesvirus Replication and Transcription Activator (RTA) and Epstein-Barr Virus Latent Membrane Protein 1 (LMP-1)
D. Xu, J. Zhang, T. Coleman, A. Fagot, K. Kotalik, L. Zhao, C. Jones, **L. Zhang**
Saint Louis, MO, Lincoln, NE (USA)

5.018 Raji EBV's Alternate Origin: A Paradigm for Mammalian ARSs?
**C. Wang**, B. Sugden
Madison, WI (USA)

5.019 Intracellular Localization of Ribosomal Protein L22 in EBV-infected Cells May Be Determined by Competition Between RNA Ligands
**J.L. Houmani**, I.K. Ruf
Irvine, CA (USA)

5.020 Cell Contact Mediated Regulation of LMP-1 Expression in EBNA-2 Negative Cell Lines. Relevance to the EBV Encoded Protein Expression in Type II Tumors
**E. Klein**, L.L. Kis, M. Takahara, N. Nagy, H. Ishii
Stockholm (Sweden)

5.021 Epstein-Barr Virus Non-coding RNAs are Confined to the Nucleus and do not Undergo Nucleocytoplasmic Shuttling
**C.C.V. Fok**, J.K. Friend, J.A. Steitz
New Haven, CT (USA)

## Session 6: Oncogenesis & Cell Cycle

6.001 Inhibitory Effects of Epstein-Barr Virus BARF1 Gene on the Growth of Macaque Cells at Preimmortalized Stage
**Y. Shimizu**, T. Ishida, T. Ooka
Lyon (France); Tokyo (Japan)

6.002 An Evaluation of the Contribution of EBNA-1 Expression to Transgenic Mouse Lymphomagenesis
P. Tsimbouri, Y. Al-Sheikh, A. Mclean, M. Drotar, **J.B. Wilson**
Glasgow (Scotland, United Kingdom)

6.003 EBNA-5 Binds to MDM2 and Decreases the Half-Life of p53
A. Savchenko, M. Yurchenko, J. Dinga, G. Klein, **E. Kashuba**
Stockholm (Sweden); Kyiv (Ukraine)

6.004 EBV-encoded EBNA-6 Protein Binds to MRS18-2 and Controls pRb-E2F Association
M. Yurchenko, B. Snopok, L. Szekely, G. Klein, **E. Kashuba**
Stockholm (Sweden); Kyiv (Ukraine)

6.005 hTERT Contributes to the Establishment of EBV Latency In B Lymphocytes by Inhibiting Viral Replication and Promoting Cell Proliferation
**R. Dolcetti**, L. Terrin, I. Corradini, S. Indraccolo, J. Dal Col, R. Bertorelle, L. Bonaldi, G. Esposito, A. De Rossi
Aviano, Padova (Italy)

6.006 The Epstein-Barr Virus Nuclear Antigen (EBNA) -3C Interacts with Homeodomain Interacting Protein Kinase 2 (HIPK2)
**A.G. Burgess**, M. Buck, T. Hofmann, T.B. Sculley
Brisbane (Australia); Heidelberg (Germany)

6.007 LMP1 Induces an Epithelial Mesenchymal Transition (EMT) in MDCK Epithelial Cells via Activation of the ERK-MAPK and PI3-K Pathways
**C.W. Dawson**, L. Laverick, S. Beighton, M.A. Chidgey, L.S. Young
Birmingham, UK (United Kingdom)

6.008 Epstein-Barr Virus Nuclear Protein EBNALP is Critical for Maintaining Lymphoblastoid Cell Line Growth
**S. Harada**
Tokyo (Japan)

6.009 Upregulation of the Novel Cell-cycle Regulatory Gene RGC-32 by EBNA 3C Disrupts Cell-cycle Checkpoints
**A. Gunnell**, H.M. Webb, S.N. Schlick, M.J. West
Brighton (United Kingdom)

6.010 Crystal Structure of the BARF1 Oncogene
**N. Tarbouriech**, F. Ruggiero, M. De Turenne-Tessier, T. Ooka, W. Burmeister
Grenoble, Lyon (France)

6.011 The Placental Specific Gene, PLAC1, is Induced by the Epstein Barr Virus and is Expressed in Human Tumor Cells
**Q. Yin**, M. Fant, E. Flemington
New Orleans, LA, Houston, TX (USA)

6.012 The Epstein-Barr Virus (EBV) Encoded Gene BDLF2 is an Early Gene Encoding a Membrane Associated Protein Essential for the Immortalization of B-lymphocytes
**M. Kleines**, K. Schellenberg, K. Ritter, S. Scheithauer
Aachen (Germany)

6.013 Analysis of EBV-mediated Resistance to Cytotoxic Agents in BI Cells Using Recombinant EBNA3-knockout Viruses
**E. Anderton**, J. Yee, R.E. White, M.J. Allday
London (United Kingdom)

6.014 Long-term Retention of Defective Het DNA with Concurrent Loss of Prototype EBV Genomes Upon Infection of Cultured Epithelial Cells
M. Ding, R.S. Scott, **K. Ikuta**, J.W. Sixbey
Shreveport, LA (USA)

6.015 Proliferative Versus Cytostatic Effect of EBV LMP1 is Linked to Reiteration of Terminal Repeat Sequences
**A.M. Repic**, R.S. Scott, J.W. Sixbey
Shreveport, LA (USA)

6.016 A Technical Approach to the Reevaluation of Epstein-Barr Virus Clonality
**K. Ikuta**, R.S. Scott, J.W. Sixbey
Shreveport, LA (USA)

6.017 A Proteomic Investigation Into the Role of LMP1Cao in the Early Stages of Carcinogenesis
**A. McLean**, J. Wilson
Glasgow (Scotland, United Kingdom)

6.018 EBER1, Oncogenic in Vivo?
**C.E. Repellin**, J.B. Wilson
Glasgow (Scotland, United Kingdom)

6.019 Epstein-Barr Virus (EBV) Latent Membrane Protein-1 (LMP-1) Oncogenic Activity is Down-Regulated by Lytic LMP-1 (LyLMP-1)
**J. Pandya**, D. Walling
Galveston, TX (USA)

6.020 EBNA3C Disrupts the G2 Phase of Cell Cycle by Interacting with Chk2 in the ATM/ATR Pathway
**T. Choudhuri**, S.C. Verma, K. Lan, E.S. Robertson
Philadelphia, PA (USA)

6.021 Proteomic Analysis of B Lymphocytes Expressing Latent Membrane Protein-1
**J. Cameron**, M. Baddoo, Q. Yin, E. Flemington
New Orleans, LA (USA)

6.022 Increase of Genomic Instability by EBV DNase in Human Epithelial Cells and B Lymphocytes
M.T. Liu, **C.C. Wu**, Y.T. Chang, Y.C. Chuang, C.Y. Fang, Y. Chang, C.H. Tsai, K. Takada, J.Y. Chen
Taipei (Taiwan)

6.023 (cancelled) LMP1, LMP2A and CD40 Are All Essential for EBV-associated Lymphoma Cell Survival
I. Guasparri, **E. Cesarman**
New York, NY (USA)

## Session 7: Signal Transduction

7.001 BS69, a Specific Adaptor in the Latent Membrane Protein 1-Mediated c-Jun N-Terminal Kinase Pathway
**W. Zhang**
Hongkong (China)

7.002 The Putative Leucine Heptad Motif in the First Membrane-spanning Domain of LMP1 is Important for LMP1's Trafficking and Signaling
**J. Lee**, B. Sugden
Madison, WI (USA)

7.003 Phosphorylation and Nuclear Translocation of STAT3 Regulated by EBV-LMP1
Y.P. Liu, **Y. Cao**
Chang Sha (China)

7.004 Blockade of AP-1 Activity by Dominant-negative TAM67 Can Abrogate the Oncogenic Phenotype in Latent Membrane Protein 1-positive Human Nasopharyngeal Carcinoma
X. Jin, **Y. Cao**
Chang Sha (China)

7.005 Identification of Novel Phosphoproteins in Signaling Pathways Triggered by Latent Membrane Protein 1 Using Functional Proteomics Technology
G.R. Yan, **Y. Cao**
Chang Sha (China)

7.006 Induction of EBV Lytic Cycle Alters the Program of Cellular Gene Regulation Stimulated Following Ligation of Surface Immunoglobulin
P. Broderick, M. Hubank, **A.J. Sinclair**
Brighton, London (United Kingdom)

7.007 Zta Physically Interacts with the Cellular DNA-Damage Response Protein 53BP1 In Vivo
**S.G. Yarranton**, M. Meyer, A. Doherty, A.J. Sinclair
Brighton (United Kingdom)

7.008 The Effect of MAP Kinase Pathway on p53 Phosphorylation Triggered by Epstein-Barr Virus Latent Membrane Protein 1
**L.L. Li**, Y.G. Tao, G.R. Yan, M. Ye, H. Zheng, M. Tang, S.W. Tsao, Y. Cao
Changsha, Hunan, Hong Kong (China)

7.009 Transforming Growth Factor-Beta 1 Stimulates Epstein-Barr Virus Reactivation by Mechanisms which Require ERK1/2 MAPK and NF-kB Pathways
**V. Ramirez**, W. Zhang, C. Cochet, H. Arbach, A. Mauviel, R. Sierra, I. Joab
San José (Costa Rica); Paris (France)

7.010 LMP2A and LMP2B Modulate Interferon Signalling in Human Epithelial Cells Through Selective Targeting of p48, a Component of the ISGF3 Complex
**K.M. Shah**, S.E. Stewart, C.W. Dawson, L.S. Young
Birmingham (United Kingdom)

7.011 EBV Latency III immortalization Program Sensitizes B-Cells to Induction of CD95 Mediated Apoptosis via LMP1: Role of NF-κ, STAT1 and p53
**C. Le Clorennec**, I. Youlyouz-Marfak, E. Adriaenssens, J. Coll, G.W. Bornkamm, J. Feuillard
Lille, Limoges (France); Munich (Germany)

7.012 Myc Impairs Interferon Induction in EBV Infected B Cells by Downregulating IRF7
**G.W. Bornkamm**
Munich (Germany)

7.013 EBV Induces a Unique Form of DNA Bound STAT1
**J.E. McLaren**, M. Rowe, P. Brennan
Birmingham, Cardiff (United Kingdom)

7.014 High Physiological Levels of LMP1 Activate PERK to Induce Phosphorylation of eIF2alpha: Is This How LMP1 Regulates Itself?
**D. Lee**, B. Sugden
Madison, WI (USA)

7.015 LMP1 Strain Variants: Biologic and Molecular Properties
**B. Mainou**, N. Raab-Traub
Chapel Hill, NC (USA)

7.016 The Deubiquitinating Enzyme UCH L1 Mediates Activation of Beta-catenin by EBV
**W. Yue**, J. Shackelford, J.S. Pagano
Chapel Hill, NC (USA)

7.017 Phosphatidylinositol 3-kinase/Akt Pathway Cell Growth and Lytic Cycle of Epstein-Barr Virus in the Burkitt's Lymphoma Cell Line, P3HR-1
**T. Sairenji**, T. Mori
Yonago (Japan)

7.018 Overexpression of Epstein-Barr Virus Latent Membrane Protein 1 (LMP1) in EBV Latency III B-cells Induces Caspase 8 Mediated Apoptosis: Role of CD95
**C. Le Clorennec**, T.S. Ouk, C. Jayat-Vignolles, I. Youlyouz-Marfak, E. Adriaenssens, J. Coll, G.W. Bornkamm, J. Feuillard
Lille, Limoges (France); Munich (Germany)

7.019 LMP2a Induction of Epithelial Cell Invasion by Recruitment of Syk
**F. Chen**, G. Gish, R. Ingham, L.F. Hu, C. Lim, L. Matskova, G. Winberg, T. Pawson, I. Ernberg
Toronto, Victoria (Canada); Stockholm (Sweden)

7.020 Epstein-Barr Virus Latent Membrane Protein 1 C-Terminal Sites 1 and 2 Activate NF-kB through TRAF2/NIK/IKKa/p52 and TRAF6/TAK1/Another Kinase Respectively
**V. Soni**, Y.J. Song, E. Cahir-McFarland, M.S. Kang, E. Kieff
Boston, MA (USA)

7.021 Role of TRAF3 in EBV LMP1 Signaling and B Lymphocyte Development
**T. Yasui**, E. Kieff, H. Kikutani
Osaka (Japan); Boston, MA (USA)

7.022 The EBV Oncoprotein Latent Membrane Protein 1 Affects the Expression of Suppressors of Cytokine Signaling in Transformed B Lymphocytes
**D. Kube**, D. Pinkert, A. Kieser, M. Vockerodt
Göttingen, Munich (Germany)

7.023 RIP Mediates LMP1 Ubiquitination and Activation of IRF7
L. Huye, **S. Ning**, J. Pagano
Chapel Hill, NC (USA)

7.024 EBV LMP1 Upregulates HIF1Alpha through Siah1 E3 Ligase-mediated Down-regulation of Prolyl Hydroxylases 1 and 3
S. Kondo, S.Y. Seo, K.L. Jang, **J. Pagano**
Busan (Republic of Korea); Chapel Hill, NC (USA)

7.025 LMP2B Modulates LMP2A Signaling
**M. Rovedo**, R. Longnecker
Chicago, IL (USA)

## Session 8: Viral Replication

8.001 The Epstein-Barr Virus BNRF1 Protein Allows Efficient Transfer from the Endosomal Compartment to the Nucleus of Primary B Lymphocytes
**R. Feederle**, B. Neuhierl, G. Baldwin, H. Bannert, J. Mautner, H.J. Delecluse
Heidelberg, Munich (Germany); Birmingham (United Kingdom)

8.002 Inter-Regulation of Zta, Rta, and Egr-1 as a Positive-Feedback Network in Spontaneous Reactivation of Epstein-Barr Virus
**Y. Chang**, Y.T. Chen, H.H. Lee, S.Y. Wu, C.W. Chen, C.H. Tsai
Tainan, Taipei (Taiwan)

8.003 (cancelled) Down-Regulation of Cellular Proteolytic Systems Following Epstein Barr Virus Activation in Burkitt's Lymphoma Cells
G. Matusali, A. De Leo, L. Di Renzo, F. Cuozzo, M. D'Erme, **E. Mattia**
Rome (Italy)

8.004 An Essential Role for Zta Cysteine 171 in C/EBP Binding Site Recognition and Lytic Cycle Replication
**P. Wang**, L. Day, P.M. Lieberman
Philadelphia, PA (USA)

8.005 EBV BGLF4 Is a Virion Tegument Protein that Dissociates from Virions in a Phosphorylation Dependent Process and Phosphorylates BZLF1
R. Asai, K. Sugimoto, T. Sairenji, **Y. Kawaguchi**
Tokyo, Yonago (Japan)

8.006 The KSHV Lytic Gene ORF57 is Essential for Infectious Virion Production
Z. Han, **S. Swaminathan**
Gainesville, FL (USA)

8.007 Postreplicative Mismatch Repair Factors are Recruited to Epstein-Barr Virus Replication Compartments
**T. Tsurumi**, A. Kudoh, T. Daikoku
Nagoya (Japan)

8.008 BZLF1 Induces Retinoic Acid Production in Epithelial Cells by Enhancing the Conversion of Retinol Into Retinoic Acid
**R. Jones**, I. Sandoval, P. Bhende, D. Jones, H. Delecluse, S. Kenney
Heidelberg, (Germany); Chapel Hill, NC, Salt Lake City, UTAH (USA)

8.009 Phosphorylation of MCM4 at Sites Inactivating DNA Helicase Activity of the MCM4-6-7 Complex during Epstein-Barr Virus Productive Replication
**A. Kudoh**, T. Tsurumi
Nagoya (Japan)

8.010 Phosphorylation of the EEBV ZEBRA Protein at S173 in the Regulatory Domain is Required for Viral Lytic DNA Replication but not for Transcriptional Activation of Viral Early Genes
**A. El-Guindy**, L. Heston, H.J. Delecluse, G. Miller
Heidelberg (Germany); New Haven, CT (USA)

8.011 Characterization of Binding of Soluble gH to Epithelial Cells
**L.S. Chesnokova**, A.J. Morgan, L.M. Hutt-Fletcher
Bristol (United Kingdom); Shreveport, LA (USA)

8.012 Characterisation of a Docking Site for Protein Kinase Ii (CK2) in the EBV mRNA Export Factor, EB2
C. Medina-Palazon, O. Filhol, C. Cochet, A. Sergeant, **E. Manet**
Grenoble, Lyon (France)

8.013 Characterization of Two New EBV Producing Cell Lines
**R. Bagni**, P. Tuma, M. Carrington, K. Nagashima, D. Dittmer, B. Ortiz-Conde, F. Ruscetti, D. Whitby
Washington, DC, Frederick, MD, Chapel Hill, NC (USA)

8.014 Accumulation of EBV BMRF1 Protein EA-D in EBV Reactivated Raji Cells
**M. Ohashi**, Y. Hoshikawa, K. Nagata, M. Osaki, H. Ito, T. Sairenji
Yonago (Japan)

8.015 Characterization of EBV BKRF3 Product
C.C. Lu, H.T. Huang, J.T. Wang, M.C. Wu, **M.R. Chen**
Taipei (Taiwan)

8.016 Identification of EBV-Lytic-Cycle-Associated MicroRNAs
**Z. Lin**, E. Flemington
New Orleans, LA (USA)

8.017 Bortezomib: Most Potent Inducer of Lytic Infection in FDA Drug Library
**J. Chen**, D. Fu, C. Chong, J. Liu, W. Hsieh, M. Lemas, R. Ambinder
Singapore (Singapore); Baltimore, MD (USA)

| Invited Presentation | Monday, July 10, 2006<br>08:30–09:00 | Grand Ballroom (Level 2) |
|---|---|---|

**Chair:** Lindsey Hutt-Fletcher

How to Enter Cells: Lessons from Epstein-Barr Virus
**Richard Longnecker**
Chicago, IL (USA)

| Session 9 | Monday, July 10, 2006<br>09:00–10:00 | Grand Ballroom (Level 2) |
|---|---|---|

## Session 9: Replication II

**Co-Chairs:** Paul Farrell
Paul Lieberman

9.001 Amino Acids in the Basic Domain of Epstein-Barr Virus ZEBRA Protein Play Distinct Roles in Binding DNA, Activating Early Lytic Gene Expression and Promoting Viral DNA Replication
L. Heston, A. El-Guindy, J. Countryman, C. Dela Cruz, H.J. Delecluse, **G. Miller**
Heidelberg (Germany); New Haven, CT (USA)

9.002 Region Adjacent to Zta bZIP (CT Region) Contributes to the Specificity of DNA Binding in Addition to Extending Dimerisation Interface
**C. Schelcher**, S. Al Mehairi, E. Verrall, M.J. West, A.J. Sinclair
Brighton (United Kingdom)

9.003 Structural Basis of the Preferential Recognition of Methylated DNA Target Sites by the EBV Lytic Switch Protein ZEBRA
**C. Petosa**, P. Morand, P. Pagniez, M. Perrissin, F. Baudin, C. Mueller
Grenoble (France)

9.004 Binding of ZEB1 to ZV of the BZLF1 Promoter Plays Central Roles in Maintenance of Latency and Reactivation of EBV
**X. Yu**, Z. Wang, J.E. Mertz
Madison, WI (USA)

10:00–10:30 Coffee Break

| Invited Presentation | Monday, July 10, 2006<br>10:30–11:00 | Grand Ballroom (Level 2) |
|---|---|---|

**Chair**: Martin Allday

EBV Persistence and Oncogenesis: Viral Modulation of Cytokine and Cell Signalling Pathways
**Lawrence S. Young**
Birmingham (United Kingdom)

| Session 10 | Monday, July 10, 2006<br>11:00–12:00 | Grand Ballroom (Level 2) |
|---|---|---|

## Session 10: Latent Infection III

**Co-Chairs:** Ih-Jen Su
Jaap Middeldorp

10.001 LMP1 Transgenic Mice Develop Germinal Center Lymphomas that are IL4-Independent
**K.H.Y. Shair**, K.M. Bendt, E.C. Bedford, N. Raab-Traub
Chapel Hill, NC (USA)

10.002 LMP2A Confers Resistance to Anoikis Through ERK Activation in Epithelial Cells
**D. Iwakiri**, M. Samanta, S. Maruo, H. Yoshiyama, K. Takada
Sapporo (Japan)

10.003 Epstein-Barr Virus Protein Interaction Networks
**M.A. Calderwood**, K. Venkatesan, L. Xing, M.R. Chase, A. Holthaus, S. Ewence, N. Li, T. Hirozane-Kishikawa, D.E. Hill, M. Vidal, E. Kieff, E.C. Johannsen
Boston, MA (USA)

10.004 Epstein-Barr Virus EBER2 Can Prevent a Stress Response in Transfected Human Cells
S. Pattle, **G. MacArthur**, P.J. Farrell
London (United Kingdom)

12:00–13:30 Lunch in Grand Ballroom/Level 2

12:00–13:30 **EBV Board Meeting for all Board Members**
(Upstairs at the MC2 Restaurant/Level 2)

| Invited Presentation | Monday, July 10, 2006<br>13:30–14:30 | Grand Ballroom (Level 2) |
|---|---|---|

**Chair:** Alan Rickinson

**HENLE LECTURE:**
Are We Reaching a Clinical Application Era After 40 Years of Basic EBV Research?
**Denis Moss**
Herston (Australia)

| Session 11 | Monday, July 10, 2006<br>14:30–15:30 | Grand Ballroom (Level 2) |
|---|---|---|

## Session 11: Therapy

**Co-Chair:** John Sixbey
Kenzo Takada

11.001 Targeting Dendritic Cells for Vaccination Against EBV-Associated Tumors
**C. Gurer**, C. Munz
New York, NY (USA)

11.002 Treatment of Epstein Barr Virus Positive Nasopharyngeal Carcinoma with Adoptively Transferred Cytotoxic T cells
C.U. Louis, K. Straathof, V. Torrano, C.M. Bollard, A.M. Leen, M.H. Huls, M.V. Gresik, H. Weiss, A. Gee, M.K. Brenner, C.M. Rooney, H.E. Heslop, **S. Gottschalk**
Houston, TX (USA)

11.003 Lytic Cycle Gene Activation for Targeted Radiotherapy
**D. Fu**, J. Chen, M. Pomper, R. Ambinder
Baltimore, MD (USA)

11.004 Epstein Barr Virus-Specific Cytotoxic T Lymphocytes Expressing an Anti-CD30 Chimeric T Cell Receptor (cTcR) for the Treatment of Hodgkin's Disease.
**B. Savoldo**, C. Rooney, H. Heslop, H. Abken, A. Hombach, L. Zhang, M. Pule, M. Brenner, G. Dotti
Cologne (Germany); Houston, TX (USA)

15:30–16:00 Coffee Break

| Invited Presentation | Monday, July 10, 2006<br>16:00–16:30 | Grand Ballroom (Level 2) |
|---|---|---|

**Chair:** Jeffrey Cohen

Aspects of Hodgkin Lymphoma Pathogenesis and the Biology of EBV
**Ralf Kuppers**
Duisburg (Germany)

| Session 12 | Monday, July 10, 2006<br>16:30–17:30 | Grand Ballroom (Level 2) |
|---|---|---|

## Session 12: Human Infection

**Co-Chairs:** Tom Sculley
Riccardo Dolcetti

12.001 Expression of LAG-3 by Tumor-infiltrating Lymphocytes is Co-incident with the Suppression of Latent Membrane Antigen-specific CD8+ T-Cell Function in Hodgkin Lymphoma Patients
**M.K. Gandhi**, E. Lambley, J. Duraiswamy, U. Dua, C. Smith, S. Elliott, D. Gill, P. Marlton, J.F. Seymour, R. Khanna
Brisbane (Australia)

12.002 BMI-1 is Induced by LMP1 and Regulates Cellular Gene Expression and Survival in Hodgkin's Lymphoma Cells
**A. Dutton**, M.B. Chukwuma, J.I.K. Last, W. Wei, M. Vockerodt, S. Morgan, C.B. Woodman, L.S. Young, P.G. Murray
Birmingham (United Kingdom)

12.003 Epstein-Barr Virus Shed in Saliva is High in the B Cell Tropic Glycoprotein gp42
**R. Jiang**, R.S. Scott, L.M. Hutt-Fletcher
Shreveport, LA (USA)

12.004 EBV Infection of Oral Mucosal Epithelium is Mediated by Monocytes and is Spread Unidirectionally Toward the Mucosal Surface
**S. Tugizov**, R. Herrera, P. Veluppillai, J. Greenspan, D. Greenspan, J. Palefsky
San Francisco, CA (USA)

**Poster Session II**

**Poster Area/Level 2:**
Session 13: PTLD & Other Malignancies
Session 14: Diagnostics, Vaccines, and Therapy
Session 15: Gene Regulation

**Poster Area/Level 3:**
Session 16: Immune Mechanisms
Session 17: Comparative Systems
Session 18: Human Infection
Session 19: Burkitt & Hodgkin Lymphoma
Session 20: Nasopharyngeal Carcinoma

## Session 13: PTLD and Other Malignancies

13.001 Persistently Elevated Cell-Associated EBV DNA Loads, Restricted EBV Transcription and Presence of Anti-EBV IgA in the Circulation of Asymptomatic HIV-Carriers
S.J.C. Stevens, P.H.M. Smits, S.A.M.W. Verkuijlen, D. Rockx, J.W. Mulder, **J.M. Middeldorp**
Amsterdam (Netherlands)

13.002 Differential Internal Translation Efficiency of Epstein-Barr Virus Present in Solid Organ Recipients
**A. Isaksson**, M. Berggren, A. Ricksten
Gothenburg (Sweden)

13.003 Expression of Interleukin 9 in The EBV-positive Nasal Natural Killer (NK)/T-Cell Lymphoma Cell Lines and Patients
**K. Kishibe**, T. Nagato, H. Kobayashi, M. Takahara, N. Bandoh, T. Ogino, H. Ishii, N. Shimizu, M. Tateno, Y. Harabuchi
Asahikawa, Tokyo (Japan)

13.004 EBV Status in an Organ Transplant Patient with Ulterior Autoimmune Disease
**M. Berggren**, Isaksson, F. Nilsson, A. Ricksten
Gothenburg (Sweden)

13.005 Variable EBV DNA Load Distribution and EBV RNA Expression in the Circulation of Stem Cell (SCT) and Solid Organ Transplant (SOT) Recipients: Consistent Expression of Barts
S.J.C. Stevens, S.A.M.W. Verkuijlen, E.A.M. Verschuuren, J.J. Cornelissen, **J.M. Middeldorp**
Amsterdam, Groningen, Rotterdam (Netherlands)

13.006 Concomitant Increase of LMP1 and CD25 Expression Induced by IL-10 in the EBV-positive NK Lines SNK6 and KAI3
**M. Takahara**, L.L. Kis, N. Nagy, A. Liu, Y. Harabuchi, E. Klein
Asahikawa (Japan); Stockholm (Sweden)

13.007 Association of Serum Epstein-Barr Virus DNA with Clinical Course of Nasal NK/T-cell Lymphoma Patients
**Y. Harabuchi**, H. Ishii, S. Moriai, M. Takahara, T. Ogino
Asahikawa (Japan)

13.008 Characterization of an EBV-Positive Intestinal Smooth Muscle Tumor Occurring Synchronously with Posttransplant Lymphoproliferative Disease in a Renal Transplant Patient
**C. Alfieri**, A.L. Rougemont, E. Papp, R. Fetni, I. Gorska, J. Champagne, V. Phan, J.C. Fournet, H. Sartelet
Montreal (Canada)

13.009 Tumorigenesis of Epstein-Barr Virus (EBV) Positive Gastric Carcinoma Cell Line, SNU-719 in Nude Mouse
**S.T. Oh**, J.H. Cha, D.J. Shin, S.K. Yoon, S.K. Lee
Seoul (Republic of Korea)

13.010 Viral microRNA Expression in EBV-associated Gastric Carcinoma
D.N. Kim, S.T. Oh, J.H. Kang, H.S. Chae, K. Takada, J.M. Lee, W.K. Lee, **S.K. Lee**
Sapporo (Japan); Seoul, Yongin, Kyunggi-do (Republic of Korea)

13.011 EBV-DNA Survey in Saliva for Early Diagnosis in Solid Tumour Patients After Allogeneic Stem Cell Transplantation
**A. Mollbrink**, K.I. Falk, A. Linde, L. Barkholt
Stockholm (Sweden)

13.012 The XLP Gene SAP is Involved in the Control of Cell Cycle/Apoptosis
**N. Nagy**, M. Takahara, L.L. Kis, G. Klein, E. Klein
Stockholm (Sweden)

13.013 Induction of Distinct Gene Expression Patterns by the BARF1 Gene of Epstein-Barr Virus in Epithelial and Lymphoid Cells
**T. Heidt**, T. Wiech, E. Nikolopoulos, S. Lassmann, M. Sarbia, A. Walch, M. Werner, T. Ooka, A. zur Hausen
Lyon (France); Freiburg, Munich (Germany)

13.014 CGH DNA Microarray Analysis as a Tool to Identify Distinct Aberrations Between EBV-Associated and EBV-Negative Gastric Carcinomas
**T. Heidt**, A. Eiserbeck, E. Nikolopoulos, T. Wiech, S. Lassmann, T. Keck, U. Hopt, M. Werner, A. zur Hausen
Freiburg (Germany)

## Session 14: Diagnostics, Vaccines, and Therapy

14.001 The *E. coli* Heat Labile Enterotoxin B-Subunit Enhances CD8+ T-Cell Killing of Epithelial Tumour Cells Expressing EBV LMP2
**O. Salim**, A.D. Wilson, A.J. Morgan
Bristol (United Kingdom)

14.002 Expansion of Antigen-specific T Cells Following Stimulation with Adenoviral Polyepitope and in Vivo Modelling of Immunotherapy for EBV-associated Malignancies
**C. Smith**, M. Burgess, L. Cooper, M. Rist, N. Webb, E. Lambley, U. Dua, J. Seymour, M. Gandhi, R. Khanna
Brisbane, Melbourne (Australia)

14.003 Evaluation of a New Commercial Assay: the 'EBV R-Gene Quantification Kit'
**S. Fafi-Kremer**, G. Bargues, S. Magro, C. Barranger, J. Bes, P. Bourgeois, M. Joannes, P. Morand, J.M. Seigneurin
Grenoble, Strasbourg, Varilhes (France)

14.004 Extreme Elevations of Epstein-Barr Viral Load in Pediatric Transplant Recipients: Consequences for Monitoring of the Development of Post-Transplant Lymphoproliferative Disease
A. Schuster, M.J. Dechant, U. Bartram, J. Bauer, **H.J. Wagner**
Giessen, Luebeck (Germany)

14.005 Molecular and Serological Monitoring of Pediatric Patients with Nasopharyngeal Carcinoma Treated by the Protocol NPC-2003-GPOH of the German Society for Pediatric Oncology
F. Hermann, R. Mertens, **H.J. Wagner**
Aachen, Giessen (Germany)

14.006 A Mouse Monoclonal Antibody Against Epstein-barr Virus (EBV) Envelope Glycoprotein 350 Prevents EBV Infection Both in Vitro and in Vivo
T. Haque, **I. Johannessen**, D. Dombagoda, C. Sengupta, D.M. Burns, P. Bird, G. Hale, G. Mieli-Vergani, D.H. Crawford
Edinburgh, London, Oxford (United Kingdom)

14.007 The Anti-cancer Drug Flavopiridol Inhibits EBNA 2-activated Transcription through Specific Inhibition of CDK9
S.J. Bark-Jones, H.M. Webb, **M.J. West**
Brighton (United Kingdom)

14.008 'LCL-specific' CD4+ T Cells from EBV-immune and EBV-naïve Donors May Have Therapeutic Potential Against a Range of EBV-associated B Cell Lymphomas
**J. Zuo**, H.M. Long, N.H. Gudgeon, H. Jia, A.B. Rickinson
Birmingham (United Kingdom)

14.009 Valproic Acid Enhances the Efficacy of Chemotherapy in EBV-positive Tumors by Increasing Lytic Viral Gene Expression
**W.H. Feng**, S. Kenney
Chapel Hill, NC (USA)

14.010 Re-Targeting Cytotoxic T Lymphocytes to LMP2 for Immunotherapy of EBV-associated Tumours
**A.M. Swanson**, I. Johannessen, D.H. Crawford
Edinburgh (United Kingdom)

14.011 Successful Stem Cell or Bone Marrow Transplantation for Severe Chronic Active Epstein-Barr Virus Disease in the United States
**J.I. Cohen**, J. Dale, E. Jaffe, S. Pittaluga, A. Marques, K. Rao, H. Heslop, C. Rooney, S. Gottschalk, M. Bishop, W. Wilson, S. Straus
Bethesda, MD, Houston, TX (USA)

14.012 A Latency-Null Gammaherpesvirus Generates Protective Immunity Independent of Latent Antigens
**Q. Jia**, I. McHardy, D. Martinez-guzman, S. Hwang, T. Rickabaugh, L. Tong, R. Sun
Los Angeles, CA (USA)

14.013 Efficient Gene Transduction of Unstimulated Normal B-lymphocytes Using an Epstein-Barr Virus-based Vector
**H. Yoshiyama**, T. Kanda, D. Iwakiri, K. Takada
Sapporo (Japan)

## Session 15: Gene Regulation

15.001 Dual Regulation of LMP1-Augmented Kappa Light Chain Expression in Nasopharyngeal Carcinoma Cells by NFκB and AP-1
**H.D. Liu**, H. Zheng, M. Li, Y.G. Tao, Z.X. Lu, D.S. Hu, Y. Cao
Changsha (China)

15.002 NF-κB Binds and Activates the LMP1 Promoter in B Cells
**P. Johansson**, A. Jansson, A. Sjöblom-Hallen, L. Rymo
Gothenburg (Sweden)

15.003 Characterization of a Cellular Protein-associated with the Q Promoter of Epstein-Barr Virus
**C.L. Liang**, P.J. Chung, Y.S. Chang
Taichung, Taoyuan (Taiwan); New York, NY (USA)

15.004 EBNA3A Induces the Expression of, and Interacts with, a Subset of Chaperones and Co-chaperones
**P. Young**, E. Anderton, M.J. Allday
London (United Kingdom)

15.005 Regulation of Transcription by EBNA3B
**P. Young**, M. Leao, M.J. Allday
London (United Kingdom)

15.006 HLARK/RBM4 Strongly Co-stimulates Gene Activation by the Epstein-Barr Virus Nuclear Antigen 2 (EBNA2) and its Cellular Counterpart Notch-IC
**T. Pfuhl**, A. Mamiani, E. Kremmer, T. Dobner, F.A. Grässer
Homburg/Saar, Munich, Regensburg (Germany)

15.007 DNA Mthylation Status of the EBV BamHI W Promoter and Promoter Activity in Novel EBV-positive Burkitt and Lymphoblastoid Cell Lines
I. Hutchings, **A.I. Bell**, G.L. Kelly, A.B. Rickinson
Birmingham (United Kingdom)

15.008 EBV Exploits the BSAP/Pax5 Cellular Transcription Factor to Ensure B Cell-specific Activation of Its Growth-transforming Programme
R. Tierney, J. Nagra, **A.I. Bell**, A.B. Rickinson
Birmingham (United Kingdom)

15.009 EBNA5 Enhances the Formation of Insoluble Protein Aggregates from Transiently Expressed Reporter Genes
**J. Ekholm**, L. Rymo
Gothenburg (Sweden)

15.010 BZLF1 Regulates the TNF-alpha Receptor Promoter through its Effects on C/EBPα and C/EBPβ
**J. Bristol**, T. Morrison, S. Kenney
Chapel Hill, NC (USA)

15.011 Decoy Receptor 3 Expression is Upregulated Upon EBV Reactivation
C.H. Ho, C.F. Hsu, P.F. Fong, **C.J. Chen**
Taipei (Taiwan)

15.012 Epstein-Barr Virus Nuclear Antigen 3C May Modulate Nm23-H1's Metastasis Suppression Potential
**M. Murakami**, K. Lan, E. Robertson
Philadelphia, PA (USA)

15.013 EBNA-LP Transcriptional Coactivation through Interaction with Histone Deacetylase 4
**D. Portal**, E. Kieff
Boston, MA (USA)

15.014 Downregulation of the Pro-apoptotic Nbk/Bik Gene During the EBV Growth Programme
E.M. Campion, S.T. Loughran, B.N. D'Souza, S. Phelan, B. Kempkes, G. Bornkamm, S.D. Hayward, **D. Walls**
Munich (Germany); Dublin (Ireland); Baltimore, MD (USA)

15.015 Biogenesis of EBV MicroRNAs and Regulation of BHRF1 Expression
**L. Xing**, E. Kieff
Boston, MA (USA)

15.016 Epstein-Barr Virus EBNA-3A Inhibits Differentiation of Muscle Cells
E. Stigger-Rosser, **C. Sample**
Memphis, TN (USA)

15.017 LMP2A Auto-regulates Its Expression Independent of EBNA2
**L. Anderson**, T. Portis, R. Longnecker
Chicago, IL (USA)

15.018 Regulation of Sp100 Intracellular Localization by EBNA-LP and Cellular Factors
**C. Echendu**, R.S. Peng, J. Tan, P.D. Ling
Houston, TX (USA)

## Session 16: Immune Mechanisms

16.001 Tonsillar Natural Killer Cells Restrict Epstein-Barr Virus-induced B Cell Transformation after Activation by DCs
**T. Strowig**, G. Bougras, F. Brilot, D. Thomas, W.A. Muller, C. Munz
New York, NY (USA)

16.002 Identification of Novel CD4+ T Cell Epitopes within Epstein-Barr Virus Proteins
**E.K. Vetsika**, M.F. Callan
London (United Kingdom)

16.003 LMP1 and LMP2 Epitope-Specific CD4+ T Cell Clones able to Recognise and Kill EBV Immortalised Lymphoblastoid Cell Lines (LCLs)
T.A. Haigh, X. Lin, E.P. Hui, A.T.C. Chan, A.B. Rickinson, **G.S. Taylor**
Hong Kong (China); Birmingham (United Kingdom)

16.004 Distinct Memory CD4+ T Cell Subsets Mediate Immune Recognition of Epstein Barr Virus Nuclear Antigen 1 in Healthy Virus Carriers
**K.N. Heller**, C. Munz
New York, NY (USA)

16.005 Epstein-Barr Virus Immediate-Early Protein RTA Negatively Regulates the Function of Interferon Regulatory Factors
R. Liu, A.M. Hahn, **S. Ning**, J. Shackelford, J.S. Pagano
Chapel Hill, NC (USA)

16.006 The Latent Membrane Protein 1 Primes EBV Latency Cells for Interferon Production: Implication for the Pathogenesis of Systemic Lupus erythematosus
D. Xu, K. Brumm, **L. Zhang**
Lincoln, NE (USA)

16.007 Nk Cells Produced IFN$\gamma$ and Potentiated T Cell Activation and Cytotoxic T Cell Generation in EBV-infected Cord Blood Cell Cultures
**A. Liu**, A. Holmgren, G. Klein, E. Klein
Stockholm (Sweden)

16.008 Control of Epstein-Barr Virus Infection in Vitro by T Helper Cells Specific for Virion Glycoproteins
**J. Mautner**
Munich (Germany)

## Session 17: Comparative Systems

17.001 Comparative Analysis of Cercopithicine Herpes Virus 15 Encoded Zta with EBV-encoded Zta
**Q. Hope**, M. Meyer, A.J. Sinclair
Brighton (United Kingdom)

17.002 Differential Translation Efficiency of EBNA-1 Encoded by Lymphocryptoviruses influences Endogenous Presentation of CD8+ T cell Epitopes
**J. Tellam**, G. Connolly, M. Rist, N. Webb, C. Fazou, F. Wang, R. Khanna
Brisbane (Australia); Boston, MA (USA)

17.003 MHV-68 Inoculation of Neonatal Mice Causes Persistent Infection and Inflammation of the CNS Mimicking Cerebral EBV Infection
**M. Kleines**, S. Scheithauer, B. Sellhaus, K. Ritter, M. Hausler
Aachen (Germany)

17.004 EBV Nuclear Antigens EBNA3C & EBNA1 Promote Metastasis and Can Overcome Metastasis Suppressor Effect of Nm23H1 in Nude Mice Model
**R. Kaul**, M. Murakami, T. Choudhuri, E.S. Robertson
Philadelphia, PA (USA)

17.005 Real-time Monitoring of Murine Gamma-Herpesvirus 68 Replication in vivo Using Molecular Imaging
**S. Hwang**, L.M. Tong, T.T. Wu, R. Sun
Los Angeles, CA (USA)

17.006 Phylogenetic Relationships and Geographic Distribution of the Japanese Macaque (Macaca fuscata) Lymphocryptovirus, Inferred from Glycoprotein B Sequences
**V. Saechan**, T. Ishida
Tokyo (Japan)

17.007 Rhesus Lymphocryptovirus Latent Membrane 2A Activates Beta-catenin Signaling and Inhibits Differentiation in Epithelial Cells
**C.A. Siler**, N. Raab-Traub
Chapel Hill, NC (USA)

## Session 18: Human Infection

18.001 Sequence Variations in the EBER Locus Do Not Correlate with the EBNA2 Type of Epstein-Barr Virus
K.B. Chang, Y.K. Jeon, M.K. Kim, **W.K. Lee**
Yongin (Republic of Korea)

18.002 Heterophile Antibodies Trigger Phagocytosis of Red Cells by Macrophages in Epstein-Barr Virus-Associated Hemophagocytic Syndrome
W.C. Hsieh, I.J. Su
Tainan (Taiwan)

18.003 Increased Expression of EBV Genes in Systemic Lupus erythematosus
**B.D. Poole**, E.J. Brown, J.M. Guthridge, J.B. Harley, J.A. James
Oklahoma City, OK (USA)

18.004 HERV-K18 Superantigen as a Potential Risk Factor in Multiple Sclerosis
A.K. Tai, **E.J. O'Reilly**, K. Alroy, K.L. Munger, B.T. Huber, A. Ascherio
Boston, MA (USA)

18.005 Tracking EBV Infection of B Cell Subsets in Vivo in Primary and Persistent Infection
**S. Chaganti**, A.I. Bell, A.B. Rickinson
Birmingham (United Kingdom)

18.006 CD8-mediated EBV-specific IFN-γ Responses Tend to be Suppressed While EBV Viral Loads are Increased after an Episode of Acute Clinical Malaria in Kenyan Children
**A.M. Moormann**, P.S. Ogolla, K. Chelimo, P.O. Sumba, D.J. Tisch, R.W. Novince, J.W. Kazura, R. Rochford
Kisumu (Kenya); Syracuse, NY, Cleveland, OH (USA)

18.007 Alterations in B Cell Subsets Following Acute Malaria in Children: Implications for EBV Persistence and the Etiology of Burkitt's Lymphoma
**R. Rochford**, A. Amolo, K. Chelimo, R. Ploutz-Snyder, A. Moormann
Kisumu (Kenya); Syracuse, NY, Cleveland, OH (USA)

18.008 Plasma Titers of Antibodies Against Epstein-Barr Virus BZLF1 and Risk of Multiple Sclerosis
**J. Massa**, K.L. Munger, E. O'Reilly, K.I. Falk, A. Ascherio
Solna (Sweden); Boston, MA (USA)

18.009 Multiple Sclerosis Patients Make a Unique Antibody Response to EBNA-1 Prior to the Diagnosis of MS
**L. Heinlen**, E. O'Reilly, R. May, K. Munger, J. Harley, J. James, A. Ascherio
Boston, MA, Oklahoma City, OK (USA)

18.010 Frequency and Phenotype of EBV-specific Cytotoxic T Lymphocytes during Intermittent EBV Reactivation in Healthy Individuals
**B. Vogl**, M. Larsen, A. Willemsen, N. Gudgeon, P. Schlenke, A. Hislop, A.B. Rickinson, **W.J. Jabs**
Luebeck (Germany); Birmingham (United Kingdom)

18.011 Comparison of Serological and Virological Parameters Between Infectious Mononucleosis and Asymptomatic Primary Epstein-Barr Virus Infection
**A.K.S. Chiang**, K.H. Chan
Hong Kong (China)

18.012 Mannose-binding Lectin Genotypes and Susceptibility to Epstein-Barr Virus Infection in Infancy
**J. Friborg**, R.F. Jarrett, A. Koch, P. Garred, J. Freeland, A. Andersen, M. Melbye
Copenhagen (Denmark); Glasgow (Scotland, United Kingdom)

## Session 19: Burkitt & Hodgkin Lymphoma

19.001 Specific Killing of EBV-positive Hodgkin`s Lymphoma Cells by a CD30 Promoter-dnEBNA1 Gene Therapy Vector
**G. Kapatai**, H. Parry, R.J. Jones, A.T.H. Burns, L.S. Young, P.G. Murray
Birmingham (United Kingdom)

19.002 Expression Profiling of Hodgkin's Lymphoma Identifies CCL20, an EBV Target that Modifies T Cell Recruitment to the Tumor Site
**K.R.N. Baumforth**, A. Birgersdotter, G.M. Reynolds, W.B. Wei, M. Vockerodt, C.B. Woodman, L.S. Young, I. Ernberg, P.G. Murray
Goettingen (Germany); Stockholm (Sweden); Birmingham (United Kingdom)

19.003 Three Restricted Forms of EBV Latency Counteracting Apoptosis and Affecting the Cellular Differentiation Status of c-myc Expressing Burkitt Lymphoma Cells
**G.L. Kelly**, A.E. Milner, G.S. Baldwin, A.I. Bell, P. Kellam, J. Rasaiyaah, J. Arrand, W. Wei, A.B. Rickinson
Birmingham, London (United Kingdom)

19.004 EBV Genome Loss from Endemic Burkitt Lymphoma Cell Lines and Its Effects on Cell Phenotype
**A. Boyce**, A. Bell, A. Rickinson, G. Kelly
Birmingham (United Kingdom)

19.005 Plasma Epstein-Barr Virus (EBV) DNA Is a Biomarker for EBV-Positive Hodgkin's Lymphoma
**M.K. Gandhi**, E. Lambley, J. Burrows, U. Dua, S. Elliott, P.J. Shaw, H.M. Prince, M. Wolf, C. Underhill, A. Mills, P. Marlton, P. Mollee, D. Gill, J.F. Seymour, R. Khanna
Brisbane, Melbourne, Sydney, Wodonga (Australia)

19.006 The EBV-encoded Latent Membrane Protein -1 Imposes on Germinal Centre B Cells, a Hodgkin Reed-Sternberg-like Gene Expression Signature
**M. Vockerodt**, S. Morgan, M. Kuo, K.R. Baumforth, J. Arrand, D. Kube, J. Gordon, W. Wei, L.S. Young, P.G. Murray
Goettingen (Germany); Birmingham (United Kingdom)

## Session 20: Nasopharyngeal Carcinoma

20.001 DNAzymes Targeted to EBV-encoded Latent Membrane Protein-1 Induce Apoptosis and Enhance Radiosensitivity in Nasopharyngeal Carcinoma
**Z.X. Lu**, L.Q. Sun, Y. Cao
Sydney (Australia); Chang Sha (China)

20.002 Immortalization of Nasopharyngeal Epithelial Cells and Their Applicatons in EBV Study
**S.W. Tsao**, C. Man, H.M. Li, Y.L. Yip, C.M. Tsang, K.W. Lo, A.K.F. Lo, Z.G. Wu, M.S. Zeng, Y.X. Zeng
Guangzhou, Hong Kong SAR (China); Baltimore (USA)

20.003 Regulation of EBV- Encoded Latent Membrane Protein1 Expression in Nasopharyngeal Carcinoma
D. Sun, D.N. Van, X.N. Zhang, I. Ernberg, **L.F. Hu**
Karolinska Institute (Sweden)

20.004 Nasopharyngeal Carcinoma in Children and Adolescents – A Multicenter Study
**R. Mertens**, B. Granzen, M. Zwaan, L. Lassay, P. Bucsky, A. Jessen, H.J. Wagner, G. Gademann, C.F. Hess
Aachen, Gießen, Göttingen, Lübeck, Magdeburg (Germany); Maastricht, Rotterdam (Netherlands)

20.005 Epstein-Barr Virus BART Gene Products
**M.A. Al-Mozaini**, G. Bodelon, C. Elgueta, B. Jin, M.N. Al-Ahdal, P.J. Farrell
Xi'an (China); Riyadh (Saudi Arabia); London (United Kingdom)

20.006 EBV Serology, not EBV-DNA Load, Predicting Distant Metastases in a Juvenile Caucasian NPC Patient: Response on EBV Lytic Cycle Induction Therapy
S.J.C. Stevens, C.M. Zwaan, S.A.M.W. Verkuijlen, **J.M. Middeldorp**
Amsterdam (Netherlands)

20.007 Sequence Variation and Functional Significance of the EBV-encoded Genes
D.J. Li, S.J. Mai, **Y.X. Zeng**
Guangzhou (China)

20.008 Antibodies to Gp350/220 Can Enhance Infection of a CR2-negative Epithelial Cell
S.M. Turk, R. Jiang, L.S. Chesnokova, **L.M. Hutt-Fletcher**
Shreveport, LA (USA)

20.009 Induction of Twist by Latent Membrane Protein-1 Causes Epithelial-Mesenchymal Transition and is Associated with Metastasis of Nasopharyngeal Carcinoma
**T. Horikawa**, J.S. Pagano
Chapel Hill, NC (USA)

20.010 Expression and Regulation of Discoidin Domain Receptor Gene Family in Nasopharyngeal Carcinoma
**C.H. Tsai**, H.H. Chua, T.S. Sheen
Taipei (Taiwan)

20.011 (cancelled) Functional Advantage of NPC-related V-val Subtype of Epstein-Barr Virus Nuclear Antigen 1 Compared with Prototype in Epithelial Cell Line
**S.J. Mai**, T. Ooka, D.J. Li, M.S. Zeng, R.C. Jiang, X.J. Yu, R.H. Zhang, S.P. Chen, Y.X. Zeng
Guang Zhou (China); Lyon (France)

20.012 Increased Intraepithelial Regulatory T-cells in Undifferentiated Nasopharyngeal Carcinoma
**J.W.Y. Hui**, K.F. To, M.H.L. Ng, K.M. Lau, A.S.H. Cheng, J.K.S. Woo, C.A. van Hasselt, K.W. Lo
Hong Kong (China)

20.013 Efficient EBV Infection of Epithelial Cells by Transfer from Resting B Cells: Monitoring Early Events Post-infection
**C.D. Shannon-Lowe**, H.J. Delecluse, A.B. Rickinson
Heidelberg (Germany); Birmingham (United Kingdom)

20.014 Consistent Overexpression of c-IAP2 Protects NPC Cells against an Unusual Form of Apoptosis
L. Friboulet, S. Rodriguez, C. Durieu, J. Bosq, A. Valent, L. Li, P. Harran, G. Tsao, K. Lo, **P. Busson**
Hong-Kong (China); Villejuif (France); Dallas, TX (USA)

20.015 (cancelled) Detection of Aberrant Methylation in Patients of Nasopharyngeal Cancer by Multiplex Methylation-Specific PCR
**S.H. Hutajulu**, D.N. Van, R. Susilowati, B. Hariwijanto, Harijadi, S.M. Haryana, I. Ernberg, L.F. Hu
Yogyakarta (Indonesia); Stockholm (Sweden); Hanoi (Vietnam)

| Session 21 | Tuesday, July 11, 2006<br>08:45–10:00 | Grand Ballroom (Level 2) |
|---|---|---|

## Session 21: Signal Transduction

**Co-Chairs:** Martin Rowe
Erle Robertson

21.001 LMP1-CTAR2 Determines a Unique Type of TRADD Signaling which is Required for IKKbeta Activation by LMP1
J. Neugebauer, F. Schneider, C. Briseño, N. Liefold, H. Kutz, **A. Kieser**
Munich (Germany)

21.002 LMP1 Signaling in IKKg Deficient Cells
D. Boehm, **E. Cahir-McFarland**
Boston, MA (USA)

21.003 LMP Interacts with UBE1L (Ubiquitin Activating Enzyme E1-like Protein) to Mediate NF-kappaB Activating Signals
**K.M. Izumi**
San Antonio, Texas (USA)

21.004 LMP1 Transmembrane Intermolecular Interactions that Mediate Nf-kB Activation
**V. Soni**, T. Yasui, E. Kieff
Osaka (Japan); Boston, MA (USA)

21.005 The EBV Encoded LMP-1 Protein Blocks Interferon-alpha Signaling in Human B Cells by Interacting with and Inhibiting Tyk2 Phosphorylation
T. Geiger, **J. Martin**
Boulder, CO (USA)

10:00–10:30 Coffee Break

| Invited Presentation | Tuesday, July 11, 2006<br>10:30–11:30 | Grand Ballroom (Level 2) |
|---|---|---|

**Chair:** Elliott Kieff

Structures and Interactions of Rel-Family Proteins
**Stephen C. Harrison**
Boston, MA (USA)

| Special Meeting | Tuesday, July 11, 2006<br>11:30–12:00 | Grand Ballroom (Level 2) |
|---|---|---|

**EBV Association General Meeting**
All EBV Association members: Please attend!

12:00–13:30 Lunch in Grand Ballroom/Level 2

| Invited Presentation | Tuesday, July 11, 2006<br>13:30–14:00 | Grand Ballroom (Level 2) |
|---|---|---|

**Chair:** Richard Ambinder

Effects of Allogeneic Donor-derived Unselected and EBV-specific T Cells in the Treatment of EBV Lymphomas Developing Following Marrow and Organ Allografts
**Richard O'Reilly**
New York, NY (USA)

| Session 22 | Tuesday, July 11, 2006<br>14:00–15:30 | Grand Ballroom (Level 2) |
|---|---|---|

## Session 22: Immunology

22.001 (cancelled) The Intertwinement of Epstein-Barr Virus Infection and T Cell Receptor Predictability
**J.J. Miles**, N.A. Borg, F.E. Tynan, J. Rossjohn, A.D. Hislop, J. McCluskey, S.R. Burrows
Brisbane, Melbourne (Australia); Birmingham (United Kingdom)

22.002 Epstein-Barr Virus-associated Infectious Mononucleosis Leads to Long-term Global Deficit of IL-15ralpha Expression and IL-15 Responsiveness in the T Cell Pool
**D. Sauce**, M. Larsen, S.J. Curnow, A.M. Leese, P. Moss, A.D. Hislop, M. Salmon, A. Rickinson
Birmingham (United Kingdom)

22.003 Increased Frequency and Broadened Specificity of Latent EBV Nuclear Antigen 1-Specific T Cells in Multiple Sclerosis
**J.D. Lunemann**, N. Edwards, P.A. Muraro, S. Hayashi, J.I. Cohen, R. Martin, C. Munz
Bethesda, MD, New York, NY (USA)

**Session 22** continued | **Tuesday, July 11, 2006 14:00–15:30** | **Grand Ballroom (Level 2)**

22.004 The Switch from Latent to Productive Infection in EBV Infected B Cells Is Associated with Sensitization to NK Cell Killing
**M. Rowe**, I.Y. Pappworth, E.C. Wang
Birmingham, Cardiff (United Kingdom)

22.005 EBV and Old World Gamma-1 Herpesviruses Encode a Cd8 T Cell Immune Evasion Gene Expressed During Lytic Cycle Replication
**A.D. Hislop**, M.E. Ressing, V.A. Pudney, D. van Leeuwen, N.P. Croft, E.J. Wiertz, A.B. Rickinson
Leiden (Netherlands); Birmingham (United Kingdom)

22.006 Impaired T Cell Recognition during Productive EBV Infection: Involvement of ORFs BGLF5 and BNLF2a
M.E. Ressing, A. Hislop, D. Van Leeuwen, V. Pudney, B. Glaunsinger, D. Ganem, M. Rowe, A. Rickinson, **E.J. Wiertz**
Leiden (Netherlands); Birmingham (United Kingdom); San Francisco, CA (USA)

15:30–16:00 Coffee Break

**Invited Presentation** | **Tuesday, July 11, 2006 16:00–17:00** | **Grand Ballroom (Level 2)**

**Chair:** Fred Wang

Gained in Translation: Generation of MHC Class I Peptide Ligands from Biosynthesized Viral Proteins
**Jonathan Yewdell**
Bethesda, MD (USA)

**Closing** | **Tuesday, July 11, 2006 17:00–17:15** | **Grand Ballroom (Level 2)**

**Fred Wang**
Boston, MA (USA)

18:30–22:00 Banquet at the Museum of Science
Buses will leave at 18:00hrs from the Marriott Cambridge Hotel


# Abstracts

**Sunday, July 9, 2006**
**Room: Grand Ballroom (Level 2)**

## Session 1: Latent Infection I
## 08:30-10:00

**1.001**

**Negative Autoregulation by EBV EBNA-1 Protein is Mediated through a Posttranscriptional Mechanism**

**M. Yoshioka**, M.M. Crum, J.T. Sample. St. Jude Children's Research Hospital, Memphis, TN, USA

EBNA-1 is required for maintenance of the episomal EBV genome within proliferating latently infected cells, and thus is essential to the oncogenic potential of EBV. During the restricted latency programs of EBV gene expression maintained within EBV-positive tumors and normal B cells, EBNA-1 is expressed from the promoter Qp that is negatively autoregulated through two EBNA-1 binding sites immediately downstream (+10) of the transcription start site and which span the cap-proximal exon/intron junction. Here we addressed the mechanism by which EBNA-1 functions in this regard. Results from electrophoretic mobility shift assays indicated that EBNA-1 binding to Qp does not preclude nearby binding by IRF-2, the major transactivator of Qp. Further, nuclear run-on assays of transcription from Qp within reporter plasmids, in the absence and presence of EBNA-1, revealed that EBNA-1 represses Qp posttranscriptionally. Analysis by quantitative RT-PCR of primary vs. mRNA levels in these transient reporter assays indicated that EBNA-1 repression results in accumulation of unspliced transcripts. This effect of EBNA-1 could be duplicated with an N-terminal deletion mutant of EBNA-1 that consists of the nuclear localization signal and C-terminal DNA-binding and dimerization domain. Notably, this mutant does not contain the previously identified RGG RNA-binding domains of EBNA-1 that are required for its nonspecific RNA-binding activity. Thus, the autoregulatory function of EBNA-1 is unlikely to require a direct interaction with its nascent transcript via these domains. Consistent with our analysis of Qp regulation in reporter assays, we observed primary but not fully processed Qp-specific EBNA-1 mRNAs within Burkitt lymphoma (BL) cells that do not use Qp for EBNA-1 expression, suggesting that the autoregulatory property of EBNA-1 is indeed active within latently infected cells. Finally, chromatin immunoprecipitation (ChIP) was used to demonstrate EBNA-1 occupancy of Qp within the endogenous EBV genomes of BL cells that use Qp for EBNA-1 expression and those that do not, suggesting that EBNA-1 autoregulation may play a role in regulating its expression from Qp in both the restricted and growth programs of latency. In summary, we conclude that EBNA-1 autoregulates its expression posttranscriptionally by inhibiting splicing and potentially other pre-mRNA processing events that occur cotranscriptionally, as its nascent transcript is synthesized by RNA polymerase II.

**1.002**

**Regulation of Epstein-Barr Virus Latency Type by the Chromatin Boundary Factor CTCF**

**C.M. Chau**, X. Zhang, S.B. McMahon, P.M. Lieberman. The Wistar Institute, Philadelphia, PA, USA

Epstein Barr virus (EBV) can establish distinct latency types with different growth transforming properties. Type I and type III latency can be distinguished by the expression of EBNA2, which has been shown to be regulated, in part, by the EBNA1-dependent enhancer activity of OriP. Here, we report that CTCF, a chromatin boundary factor with well-established enhancer-blocking activity, binds to EBV sequences between OriP and the RBP-Jk response elements of Cp and regulates transcription levels of EBNA2 mRNA. Using DNA affinity, EMSA, DNase I footprinting, and chromatin immunoprecipitation (ChIP), we found that CTCF binds both in vitro and in vivo to the EBV genome between OriP and Cp, with ~50 bp footprint at EBV coordinate 10515-10560. Deletion of this CTCF binding site in recombinant EBV BAC increased EBNA2 transcription by 3.5 fold when compared to wild-type EBV BAC. DNA affinity and ChIP showed more CTCF binding at this site in type I latency cell lines (MutuI and KemI) than in type III latency cell lines (LCL3456 and Raji). CTCF protein and mRNA expression levels were higher in type I, than type III cell lines. siRNA depletion of CTCF in type I MutuI cells stimulated EBNA2 mRNA levels, while overexpression of CTCF in type III Raji cells inhibited EBNA2 mRNA levels. These results indicate that increased CTCF can repress EBNA2 transcription. We also show that c-MYC, as well as EBNA2, can stimulate CTCF mRNA levels, suggesting that CTCF levels may contribute to B-cell differentiation as well as EBV latency type determination.

**1.003**

**Conceptual Promoter Analysis and Subsequent Validation Reveals a Role for EBNA1 in Enhancing the Expression of Angiogenic Factors in an NPC Cell Model**

**J.D. O'Neil**, V.H.J. Wood, K.L. Date, C.W. Dawson, L.S. Young. Cancer Research UK (Birmingham) Institute for Cancer Studies, University of Birmingham, Birmingham, United Kingdom

**Background:** While the role of EBNA1 in viral genome maintenance and viral gene transcription is well established, the possible influence of EBNA1 on the host cell phenotype remains controversial. We have recently used transcriptional profiling to confirm that EBNA1 modulates cellular gene transcription in an NPC model cell line, Ad/AH. Many of the transcriptional targets influenced by EBNA1 in these carcinoma cells impact upon key pathways relevant to the carcinogenic process. The current study set out to identify transcriptional networks that EBNA1 utilizes in order to achieve modulation of cellular genes and to determine how this contributes to NPC pathogenesis.

**Methods:** Conceptual PAINT (Promoter Analysis and Interaction Network Toolset) batch promoter analysis was performed on 2kb of DNA upstream of the genes found to be differentially regulated by EBNA1 in Ad/AH cells. Luciferase reporter assays, RT-PCR analysis, DNA binding assays, pharmacological inhibitor studies and cytokine analyses were performed upon the Ad/AH cell panel (control, EBNA1-transfected, EBV-infected) in order to validate and explore selected results from the PAINT analysis.

**Results:** PAINT analysis identified the AP-1 transcription factor pathway as a potential target of EBNA1. This was confirmed by reporter assays and EMSAs. Furthermore, the ability of EBNA1 to enhance AP-1 activity could be abrogated by expression of a dnc-Jun. RT-PCR identified that EBNA1 expression lead to up-regulation of the AP-1 subunits c-Jun and ATF2 and elevated DNA binding activity of these proteins was confirmed. Inhibitor studies demonstrated that EBNA1 enhanced AP-1 activity via potentiation of the PKC, p38 and MEK pathways but in a JNK-independent manner. The enhanced AP-1 activity induced by EBNA1 resulted in up-regulation and secretion of the angiogenic cytokines IL-8, VEGF and angiogenin. Furthermore, up-regulation of these cytokines was demonstrated in EBV-positive but LMP1 negative Ad/AH cells.

**Conclusions:** Detailed analysis of the transcriptional networks targeted by EBNA1 in a carcinoma cell line revealed a contribution of the AP-1 pathway and it was confirmed that EBNA1-induced activation of AP-1 results in enhanced expression of angiogenic cytokines. This explains previously published data suggesting a role for EBNA1 in the metastatic behaviour of carcinoma cells and highlights a potential target for the development of novel therapeutic interventions.

**1.004**

**EBNA-1 Binds the Promoters of Cellular Genes Whose Transcript Levels Are Altered in the Presence of Dominant Negative EBNA-1**

**D. Vereide**, B. Sugden. University of Wisconsin-Madison, Madison, WI, USA

We are studying the mechanism by which EBNA-1 contributes to the survival of EBV+ Burkitt lymphoma (BL) cell lines. We have demonstrated that efficient expression of a dominant negative derivative of EBNA-1 (deltaUR1) that inhibits transcriptional activation by EBNA-1 prompts apoptosis and reduces cell survival. DeltaUR1 induces these phenotypes in both BL Type III and Type I cell lines. Because the latter cell type expresses few viral genes, we hypothesize that EBNA-1's survival function is mediated through its transcriptional regulation of cellular genes. To search for cellular promoters bound by EBNA-1 in vivo we treated cells with formaldehyde, immunoprecipitated EBNA-1 cross-linked to DNA, isolated bound DNA, and used it to probe 27000 human promoter sequences. Promoter DNAs identified in this analysis were confirmed by testing them in vitro by EMSA. To determine if any of these EBNA-1 binding sites could have a functional role in regulating transcription, we analyzed the levels of their transcripts in EBV+ LCL 721 cells in the presence or absence of a dominant negative EBNA-1 using an Affymetrix expression array. We found 50 regions within or nearby cellular promoters that EBNA-1 apparently binds in vivo. Of the five regions so far tested by EMSA, all were bound by EBNA-1. Analysis with microarrays revealed a differential expression in 21 transcripts (corresponding to 17 EBNA-1 binding regions) induced by expression of a dominant negative derivative of EBNA-1. Thus, we have found cellular genes that are candidates for being regulated by EBNA-1. We are examining them functionally to determine if they contribute to the survival or maintenance of EBV-associated tumor phenotypes.

**1.005**

## Functional Characterization of the EBNA1-USP7 Interaction

**F. Sarkari**[1], T.S. Alcaraz[1], Y. Sheng[2], V. Saridakis[1], C. Arrowsmith[2], L. Frappier[1].
[1]Department of Medical Genetics and Microbiology, Toronto, Canada; [2]Banting and Best Department of Medical Research, Toronto, Canada

**Background:** We have previously shown that EBNA1 interacts specifically with a cellular ubiquitin-specific protease, USP7. USP7 is a key regulator of the p53 protein and its E3-ubiquitin ligase, MDM2. We recently determined the crystal structure of the USP7 N-terminal domain (NTD) bound to EBNA1 and p53 peptides, showing binding to each is mutually exclusive. In keeping with these structures, we found that EBNA1 can interfere with the stabilization of p53 by USP7 and hence protect cells from apoptosis. Since MDM2 is also a target of USP7, it is important to understand the basis for MDM2 recognition by USP7 and investigate whether EBNA1 alters MDM2 function through USP7. USP7 likely has cellular targets outside of the p53 pathway as well, since Drosophila USP7 has been shown to affect gene expression by cleaving monoubiquitination from histone H2B. It is yet to be determined if human USP7 also regulates gene expression through histone deubiquitination and whether this role is affected by EBNA1. Furthermore, it is also not known whether EBNA1 recruits USP7 to oriP sequences, or alternatively if the USP7 interaction inhibits EBNA1 assembly on DNA.

**Methods:** The USP7-binding region in MDM2 was determined by pull-down assays and peptides recognized were further delineated by measuring their ability to alter the intrinsic fluorescence of the USP7-NTD. A co-crystal structure of the USP7-NTD bound to an MDM2 peptide was determined. Deubiquitination of histones by USP7 was measured in vitro using purified USP7 and histone preparations. Electrophoretic mobility shift assays were used to determine the effect of USP7 on EBNA DNA-binding activity.

**Results and Conclusions:** Both p53 and MDM2 have two closely-spaced 4-residue sites that bind the same pocket on the USP7-NTD previously shown to bind EBNA1. Mutagenesis and structural analysis show a preference for a P/AXXS motif in USP7-binding peptides. Gel-filtration analyses show that EBNA1 can compete with MDM2 for binding USP7. These results suggest that EBNA1 might directly regulate MDM2 levels under some circumstances and hence play a complex role in altering the p53-MDM2 pathway. USP7 was also shown to cleave monoubiquitin from histone H2B in vitro, suggesting a role for USP7 in affecting chromatin structure in human cells. EBNA1-USP7 complexes were found to bind EBNA1 recognition sites in vitro and the affinity of EBNA1 for its sites was greatly stimulated by USP7. This suggests that EBNA1 may recruit USP7 to the EBV origin, perhaps to modify chromatin and or the replication machinery.

**1.006**

## Identification of a Specific, Non-random Mechanism for the Partitioning of Epstein-Barr Virus Plasmids

**A. Nanbo**, B. Sugden. University of Wisconsin, Madison, Madison, WI, USA

In latent infection Epstein-Barr virus (EBV) is maintained as an extrachromosomal plasmid. EBV plasmids usually replicate once per cell cycle but are slowly lost from proliferating cells in the absence of selection. We have examined the partitioning of EBV plasmids by studying both populations and single, live cells. Fluorescent in situ hybridization (FISH) was used to measure the distribution of EBV plasmids in the EBV-transformed lrmphoblastoid 721 parental cells and four of its subclones propagated for more than 100 or 25 generations, respectively. The distributions of the viral plasmids in all five cell populations were similarly broad with the mean number of plasmids centered on 6 to 9 plasmids/cell. The constancy of these mean numbers of plasmid indicates that there is likely a selection for this number of EBV genomes in 721 cells. We have also analyzed the distribution of EBV plasmids in individual, live HeLa cells by detecting the wild type Lac repressor fused to a tandem dimer of RFP bound to the plasmids site-specifically. Both the number of plasmids visualized as dots in the cells and their average fluorescence intensities before and after mitoses was measured. 70% of plasmids examined in 106 mitoses partitioned equally and this partitioning usually reflected a mechanism in which pairs of plasmids co-localized in G2, separated, and segregated faithfully during mitosis. These pairs of plasmids co-localized in G2 had intensities similar to twice that of the single plasmids derived from them at the end of mitosis. 19% of all of the plasmids were present in odd number (2n+1) in G2 and yielded distributions of n and n+1 plasmids in the daughter cells following mitosis. This partitioning is thus as equal as was possible. Most of the remaining 11% of plasmids were also present as co-localized pairs in G2 but both segregated to one daughter cell. Strikingly all plasmids detected in cells at G2 survived mitosis and were found in daughter cells.
These findings indicate that EBV plasmids encode some unknown, non-random, and fascinating mechanism for distributing themselves into daughter cells efficiently. In particular most EBV plasmids co-localize as pairs in G2 and segregate equally to daughter cells by the end of mitosis.

## Notes


# Abstracts

**Sunday, July 9, 2006**
**Room: Grand Ballroom (Level 2)**

## Session 2: Latent Infection II
## 11:00-12:00

**2.001**

**Epstein-Barr Virus Nuclear Protein EBNA3C is Required for Continuous Cell Cycle Progression and Growth Maintenance of Lymphoblastoid Cells**

**S. Maruo**, Y. Wu, S. Ishikawa, T. Kanda, K. Takada. Hokkaido University, Sapporo, Japan

To examine the role of Epstein-Barr virus (EBV) nuclear antigen 3C (EBNA3C) in the continuous proliferation of lymphoblastoid cell lines (LCLs), we established LCLs infected with an EBV recombinant that expresses EBNA3C with its C terminus fused in frame to a 4-hydroxytamoxifen (4HT)-dependent mutant estrogen receptor (E3C-HT). In the presence of 4HT, the LCLs expressed E3C-HT protein and grew similarly to wild-type LCLs. When E3C-HT EBV-infected LCLs were transferred to medium without 4HT, E3C-HT protein slowly disappeared and the LCLs gradually ceased growing. Wild-type EBNA3C expression from an oriP plasmid transfected into the E3C-HT LCLs protected the LCLs from growth arrest in media without 4HT, whereas EBNA3A or EBNA3B expression was unable to protect the LCLs from growth arrest. Expression of other EBNAs, LMP1, CD21, CD23, and c-myc was unaffected by EBNA3C inactivation. EBNA3C inactivation resulted in the acumulation of p16INK4A, the decrease of hyper-phophorylated form of pRb, and the decrease of cells in S or G2/M. These results indicate that EBNA3C has an essential role for cell cycle progression and growth maintenance of LCLs.

**2.002**

**EBNA3C and the Regulation of Mitosis**

G.A. Parker, M. Leao, E. Anderton, P. Young, R.E. White, **M.J. Allday**. Imperial College London, London, United Kingdom

**Background:** EBNA3C has been reported to exert multiple effects on cell cycle regulation and cell cycle checkpoints. Here we have focused on the regulation of mitosis, since in U2OS cells over-expression of EBNA3C disrupts the mitotic spindle assembly checkpoint and also induces polyploidy (Parker et al., Oncogene, 2000).

**Methods:** Various Burkitt lymphoma (BL) cell lines expressing different combinations of the known EBV latent gene products were selected or were newly established using recombinant EBNA-knockout viruses (see accompanying abstract from EA). These cells were treated with microtubule disrupting drugs nocodazole or taxol and analysed by microscopy, flow cytometry and western blotting. In addition, recombinant adenoviruses were used to deliver individual EBNA3 proteins to U2OS osteosarcoma cells and normal human fibroblasts and also analysed by microscopy, flow cytometry and western blotting.

**Results:** We show that EBV encoding a subset of latent gene products that includes the EBNA3 proteins, but not EBNA2 nor the LMPs, can suppress the spindle checkpoint and rescue BL cells from caspase-dependent cell death associated with aberrant mitosis. Knockout viruses indicate that both EBNA3C and EBNA3A contribute to this phenotype. It was confirmed using recombinant adenoviruses, that EBNA3C (but not EBNA3A nor 3B) interferes with the spindle checkpoint and was discovered that EBNA3C also activates cellular mitotic kinases. Phospho-epitopes that are recognised by the monoclonal antibody MPM-2 are generally restricted to mitosis, but when EBNA3C was over-expressed they were induced in all phases of the cell cycle, even in G1/S-arrested cells.

**Conclusions:** Experiments using BL cells infected with recombinant EBVs carrying various EBNA-specific deletions and experiments using recombinant adenoviruses to deliver the individual EBNA3s confirm that EBNA3C can have a significant effect on regulators of mitosis and interferes with a major mitosis-associated checkpoint.

**2.003**

## EBNA-3B and EBNA-3C Regulated Cellular Genes in Epstein-Barr Virus Immortalized Lymphoblastoid Cell Lines

**A. Chen**, B. Zhao, E. Kieff, J.C. Aster, F. Wang. Brigham & Women's Hospital, Boston, MA, USA

The cellular pathways that Epstein-Barr virus (EBV) manipulates to effect its lifelong persistence within hosts and facilitate its transmission between hosts are not well understood. The EBV nuclear antigen 3 (EBNA-3) family of latent infection proteins are transcriptional regulators that influence viral and cellular gene expression in EBV infected cells. To identify EBNA-3B and EBNA-3C regulated cellular genes potentially important for virus infection in vivo, we studied an LCL infected with an unusual EBV mutant where a genetic manipulation to delete EBNA-3B also resulted in a significant decrease in EBNA-3C expression and slower than normal growth ($3B^-/3C^{low}$). Transcriptional profiling was performed on the $3B^-/3C^{low}$ LCLs, and comparison of mutant and wild-type LCL profiles resulted in a group of 21 probe sets representing 16 individual genes showing statistically significant differences in expression. Further quantitative RT-PCR analyses comparing $3B^-/3C^{low}$ LCLs to a previously described EBNA-3B mutant ($3B^-$) where EBNA-3C expression was normal revealed 3 potential EBNA-3B repressed genes, 3 potential EBNA-3C repressed genes, and 2 potential EBNA-3C activated genes. The most highly EBNA-3C repressed gene was Jagged1, a cell surface ligand and inducer of the NOTCH receptor signaling pathway that is usurped by EBV genes essential for B cell immortalization. $3B^-/3C^{low}$ LCLs expressed increased levels of Jagged1 protein, were able to more efficiently induce functional NOTCH signaling, and this signaling was dependent on NOTCH cleavage by γ-secretase. However, inhibiting γ-secretase mediated NOTCH cleavage did not rescue $3B^-/3C^{low}$ LCL growth, suggesting that EBNA-3C mediated repression of this signaling pathway did not significantly contribute to LCL growth in tissue culture. These studies show that EBNA-3B and EBNA-3C were able to differentially regulate cellular gene expression in virus immortalized primary B cells. In addition, we identified EBV manipulated cellular pathways not involved in B cell growth that may be important for virus survival or transmission in humans. These pathways provide potential targets for testing in vivo with the rhesus lymphocryptovirus animal model for EBV infection.

**2.004**

**Epstein-Barr Virus EBNA-3C is Targeted to and Regulates Expression from the Bidirectional LMP-1/2B Promoter**

C. Jimenez-Ramirez, A. Brooks, L. Plym Forshell, K. Iakimtchouk, B. Zhao, T. Fulgham, **C. Sample**. St. Jude Children's Research Hospital, Memphis, TN, USA

The EBV EBNA-3C latency-associated protein is a transcriptional regulator with an essential but poorly understood contribution to EBV-mediated immortalization. EBNA3C activates expression of LMP-1, and reporter gene assays suggest that the interaction between EBNA3C and transcription factor PU.1 as well as with SUMO are essential for activation. In reporter gene assays, EBNA3C represses transcription from the C promoter (Cp) from which all EBNA proteins are transcribed, suggesting that feedback inhibition might occur in latently infected cells. Although reporter gene assays have provided considerable information, we wanted to establish a system where we could investigate EBNA3C's effects on endogenous viral promoters in the context of latency type III proteins, because there is significant interplay between the EBNA transcription factors. We therefore generated a cell line in which EBNA-3C expression is inducible using the Burkitt Lymphoma cell line, Raji, which is EBV-positive but EBNA3C negative, and where EBNA-3C has previously been shown to activate LMP1. Induction of EBNA-3C expression led to an increase in the level of LMP-1 mRNA and protein. By contrast, levels of EBNA3A and EBNA2 did not change, suggesting that at physiological levels, EBNA3C may not repress expression from Cp. LMP-2B mRNA was also up-regulated by EBNA3C, suggesting that EBNA-3C was acting on the LMP-1/LMP-2B bidirectional promoter, and chromatin immunoprecipitation assays indicated that EBNA-3C was present at this promoter. To identify the domains of EBNA3C essential for activation of LMP1, we introduced various point mutations or deletions in EBNA3C that targeted either reported functional domains or sites of protein interactions, and examined the ability of each mutant protein to activate LMP1. Mutations or deletions in the N-terminal half of the protein disrupted activation, whereas alterations in the C-terminal half of the protein, including mutation of the CtBP binding site, had no effect. The domains required for activation of LMP1 correlate with those we have previously identified as conserved throughout evolution, suggesting that activation of LMP1 is an important function of EBNA3C.

## Notes


# Abstracts

**Sunday, July 9, 2006**
**Room: Grand Ballroom (Level 2)**

## Session 3: Malignancy
## 14:00-15:30

**3.001**

## Regulation of Tumor Suppressor-related Genes in Nasopharyngeal Carcinoma Cells

**C.T. Lin**. Institute of Pathology, School of Medicine, National Taiwan University, Taipei, Taiwan

Nasopharyngeal carcinoma (NPC) is one of the common cancers in Taiwan, South China and Singapore. The etiological factors are not well defined yet. This research is aimed to identify the pivotal genes that may have been altered during NPC tumorigenesis. We used microarray assay to compare gene expression profiles of 5 NPC cell lines and expression profiles of the primary cultures of normal nasal mucosal cells, respectively. From microarray assay, we picked out 29 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, we found that, among all the selected genes, 4 genes, FGFR1, Osteoprotegerin (OPG), Osteonectin (SPARC) and UCHL1, were significantly down regulated in NPC cell lines. Since there was no known correlation of these 4 genes functionally, we decided to check the promoter regions of these 4 genes. We found that they all have binding sites for the transcription factor SOX5. And SOX5 was significantly up regulated in NPC cell lines. We then made a stable mixed clone expressing shRNA, which blocked the expression of SOX5 in NPC-TW04. We found that knocking down SOX5 mRNA increased the expression levels of all 4 genes, FGFR1, OPG, SPARC and UCHL1, and also slow down the cell proliferation rate and migratory ability. Among these 4 genes, expression level of SPARC increased 107-folds. We thus focus on SPARC for further study. Over expression of SPARC in NPC-TW01 inhibited tumor cell proliferation, invasion and induce apoptosis both in vitro and in vivo. In SCID mice bearing SPARC (+) NPC xenograft, the tumor size was markedly suppressed and the metastasis activity was mildly inhibited. We also found that the suppressive effect of SPARC on tumor cell proliferation and invasion was due to up regulation of RECK and down regulation of bFGF. Our results suggest that SOX5 may be one of the pivotal genes changed during NPC tumorigenesis. Up regulation of SOX5 in NPC inhibits SPARC and other regulated genes, thus inhibits RECK and promotes bFGF, resulting in increase of NPC progression.

**3.002**

**Bmi-1 is a Novel Molecular Marker of Nasopharyngeal Carcinoma Progression and Immortalizes Primary Human Nasopharyngeal Epithelial Cells**

L.B. Song[1], **M.S. Zeng**[1], W.T. Liao[1], L. Zhang[1], G.S.W. Tsao[2], G. Dimri[3], V. Band[3], Y.X. Zeng[1]. [1]State Key Laboratory of Oncology in Southern ChinaCancer Center, Sun Yat-sen University, Guangzhou, China; [2]University of Hong Kong, Hong Kong, China; [3]Evanston Northwestern Healthcare Research Institute, Northwestern University, Evanston, IL, USA

The Bmi-1 oncoprotein regulates proliferation and oncogenesis in human cells. Its overexpression leads to senescence bypass in human fibroblasts and immortalization of human mammary epithelial cells. In this study, we report that compared to normal nasopharyngeal epithelial cells (NPECs), Bmi-1 is overexpressed in nasopharyngeal carcinoma (NPC) cell lines. Importantly, Bmi-1 was also found to be overexpressed in 29 of 75 NPC tumors (38.7%) by immunohistochemical analysis. In contrast to NPC, there was no detectable expression of Bmi-1 in non-cancerous nasopharyngeal epithelium. Moreover, high Bmi-1 expression positively correlated with poor prognosis of NPC patients. We also report that the overexpression of Bmi-1 lead to bypass of senescence and immortalization of NPECs, which normally express p16INK4a and exhibit finite replicative life span. Overexpression of Bmi-1 in NPECs led to the induction of human telomerase reverse transcriptase (hTERT) activity and reduction of p16INK4a expression. Mutational analysis of Bmi-1 showed that both RING finger and helix-turn-helix (HT) domains of it are required for immortalization of NPECs. Our findings suggest that Bmi-1 plays an important role in the development and progression of NPC, and that Bmi-1 is a valuable marker for assessing the prognosis of NPC patients. Furthermore, this study provides a cellular proto-oncogene immortalized nasopharyngeal epithelial cell line, which may serves as a cell model system for studying the mechanisms involved in the tumorigenesis of NPC.

**3.003**

## Dynamics of EBV-DNA Load in Nasopharyngeal (NP) Brushings and Changes in IgA VCA/EBNA1 Serology during Treatment of Nasopharyngeal Carcinoma

M. Adham[1], A. Harahap[2], G. Suhartati[3], R. Averdi[1], A.N. Kurniawan[4], A. Djumhana[5], S.A.M.W. Verkuijlen[6], I.B. Tan[7], S.J.C. Stevens[6], **J.M. Middeldorp[6]**. [1]Dr. Cipto Mangunkusumo Hospital, dept. ENT, University of Indonesia, Jakarta, Indonesia; [2]Eijkman Institute, Jakarta, Indonesia; [3]Dr. Cipto Mangunkusumo Hospital, dept. Radiotherapy, University of Indonesia, Jakarta, Indonesia; [4]Dr. Cipto Mangunkusumo Hospital, dept. Pathology, University of Indonesia, Jakarta, Indonesia; [5]Dr. Cipto Mangunkusumo Hospital, dept. Internal Medicine, University of Indonesia, Jakarta, Indonesia; [6]VU University medical center, Dept. Pathology, Amsterdam, Netherlands; [7]Anthony van leeuwenhoek Hospital, Dutch Cancer Institute, Amsterdam, Netherlands

**Objective:** This prospective study was designed to evaluate if NPC stage at diagnosis is reflected by the level of local EBV-DNA in NP brushings, and if fluctuation of local EBV DNA or plasma/serum EBV IgA levels reflect NPC treatment efficacy during follow-up. The second aim of this study is if Nasopharyngeal brushing as a noninvasive procedure can be used as alternative for biopsy to detect tumor presence.

**Methods:** Suspected nasopharyngeal cancer patients (n=110) undergo NP brushing before biopsy was taken with rigid/fiber nasoendoscope guidance at first diagnostic visit, and subsequently after every cycle of chemotherapy and every week during radiation therapy, and 3 months interval follow up. NP brushing in NASBA lysis buffer and plasma were collected and examined for DNA load with Light Cycler PCR and IgA VCA-p18 and IgA EBNA1 by peptide ELISA (Fachiroh et al. J.Clin.Microbiol. (2006) 44;1459-67). Cut-off values (COV) for EBV DNA PCR were defined as mean EBV DNA load +2SD in control NP brushings (i.e. 1666 copies/brush; Stevens et al. Int. J.Cancer (2006), In press).

**Results:** At diagnosis NP brushing from biopsy confirmed NPC patients contained extremely high EBV DNA loads compared to non-NPC control ($p< 0,0001$; 101 NPC patients, 4 T/NK Cell Lymphoma, 2 NHL, 3 non tumor controls). EBV DNA loads range from 1.079 - 94.760.000 copies/brush. Sensitivety/specificity for NPC >99%). However EBV-DNA level and NPC stage did not show direct correlation.
Sixteen patients were studies in detail during follow-up with a standardized protocol ( NPC stage (n=2), stage 3 (n=4) patients, stage 4 (n=9)). In 9 patients DNA load returned to below COV, in 7 patients levels reduced but remained above COV, and 1 patient had persistent high DNA load 300x above COV, correlating with persistent disease. EBV IgA antibodies were all positive at diagnosis and slowly reduced in some cases during follow-up, but not in all. One patient have stable disease with elevated DNA load, and EBV-IgA above COV, and had inguinal lymph node metastasis during treatment. One patient with positive DNA load and EBV-IgA below COV had asymetrical CT Scan result but biopsy was negative. Most of the patients have a complete clinical response with negative CT Scan and biopsy.

**Conclusions:** EBV DNA load measurement in NP brushings has high positive and negative predictive value. It can assist in clinical patient management and can be repeated easily and more frequently than the biopsy. The brush procedure is well tolerated by patients and well standardized providing a useful diagnostic parameter. Kinetic changes in EBV DNA load during therapy reflect treatment outcome. Dynamic changes in EBV-IgA are less pronounced and less predictive on the short term. Long term follow-up is currently ongoing.

**3.004**

## High Serum Levels of Soluble CD40 Ligand in Undifferentiated Nasopharyngeal Carcinoma Patients are Associated with Distant Metastases and Lack of LMP-1 Expression at Presentation

L. Caggiari[1], M. Guidoboni[1], E. Vaccher[1], L. Barzan[2], G. Franchin[1], A. Gloghini[1], D. Martorelli[1], P. Zancai[1], M.T. Bortolin[1], M. Mazzucato[1], D. Serraino[1], A. Carbone[3], P. De Paoli[1], **R. Dolcetti**[1]. [1]CRO-National Cancer Institute, Aviano, Italy; [2]Head and Neck Dept., Azienda Ospedaliera, Pordenone, Italy; [3]Dept. of Pathology, Istituto Nazionale Tumori, Milano, Italy

**Purpose:** Engagement of CD40 promotes survival of undifferentiated nasopharyngeal carcinoma (UNPC) cells and similar effects are induced by the EBV oncoprotein LMP-1 that is expressed in a fraction of cases. Considering that CD40 may be activated also by the soluble isoform of CD40L (sCD40L), we investigated the serum levels of sCD40L in a series of UNPC patients from Italy, a non-endemic area for this disease.

**Results:** Serum samples of Italian UNPC patients (n=61), taken at diagnosis, contained higher levels of sCD40L than age-matched healthy controls (n=71) (15.2 ± 6.4 vs. 5.4 ± 3.6 ng/ml; p18 ng/ml) were more frequently found in UNPC patients under 40 years of age (p=0.03) and in those with distant metastases at presentation (p=0.03). Serum levels of sCD40L were inversely associated with the expression of LMP-1 (p=0.03), which mimics a constitutively activated CD40. CD40L expression was not detected in peripheral blood T cells of UNPC patients, ruling out a systemic activation of the immune system. The amount of sCD40L decreased in a fraction of patients treated with local radiotherapy alone, and CD40L+ lymphoid cells admixed to neoplastic UNPC cells were detected in cases with high serum levels of sCD40L, suggesting that sCD40L is probably produced within the tumor mass.

**Conclusions:** sCD40L may contribute to CD40 activation in UNPC cells, particularly of LMP-1- cases, further supporting the crucial role of CD40 signaling in the pathogenesis of UNPC. sCD40L levels may be useful to identify UNPC patients with occult distant metastases at presentation.

**3.005**

**Survival of Epstein-Barr Virus-infected T Cells from TNFa-induced Apoptosis through Down-regulation of TNFR1 and Recruitment of TRADD by LMP1**

**H.C. Chuang**[1], J.D. Lay[2], I.J. Su[3]. [1]Graduate Institutes of Basic Medicine, National Cheng Kung University Medical College, Tainan, Taiwan; [2]Divisions of Cancer Research, NHRI, Taipei, Taiwan; [3]Divisions of Clinical Research, NHRI, Tainan, Taiwan

The infection of T or NK cells by Epstein-Barr virus (EBV) may result in a fatal hemophagocytic syndrome (HPS) through the enhanced Th1 cytokine secretion induced by EBV LMP1 protein. One bewildering phenomenon in HPS is the progression from HPS to T cell lymphoma in a substantial percentage of patients. These observations raise the question whether EBV-infected LMP1-expressed T cells may be resistant to the cytokine-mediated cytotoxicity and survive from the TNFa-induced apoptosis in the lymphoid tissues of HPS patients. In order to explore this possibility, we tested the sensitivity of LMP1-expressed T cells to apoptosis in the presence of TNFa. We demonstrated that the LMP1-expressed T cells, as compared to the control T cells, were resistant to TNFa-induced apoptosis. In the presence of LMP1, p55 TNFR1 expression was down-regulated in T cells. Furthermore, the TNFa-induced apoptotic signal molecule TRADD was constitutively recruited by LMP1. The activities of apoptotic caspases 3, 9, 8 and JNK were remarkably suppressed. Reconstitution of TNFR1 reversed the TNFa-induced apoptosis in LMP1-expressed T cells. Taken together, these data suggest that the EBV-infected, LMP1-expressed T cells are resistant to TNFa-mediated apoptosis the down-regulation of TNFR1 and recruitment of TRADD by EBV LMP1 and may survive in the cytokine milieu in lymphoid organs of HPS patients. This study provides a potential molecular mechanism to explain the progression to clonal T cell proliferation or T cell lymphoma in HPS patients.


# Abstracts

**Sunday, July 9, 2006**
**Room: Grand Ballroom (Level 2)**

## Session 4: Replication I
## 16:30-17:45

**4.001**

**Role of the Viral Protein Kinase BGLF4 in Epstein-Barr Virus Nuclear Egress**

**E. Gershburg**[1], D. Scheswohl[2], S. Raffa[3], M.R. Torrisi[4], J.S. Pagano[5]. [1]Department of Microbiology and Immunology, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [2]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; [3]Istituto Dermatologico Santa Maria e San Gallicano, IRCCS, Rome, Italy; [4]Istituto Pasteur Fondazione Cenci-Bolognetti, Dipartimento di Medicina Sperimentale e Patologia, Università di Roma La Sapienza, Rome, Italy; [5]Lineberger Comprehensive Cancer Center, Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Epstein-Barr virus (EBV) protein kinase encoded by the BGLF4 gene has been shown to phosphorylate a number of viral targets and at least one cellular target. Its biochemical properties have also been partly characterized by several groups. Despite that, the biological role of this enzyme remains enigmatic. Here we describe the phenotype generated by knocking down the expression of EBV-PK by means of RNAi. Specifically we report the following: (I) Levels of the EBV BGLF4 mRNA were reduced by about 50 to 70 percent by this approach while protein expression lessened to levels undetectable by immunoblotting. Lack of EBV-PK activity has been confirmed by abolishment of the hyperphosphorylated form of the EBV-PK substrate EBV EA-D, pp58. (II) EBV-PK knockdown significantly reduced the amount of infectious virus as measured by superinfection of Raji cells. (III) Reduced amounts of infectious virus correlate with retention of virions in the nucleus in the absence of EBV-PK. (IV) This retention appears to be related to reduced levels of EBV BFLF2 protein, a component of the primary envelopment complex. The mechanism by which the EBV-PK regulates BFLF2 levels is discussed. The results suggest that EBV-PK is essential for efficient nuclear egress of EBV.

**4.002**

## Systematic Identification of Cellular Signals Reactivating Kaposi's Sarcoma-Associated Herpesvirus

**F. Yu**[1], J.N. Harada[2], H.J. Brown[1], S. Lee[1], H. Deng[1], M.J. Song[1], T.T. Wu[1], J. Kato-Stankiewicz[1], C.G. Nelson[2], J. Vieira[3], F. Tamanoi[1], S.K. Chanda[2], R. Sun[1]. [1]UCLA, LA, CA, USA; [2]Genomics Institute of the Novartis Research Foundation, San Diego, CA, USA; [3]University of Washington, Seattle, WA, USA

The herpesvirus life cycle has two distinct phases: latency and lytic replication. The balance between these two phases is critical for viral pathogenesis. The switch from latency to lytic replication occurs in response to a number of cellular signals.

To systematically evaluate the cellular signals regulating this reactivation process in KSHV, a luciferase reporter was utilized in a genome-wide cell-based screen approach. The effects of 26,000 full-length cDNA expression constructs on viral reactivation were individually assessed in primary effusion lymphoma (PEL)-derived cells which harbor the latent virus. Multiple cellular signaling proteins were identified from the screen, which include Ras and PKA. Further analysis revealed that Ras-mediated reactivation, as well as reactivation induced by the commonly used agent 12-O-tetradecanoylphorbol-13-acetate (TPA), is transduced via the Raf/MEK/ERK/Ets-1 pathway. We also found that the Raf/MEK/ERK/Ets-1 pathway is critical for spontaneous reactivation of KSHV. Ras and Ets-1 can activate the promoter of Rta, but not a downstream viral promoters using reporter assay. We also verified that KSHV can be reactivated by epinephrine or norepineprhine via PKA pathway.

To address the question from a different perspective, we also established a GFP reporter cell line and screened a chemical library. We found that a group of dopamine derivatives reactivated KSHV from latency. The targets of this group of chemicals are dopamine receptors. It is known that dopamine receptors can activate PKA and ras pathways. Thus our screens with cDNA expression library and chemical library generated a consistent result. Our study presents a high throughout functional genomic approach to systematically identify the cellular signals regulating the herpesvirus life cycle, thus facilitating better understanding of a fundamental issue in virology and identifying novel therapeutic targets.

**4.003**

**The Plasma Cell Differentiation Factor, X-Box-binding Protein-1 (XBP-1), Activates Lytic Epstein-Barr Virus Gene Expression**

P. Bhende, E. Holley-Guthrie, **S. Kenney**. Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

**Background:** Epstein-Barr virus (EBV) establishes latent infection in B cells. However, differentiation of B cells into antibody-secreting plasma cells leads to lytic EBV replication. The cellular transcription factor XBP-1 is required for B-cell differentiation into plasma cells. Antibody production in B cells activates the unfolded protein response (UPR) in the endoplasmic reticulum and leads to IRE1 mediated splicing of XBP-1 into its active (XBP-1s) form. XBP-1s directly binds to and activates numerous cellular target genes, resulting in an expanded endoplasmic reticulum that promotes massive protein production/secretion. In this study, we have determined if XBP-1s also plays a role in activating lytic EBV gene transcription during B cell differentiation into plasma cells.

**Methods:** The ability of XBP-1s to activate two EBV immediate-early (IE) promoters [BZLF1 promoter (Zp) and BRLF1 promoter (Rp)] was examined in reporter gene assays. The XBP-1s responsive region in Rp was determined using a series of promoter deletions. Electrophoretic mobility shift assay (EMSA) was performed to determine if XBP-1s binds directly to Rp. The ability of transfected XBP-1s (with or without valproic acid or CaMK IV) to induce lytic EBV gene expression in EBV-positive HONE1 (Hone1/EBV) cells was examined by immunoblot analysis.

**Results:** In transient reporter gene assays, XBP-1s activated the EBV Rp IE promoter, but not the Zp IE promoter. The major XBP-1s-responsive element in Rp was mapped to the region of the promoter between -635 and -750. This region contains one consensus XBP-1s binding motif. However, XBP-1s did not detectably bind in EMSA to a probe containing this Rp sequence. When XBP-1s was transfected by itself in HONE1/EBV cells, it slightly increased lytic EBV gene expression. However, XBP-1s dramatically enhanced the ability of valproic acid (which inhibits type I HDACs), or transfected CaMKIV (which inhibits type II HDACs) to activate lytic EBV gene expression. Preliminary results using an XBP-1 siRNA indicate that XBP-1s expression is also required for the low level constitutive lytic EBV gene expression observed in HONE1/EBV cells.

**Conclusion:** The XBP-1s transcription factor, which is intricately linked to plasma cell differentiation, promotes lytic EBV replication by activating the Rp IE promoter. The ability of XBP-1 to induce BRLF1 transcription represents a novel mechanism by which the virus ensures that lytic EBV infection follows plasma cell differentiation.

**4.004**

**Viral Trans-factors Essential for Expression of Late Genes in a Gammaherpesvirus**

**T.T. Wu**, V. Arumugaswami, Q. Jia, D. Martinez-Guzman, H. Hana, T. Tran, L. Tong, N. Reyes, H. Deng, R. Sun. UCLA, LA, CA, USA

The coupling of viral late gene expression to genome replication is a hallmark of productive infection by DNA viruses. However, the mechanisms underlying the regulation of late gene expression in herpesviruses remain largely unknown.

Here we report the identification of viral trans factors (ORF18, ORF30, ORF31 and ORF34) crucial for activating late gene transcription during lytic infection of murine gammaherpesvirus-68 (MHV-68). These ORFs are conserved among gammaherpesviruses and essential for the completion lytic infection. Our results show that the mutant viruses lacking one of these ORFs undergo normal lytic DNA replication but were unable to express late gene transcripts. To further delineate the regulation of late gene expression, reporter constructs driven by late gene promoters were generated. We found that unlike early gene promoters, induction of late promoters requires a functional replication origin of MHV-68 in cis on the reporters for allowing DNA replication. However, replication initiated with the simian virus (SV40) replication origin was not sufficient to permit the reporters to be activated by viral infection. The results suggest a more complex cis role of MHV-68 DNA replication in late gene expression than simply increasing the copy number or providing the replicated transcriptional template. By using the reporter system, we also demonstrate that in the absence of ORF18, ORF30, ORF31 or ORF34, late promoters could not be fully induced while the activities of early promoter were not affected.

In conclusion, we provide evidence that MHV-68 late gene expression is controlled by viral DNA replication in cis as well as virally encoded trans-factors. Our discovery of the viral mutants that uncouple late gene transcription from DNA replication lays an important foundation to dissect the mechanism of this essential gene regulation step in viral life cycle.

**4.005**

**Characteristics of EBV Gene Dependence on SM Expression**

Z. Han[1], E. Marendy[2], J. Yuan[3], M. Wang[2], J. Sample[2], **S. Swaminathan**[1]. [1]University of Florida Shands Cancer Center, Gainesville, FL, USA; [2]St. Jude Children's Research Hospital, Memphis, TN, USA; [3]Harvard Medical School, Boston, MA, USA

The EBV nuclear phosphoprotein SM (EB2, Mta) binds RNA and enhances accumulation and export of target mRNAs. SM, an early lytic cycle gene, is essential for EBV replication, as recombinant EBV lacking its expression fail to produce infectious virions. The enhancing effect of SM on gene expression is target gene-specific. However, the basis of its specificity and the EBV genes that require SM for expression have not been comprehensively addressed.

Therefore, using a custom EBV DNA array representing all known and potential EBV transcripts, we performed transcriptional profiling of EBV gene expression in the presence and absence of SM. Cells latently infected with an SM-null EBV were transfected with expression vector(s) encoding the immediate-early EBV transactivator Z (to induce replication), Z plus SM, or empty vector. Two sets of comparisons were performed with RNA isolated at 24 and 48 hr after transfection: Between control vector- and Z-transfected cells, and between Z- and Z plus SM-transfected cells. The former allowed us to identify those genes expressed in the absence of SM, while the latter revealed the lytic-cycle genes dependent on SM for expression or repression.

Although most early genes were SM-independent, several early genes were highly SM dependent, including some involved in DNA replication, notably EBV DNA polymerase. In the absence of SM, there was no significant accumulation of the mRNA for these genes. Consistent with this, Gardella gel analysis revealed that production of linear DNA molecules (indicative of viral DNA replication) did not occur in the absence of SM, but could be restored by SM rescue. Intriguingly, one early gene was downregulated by SM. An additional subset of early genes was induced by Z alone but was further enhanced by SM.

As expected, the majority of late genes was not expressed without SM, but several late genes were expressed at normal levels without SM, confirming that not all late gene expression is strictly tied to DNA replication. Experiments are currently underway to determine the relative contribution of DNA replication versus SM to late gene expression. SM is therefore essential for EBV virus production in part because of the dependence of EBV DNA replication on SM. In addition SM is likely to have enhancing effects on a specific subset of late genes although late gene expression per se is not SM-dependent.


# Abstracts

**Sunday, July 9, 2006**
**Room: Poster Area/Level 2**

## Session 5: Poster Session I
## Latent Infection
## 20:00-22:00

**5.001**

**B-Cells Infected with EBNA-3C Negative EBV Mutants Have a Limited Life Span**

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**Background:** Growth transformation of primary human B-cells by Epstein Barr Virus requires the concerted action of Epstein Barr virus nuclear antigens (EBNAs) and latent membrane proteins (LMPs). EBNA-3C belongs to the subgroup of latent viral proteins, considered to be absolutely essential for this growth transformation process. EBNA-3C can act as an activator or repressor of transcription and also interacts with various elements of the cell cycle machinery.

**Methods:** In order to test, at which stages of the growth transformation process EBNA-3C is required to support this process, we have generated a tractable recombinant Epstein-Barr virus mutant deleted for EBNA-3C and measured the transformation efficiency of this virus compared to control virus.

**Results:** Unexpectedly, infection of primary B-cells by EBNA-3C negative EBV induced massive proliferation of the infected cell cultures for 30 to 40 days. Then, proliferation ceased but single cell cultures survived and could be expanded indefinitely. EBNA-3C negative EBV infected B-cells were less viable than control cells infected with wild-type virus but the cell division rate was unchanged.

**Conclusion:** Our results show for the first time, that EBNA-3C is required in a delayed phase of the growth transformation process, which has not been recognized as a critical time window in earlier studies.

**5.002**

## Origin Recognition Complex Binding to Telomere Repeat Factor 2 Stimulates OriP Replication

**Z. Deng**, C. Atanasiu, J. Norseen, P.M. Lieberman. The Wistar Institute, Philadelphia, PA, USA

**Background:** Latent cycle DNA replication of EBV involves the recruitment of the cellular origin recognition complex (ORC) to the EBV genome. The Dyad Symmetry (DS) region of OriP is an EBNA1-dependent origin of DNA replication, but the precise mechanism of its regulation and ORC recruitment has not been elucidated. We have previously shown cellular telomere repeat binding factors bind DS and modulate replication function. We now investigate the function of telomere repeat factors in the recruitment of ORC to DS.

**Methods:** EBNA1, TRF2, and ORC2 binding to the DS was assayed by DNA affinity and chromatin immunoprecipitation assays. Coimmunoprecipitation studies of both EBNA1 and TRF2 were used to identify protein complex formation with ORC in nuclear extracts. Purified GST-TRF2 and ORC1 peptides were used to map the domains of interaction with ORC. The functional importance of these interactions is confirmed by replication assay of an OriP-containing plasmid.

**Results:** ORC bound to DS, but not to other EBNA1 binding elements Qp or FR using DNA affinity purification. Substitution mutation of the nonamer sites in the DS disrupted ORC recruitment as demonstrated by both DNA affinity and chromatin immunoprecipitation (ChIP). EBNA1 was immunoprecipitated from cells in complex with ORC2 and TRF2, but the TRF2 interaction with ORC2 was not EBNA1-dependent. TRF2, but not TRF1 or hRap1, bound ORC in vitro. TRF2 also bound EBNA1, thus providing a mechanism for cooperative binding at DS. The TRF2 amino terminal basic domain was found to be essential for ORC recruitment from cell extracts. Furthermore, the basic region of TRF2 was functionally important for replication of OriP-containing plasmids. We also found that a region of ORC1 (aa 201-511) is able to interact directly with the TRF2 basic domain. This region of ORC1 is sufficient to suppress OriP dependent replication in vivo.

**Conclusion:** Our results support a model where EBNA1 and TRF2 form a stable complex with ORC at DS and cooperatively stimulate oriP-dependent replication activity.

**5.003**

**Phosphorylation of the Nuclear Localization Signal of EBNA-1 Up- and Down Regulates Its Nuclear Import Mediated by Importin Alpha5**

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EBV nuclear antigen-1 (EBNA-1) is essential for replication of episomal EBV DNAs and maintenance of latency. We have previously reported that EBNA-1 proteins interact with importin alpha5 (NPI-1), a nuclear import adaptor, (Ito et al., 2000) and EBNA-1 proteins bind to cellular chromatin (Ito et al., 2002) and replication foci (Ito et al., 2003) in the absence of episomal EBV DNA. Multifunctional EBNA-1 is phosphorylated. Here, we examined the effects on nuclear translocation of Ser-phosphorylation of the EBNA-1 nuclear localization signal (NLS) sequence, 379Lys-Arg-Pro-Arg-Ser-Pro-Ser-Ser386. We found that Lys379Ala and Arg380Ala substitution greatly reduced nuclear transport and nuclear level of green fluorescent protein (GFP)-EBNA1, whereas substituting Pro381Ala, Arg382Ala, Pro384Ala, and Glu378Ala did not. Microinjection of modified EBNA-1 NLS peptide-inserted proteins and NLS peptides cross-linked to bovine serum albumin showed that Ala substitution of three NLS Ser residues reduced the efficiency of nuclear import. Similar microinjection analyses demonstrated that phosphorylation of Ser385 accelerated the rate of nuclear import, but phosphorylation of Ser383 and Ser386 reduced it. However, transfection analyses of GFP-EBNA1 mutants with Ser to Ala substitution causing reduced nuclear import efficiency did not result in a decrease in the nuclear accumulation level of EBNA-1. The results suggest dynamic nuclear transport control of phosphorylated EBNA-1 proteins, although the nuclear localization level of EBNA-1 that binds to cellular chromatin/chromosomes seems unchanged. The importin alpha5 bound more strongly to Ser385-phosphorylated NLS than to any other phosphorylated or non-phosphorylated forms. Importin alpha1 (Rch 1) bound only weakly and importin alpha3 (Qip 1) did not bind to the Ser385-phosphorylated NLS. These findings suggest that the amino-terminal 379Lys-Arg380 is essential for the EBNA-1 NLS and Ser385-phosphorylation up-regulates nuclear transport efficiency of EBNA-1 by increasing its binding affinity to importin alpha5, while phosphorylation of Ser386 and Ser383 down-regulates it.

**5.004**

**Silencing of the Epstein-Barr Virus (EBV) Gene *LMP2B* Decreases Induction of Lytic EBV in Burkitt's Lymphoma Cells**

**M.P. Rechsteiner**, D. Nadal, C. Berger, M. Bernasconi. Experimental Infectious Diseases and Cancer Research, University Childrens Hospital of Zurich, Zurich, Switzerland, Zurich, Switzerland

**Background:** To test the role of EBV latent membrane protein 2, splice variant B (LMP2B) in the switch of EBV latent to lytic infection.

**Methods:** We engineered short hairpin (sh)RNAs systems targeting *LMP2B* mRNA in the lentiviral vector system pSICOR carrying enhanced green fluorescent protein (EGFP). EGFP-positive Akata cells with the lentiviral vector system were enriched using fluorescence activated cell sorting. Expression levels of EBV mRNAs and proteins before and 24h after surface IgG cross-linking were monitored by qPCR and by Western blotting, respectively. Akata cells overexpressing LMP2A were established and compared to the LMP2B-silenced Akata cells (Akata/LMP2B-).

**Results:** *LMP2B* mRNA expression was reduced by the shRNA-construct up to 73% while *LMP2A* mRNA was not. Surface IgG cross-linking of control Akata cells resulted in activation of lytic EBV infection as monitored by *BZLF1* and *TK* transcription. In Akata/LMP2B- cells *BZLF1* mRNA expression was reduced by 63% and *TK* mRNA expression by 77% compared to control Akata cells. Furthermore, *EBNA2A* mRNA expression was almost completely abolished (91% reduction) in Akata/LMP2B- cells. Overexpression of LMP2A in Akata cells did not alter *LMP2B* mRNA levels and resulted in a decreased transcription of *BZLF1* (97%), *TK* (99%), and *EBNA2A* (100%), respectively, as compared to control Akata cells.

**Conclusion:** LMP2B silencing decreases the induction of lytic EBV infection suggesting a regulatory function of LMP2B in switching of latent to lytic EBV infection. Since LMP2A overexpression also results in less EBV lytic infection our results imply that LMP2B interferes with LMP2A to maintain EBV latent infection.

**5.005**

**Identification of Proteins Associated with the EBV-encoded Nuclear Antigen-5 in the Cell**

**A. Stromberg**, U. Rüetschi, L. Rymo. Institution of Biomedicine, Gothenburg, Sweden

**Background:** 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. One defined function of EBNA5 is the cooperation with EBNA2 in LMP1 promoter activation. Other reported functions include regulation of the cell cycle and pre-mRNA processing. EBNA5 is known to interact with a number of cellular proteins, but in most cases the functional implications remains to be clarified.

**Methods:** A possible way to elucidate the function of EBNA5 is to identify its cellular binding partners. We have developed a protocol for isolation of EBNA5 containing protein complexes from mammalian cells with an improved tandem affinity purification procedure. The tandem affinity tag is composed of a Protein A domain and a StrepTagII sequence fused to the C-terminus of EBNA5. Proteins specifically bound to the EBNA5 part of the bait were purified by 1 dimensional polyacrylamide gel electrophoresis and identified with LC-MS/MS mass spectrometry.

**Results:** To analyze the composition of EBNA5-containing complexes, the TAP-linked EBNA5 derivative was transiently transfected into HEK293 cells and the complexes isolated by affinity chromatography. Consistent with published data from several groups, HA95, Hsp70, Hsc70, HAX-1, S3a, $\alpha$- and $\beta$-tubulin were identified as EBNA5-interacting proteins. In addition, several members of the chaperone family were detected including the Bcl2-associated Athanogene-2 (BAG2). The p54nrb, PSF, and p68 helicase proteins reported to be components of the snRNP-free U1A complex (SF-A), that has been shown to be involved in pre-mRNA cleavage and polyadenylation, were also identified.

**Conclusion:** We have demonstrated that EBNA5 interacts with components of the Hsp/Hsc70-BAG2 chaperone complex, and with the p54nrb/PSF/p68 helicase complex. This suggests that the multifunctional EBNA5 protein has functional relationships with proteins involved in the chaperone folding and the ubiquitin-proteasome degradation systems as well as with proteins linked to pre-mRNA processing. Our results confirm the usefulness of this two-step affinity purification strategy for purification of protein complexes in mammalian cells.

**5.006**

## Gene Expression Profiling of LMP1-expressing Epithelial Cells Reveals Alterations Characteristic of Hyperkeratotic or Inflamed Epithelium

**J.R. Arrand**, M. Morris, W.B. Wei, C.W. Dawson, L.S. Young. Cancer Research (UK) Institute for Cancer Studies, The Medical School, University of Birmingham, Birmingham B15 2TT, UK, United Kingdom

**Background:** Our previous studies have demonstrated that LMP1 has profound effects on the differentiation capacity of human epithelial cells inducing an undifferentiated and more motile phenotype. To better understand the mechanism(s) by which LMP1 induces these effects, we generated a stable clone of the differentiation-competent SCC12F cells in which LMP1 expression is controlled from a tetracycline inducible promoter. Upon LMP1 induction, these cells exhibited similar phenotypic alterations to those observed when LMP1 is stably expressed. This system allowed us to investigate the effects of transient LMP1 expression on pathways associated with growth, differentiation and morphology.

**Methods:** SCC12F cells were induced for LMP1 expression and, after 72 hours, RNA was prepared from LMP1 positive and negative cells and analysed using Affymetrix arrays. Lists of significantly regulated genes were produced using RMA/SAM, dCHIP and RP algorithms. Expression of differentially regulated genes was validated by Q-PCR, western blotting and immunofluorescence staining.

**Results:** We identified subsets of differentially expressed genes induced by LMP1 that are characterisitically expressed in hyperkeratotic epithelium. These include (i) chemokines, cytokines and their receptors, (ii) growth factors involved in promoting epithelial cell motility and proliferation, and (iii) signalling molecules that regulate actin filament reorganisation and cell movement.

**Conclusions:** The genes found to be significantly influenced by LMP1 help to define the pathways by which this EBV-encoded oncoprotein regulates terminal differentiation, cell motility and inflammation. These alterations in gene expression are consistent with findings obtained from hyperkeratotic epithelium (psoriasis), confirm observations made on transgenic mouse models where LMP1 targeting to epidermis induces hyperkeratosis, and support a role for LMP1 in the pathogenesis of NPC.

**5.007**

**The Expression Profile of Epstein-Barr Virus-Encoded MicroRNAs in Akata Cells**

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MicroRNAs (miRNAs) are small noncoding RNAs of ≈22 nt that enter the RNAi pathways and down-regulate expression of target genes. These RNAs are processed from long primary miRNA transcripts in a series of cleavage reactions mediated by members of the RNAse III family. Recently, it has been demonstrated that wild type EBV encodes at least 17 miRNAs, which are mapped either within the BART gene (miR-BART1-14) or nearby the BHRF1 gene. BHRF1 is abundantly expressed at early stage of lytic replication. miR-BHRF1-1 is located at the upstream of the BHRF1 transcription start site, while miR-BHRF1-2 and -3 are found at the 3'-untranslated region of BHRF1 mRNA. Thus, the expression of miR-BHRF1-2 and -3 would increase upon induction of lytic replication. We analysed the expression of a selection of EBV miRNAs in latency I Akata cells before and after lytic induction. Akata expressed miR-BART1 but not any of the BHRF1 miRNAs, whereas latency III B cell lines expressed readily detectable levels of miR-BART1 and BHRF1 miRNAs. These results are consistent with the notion that the expression of BHRF1 miRNAs is associated only with EBV latency III but not with latency I. Induction of lytic replication in Akata by surface IgG cross-linking caused slightly increased expression of miR-BART1 but not the expression of mature BHRF1 miRNAs. Interestingly, there was a robust increase in the expression and accumulation of pre-miR-BHRF1-2 and -3, beginning at 6 hr after IgG cross-linking. A small increase in the expression of pre-miR-BHRF1-1 was noticable from 12 hr after the induction. These results indicate that the primary BHRF1 miRNA transcripts are induced during lytic replication in Akata cells but they are poorly processed to the mature miRNAs.

**5.008**

## Increased B-cellular Infection with EBV Using a Novel Protocol: Improvement for Studies on Host Gene Expression and Cell Transformation

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**Background:** Conventional infection of human cells in vitro with EBV is done by coincubating target cells with supernatant of the EBV producer cell line B95.8. This method results in B-cellular infection rates of > 2-5%. Our aim was to increase the infection rate to enable gene expression studies.

**Methods:** We inoculated mononuclear cells isolated from peripheral blood or tonsils with concentrated supernatants of the producer recombinant B95.8EBfaV-GFP cell line using centrifugation. B95.8 EBfaV-GFP carries a cassette encoding enhanced green fluorescent protein (EGFP) that becomes visible 12 h post cellular infection. Infected cells were phenotypically characterized using flow cytometry. EBV mRNA expression was longitudinally quantified using quantitative polymerase chain reaction. Parental B95.8 virus was used in control experiments.

**Results:** Maximal Infection rates of B cells ranged between 35% and 40% 24 h post inoculation by centrifugation as visualized by EGFP expression. Whereas the rate of infection of naïve B cells was 60% to 70%, the rate for memory B cells was 20% to 25%. Analysis of EBV mRNA expression showed similar longitudinal patterns in naïve and in memory B cells with sequential appearance of EBNA2, EBNA1, LMP1, and LMP2 mRNA expression, respectively, but no mRNA expression of lytic EBV genes. The EBV mRNA expression pattern following infection with B95.8 EbfaV-GFP or with B95.8 using our infection method and the conventional method, respectively, did not differ. As demonstrated by blocking experiments, both gp350/220 and gp42 were involved in the infection process of the cells following centrifugation. Finally, the increased infection rates resulted in increased transformation rates.

**Conclusion:** Our infection method remarkably increases the number of infected cells without altering EBV mRNA expression patterns, and the use of EBfaV-GFP enables fluorescent-activated sorting of infected cells, a prerequisite to study host gene expression in pure EBV-infected B cells early after virus entry.

**5.009**

**Variable Methylation and Efficiencies in Splicing of Intron 1 Modulate LMP2A Expression Levels**

**R.S. Scott**, C.A. Moody, E.T. Anye, J.W. Sixbey. Louisiana State University Health Sciences Center, Shreveport, LA, USA

Rapid evolution of experimentally infected carcinoma cells towards EBV clonality has been previously reported to reflect a terminal repeat (TR) number that optimized LMP2A expression. Formed upon EBV genome circularization, the LMP2A ORF encodes a precursor mRNA that is transcribed across fused TRs of variable length, which comprise intron 1. To define mechanisms behind TR modulation of LMP2A expression, we examined infected clones of the epithelial cell line CCL20.2, each of which contained 3 episomes per cell but with a distinct TR number. Since TRs are GC-rich, we examined whether the inverse correlation between TR number and LMP2A expression might be explained by differential methylation of intronic TR sequences. Using a methylation-sensitive isochizomer technique, we showed repeats in the 20TR clone were more densely methylated than in the comparable 4TR clone. Whereas 5-azacytidine increased transcription in the 20TR clone by 10-fold, only a 2-fold increase was observed for the 4TR clone, changes that were not explained by any alteration in transcriptional initiation. Because treatment with 5-azacytidine did not completely equilibrate levels of transcript between the two clones, we tested the hypothesis that disparities in LMP2A levels may also reflect impaired processing of the 20TR pre-mRNA. We used a real-time RT-PCR strategy designed to measure relative rates of intron removal in a steady state population of partially processed pre-mRNAs by assessing splicing rates of two introns within the same gene. Using primer sets that spanned exon/exon junctions formed by excision of introns 1 and 8, we derived a ratio of splicing efficiencies within individual transcripts that allowed cross-comparison between 4TR and 20TR clones. Markedly divergent splicing kinetics of the two introns within the 20TR transcript as compared to 4TR transcript implied impaired splicing efficiency for the larger intron 1. In sum, both transcriptional and post-transcriptional mechanisms link expression of LMP2A to a widely used viral marker of tumor clonality, making TR number per se a factor in clonal selection.

**5.010**

**EBNA2-independent Induction of EBV-encoded LMP1 by CD40-ligand, IL-4 and IL-13: Involvement of Cytokines in the Expression of Type II Latency**

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**Background:** After infection Epstein-Barr virus (EBV) resides in a latent state in the infected human memory B-cells and expresses a limited number of virus-encoded genes. Whereas in type III latency EBNA2 and EBNA5 drive LMP1 and LMP2 expression, the mechanism of LMP expression in type II latency, seen in EBV-positive nasopharyngeal carcinomas (NPC), Hodgkin lymphoma (HL), and nasal NK/T cell-lymphomas, is not known.

**Methods:** We initiated experiments to study how type II latency is established focusing on the induction of LMP1. We used EBV-carrying cell lines that do not express EBNA2: our in vitro EBV-converted subline of the HL-derived cell line KMH2 (KMH2-EBV), the EBNA2-deleted Burkitt lymphoma cell lines P3HR1 and Daudi, P3HR1 virus-infected normal B cells, and the NPC-derived EBV-infected TWO3 cells. These cells were exposed to recombinant human cytokines and the expression of EBV-encoded proteins was detected by immunoblotting and immunofluorescence.

**Results:** The KMH2-EBV cells expressed EBNA1 and LMP2A, but no LMP1 (Int J Cancer. 2005;113:937). Exposure of the cells to IL-4 or IL-13 induced LMP1, but not EBNA2, expression. Inhibition of IL-4- or IL-13-induced STAT6 phosphorylation by the JAK-inhibitor AG490 prevented the induction of LMP1. IL-4 and IL-13 induced LMP-1 in the TWO3-EBV cells too. CD40-ligand (CD40L) induced the expression of LMP1 in the Daudi and P3HR1 cells, but not in the KMH2-EBV cells. IL-4 alone did not induce LMP1 in the BL cells, but when combined with CD40L LMP1 induction was enhanced. IL-4 alone or in combination with CD40L induced LMP1 in tonsillar B-cells infected with the EBNA2-deleted P3HR1 EBV strain.

**Conclusion:** We have identified several cytokines that could induce LMP1 expression in the absence of EBNA2. Cytokines could be responsible for the EBNA2-independent LMP1 expression seen in type II EBV latency. The cytokines could be provided by the tumor-infiltrating normal cells or they could be secreted by the EBV-infected malignant cells and act in an autocrine manner. In line with this idea recent studies reported that the malignant Hodgkin-Reed Sternberg cells secreted IL-13 while the surrounding, infiltrating CD4+T-cells expressed CD40L. Furthermore, cytokines provided in the germinal center of secondary follicles might be involved in the LMP1 expression seen in the type II latent GC-B cells of healthy virus carriers.

**5.011**

## EBNA2 Interferes with Germinal Center Phenotype by Downregulating Bcl6 and Tcl1

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**Background:** In vitro infection of Epstein-Barr virus (EBV) negative B lymphoma lines with the prototype virus strain has provided important clues towards understanding the role of the virus in tumor development. We asked if EBV infection could alter the germinal center phenotype which is the characteristic of most EBV associated lymphomas.

**Methods:** We infected two DLBCLs and a BL with a recombinant EBV. The resulting virus convertant lines were studied for viral latency, and the influence of the viral latent genes on cellular genes such as BCL6 and TCL1.

**Results:** The resulting virus convertant lines expressed type II-III latency. High expression of EBNA2 inversely correlated with expression of typical germinal center (GC) associated genes, BCL6 and TCL1. The decreased expression of BCL6 appeared EBNA2 dose-dependent with almost complete abrogation in high EBNA2 expressing clones. The role of EBNA2 in the downregulation of BCL6 and TCL1 was confirmed in a hormone inducible EBNA2 carrying cell line. The repression of these GC associated genes was at transcriptional level. A parallel decrease in HLADR, sIgM and class II transactivator (CIITA) expression and an increase in CCL3, a BCL6 repression target, was observed in high EBNA2 expressing clones. Conclusion:Since BCL6 is indispensable for GC formation and somatic hypermutations (SHM), we suggest that the previously reported lack of SHM seen in EBNA2 expressing GC cells from infectious mononucleosis (IM) tonsils could be due to negative regulation of BCL6 by EBNA2. These findings suggest that EBNA2 interferes with the GC phenotype.

**5.012**

**Genetic Analysis of EBNA3C Using an EBV Bacterial Artificial Chromosome**

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Department of Medicine and Department of Microbiology and Molecular Genetics, Channing Laboratory, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA

The Epstein-Barr virus (EBV) nuclear antigen 3C (EBNA3C) is essential for EBV-mediated B cell immortalization. Insertion of a chloramphenicol (CAT) tag immediately downstream of EBNA3C permitted selection of mutant EBNA3C recombinant genomes in a bacterial artificial chromosome (BAC) system, without disrupting EBNA3C expression or transformation of primary B lymphocytes to lymphoblastoid cell lines (LCLs). Using this system, we generated EBNA3CΔ249-311,Δ311-364,Δ367-380,Δ385-392, and Δ401-450, which are transcriptionally active for both repression of EBNA2 activation of RBP-Jκ/CBF1-dependent Cp promoter and coactivation of the LMP1 promoter with EBNA2. Our preliminary results suggest that deletion of amino acids 249 to 311 could produce LCL growth similar to the wild-type EBNA3C-CAT, indicating the putative leucine-zipper region of EBNA3C is dispensable. EBNA3C TFGC207 to AAAA207, which is defective for RBP-Jκ/CBF1 association and repression of EBNA2 Cp promoter activation, was unable to support LCL growth in this genetic analysis.

**5.013**

## The BZLF1 Gene is Expressed as an Immediate Early Gene Following Primary Infection of B-lymphocytes

**K. Takada**, W. Wen, D. Iwakiri, K. Yamamoto, S. Maruo, T. Kanda. Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan

**Background:** Thus far, EBNA2 and EBNA-LP have been believed to be the first viral genes expressed following infection of B-lymphocytes. Protein synthesis is not required for the transcription of these genes, suggesting that the early stages of infection do not depend on the expression of novel cell genes. We demonstrate here that the BZLF1 gene is the first gene to be expressed after EBV infection of B-lymphocytes.

**Methods:** Burkitt's lymphoma (BL)-derived EBV-negative Akata and Daudi cells and primary B-lymphocytes were used as targets for EBV infection. Akata virus-derived recombinant EBV carrying the EGFP gene was used as a source of EBV. Expression of BZLF1 was determined by RT-PCR and Western blot analysis.

**Results:** BZLF1 mRNA was detected as early as 1.5 h postinfection, peaked at 6 h in Akata cells, at 3 to 12 h in Daudi cells, and at 3 h in primary B-lymphocytes, and quickly decreased thereafter. EBNA2 mRNA was first detected at 6 h postinfection and constitutively expressed thereafter. BZLF1 protein expression was also examined for EBV-infected Akata and Daudi cells and was detected at 6 h and 12 h postinfection. UV-treated EBV could not induce BZLF1 mRNA expression, indicating that intact viral DNA was necessary for BZLF1 expression. BZLF1 mRNA was expressed even when the cells were infected with EBV in the presence of anisomycin.

**Conclusion:** We demonstrate here that BZLF1 is the first gene to be expressed after EBV infection of B-lymphocytes. Since expression of BZLF1 mRNA does not require prerequisite protein synthesis, BZLF1 is expressed as an immediate early gene following primary EBV infection of B-lymphocytes. The role of early expression of BZLF1 remains to be clarified.

**5.014**

## Expression of Viral miRNAs during Primary Infection of Epstein-Barr Virus in B Cells

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**Background:** MicroRNAs (miRNAs) are an endogenously encoded class of small RNAs that have been proposed to function as key post-transcriptional regulators of gene expression in a range of eukaryotic species, including humans. EBV has recently been shown to encode miRNAs. Although we can predict that the small size of miRNA precursors makes them potentially ideal for use by viruses as inhibitors of host cell defense pathways, the function of most of them and expression patterns are still unknown

**Methods:** In this study, to investigate the expression pattern of EBV miRNAs in early infection of B cells, Akata, EBV negative Burkitt's lymphoma cell line, was infected with B95-8 EBV and time course expression of EBV miRNAs was analyzed following infection. Cell cycle progression and apoptosis were analyzed following infection with or without miRNA inhibitor.

**Results:** Mir-BHRF1s were expressed during an initial infection step. Mir-BHRF1s were coordinately expressed with EBNA2 that was also expressed in an early infection step. Mir-BARTs were expressed late stage of infection. To determine the function of viral miRNAs, we analyzed cell cycle progression and apoptosis in EBV positive cells with or without transfecting 2'-O-Methyl oligonucleotides, miRNA inhibitor. 2'-O-Me-mir-BHRF1-3 resulted in an increase of apoptosis in EBV infected cells, implicating that mir-BHRF1-3 functions in cell survival maintenance.

**Conclusion:** According to our data, EBV miRNA, especially Mir-BHRF1-3, contributes to maintenance of EBV latent infection and EBV-induced tumorigenesis.

**5.015**

## EBNA1 Can Distribute Replicated 'Sister Viral Molecules' Evenly to Sister Chromatids

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**Background:** EBV genomes are maintained as circular extrachromosomal molecules (episomes) in nuclei of latently-infected cells. EBV episomes replicate only once during S phase of the cell cycle, and they segregate to daughter nuclei by attaching to host cell chromosomes. EBNA1 protein works as a molecular tether to keep viral episomes attached to host chromosomes. However, it remains unknown whether episomes are just randomly distributed between daughter nuclei or somehow evenly distributed. We generated a recombinant EBV expressing an epitope-tagged EBNA1 protein to clarify this issue.

**Methods:** The EBNA1 gene of Akata strain EBV genome was replaced with a transgene encoding hemagglutinin (HA)-tagged EBNA1 by using a bacterial artificial chromosome (BAC) system. The resultant BAC clone DNA was introduced into Akata cells, and cells harboring only the recombinant EBV were obtained. A combined method of fluorescence in situ hybridization (FISH) and immunofluorescence (IF) was developed to visualize the localizations of EBV genomes and EBNA1 protein simultaneously.

**Results:** The established cells expressed HA-tagged EBNA1 protein, and the recombinant EBV genomes were stably maintained as episomes. IF analyses using anti-HA antibody revealed that HA-tagged EBNA1 protein localized as intranuclear punctate dots. Combined FISH and IF analyses revealed that EBNA1 signals overlapped with the signals of EBV genomes in all phases of the cell cycle. Notably, EBNA1 signals were frequently observed as double dots in S and G2 phase-synchronized cells. Some of the FISH signals of EBV genomes were sandwiched between paired dots of EBNA1, displaying 'dumbbell-like' structures. Most importantly, we found that more than 50% of the paired dots of EBNA1 localized symmetrically on sister chromatids in prematurely condensed G2-phase chromosome spreads.

**Conclusion:** The localization of EBV episomes in G2-phase nuclei should reflect the mode of viral replication occurring during S phase. We conclude that EBNA1 can distribute replicated 'sister viral molecules' evenly to sister chromatids while host chromosomes and viral episomes are synchronously replicating during S phase. This mechanism is likely to increase the stability of EBV episomes in dividing cells.

**5.016**

**OCTs and Grg/TLEs Compete with EBNA 1 in Regulation of FR - Implications for Switching between Cp and Qp**

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The regulation of Cp and Qp is instrumental in controlling the EBV latency programs, with implications for cell cycle entry and exit. The family of repeats (FR) is the major upstream control element of the EBV latent C promoter (Cp), driving latency III and cell proliferation. It can be activated by the binding of multiple EBV nuclear protein EBNA1s. We have shown that OCT-transcription factors also can bind to and activate FR, as demonstrated by electrophoretic mobility shift assay (EMSA), by an affinity DNA-binding assay ('DNA-fishing'), chromatine immuno precipitation (ChIP) and luciferase based reporter assays. Oct-1 in combination with co-regulator Bob.1, or Oct-2 alone could drive transcription of a heterologous thymidine kinase (TK) promoter controlled by FR in both B-cells and epithelial cells, as well as FR-Cp reporter constructs.

We have also shown that OCT proteins can be turned into repressors by recruiting members of the Grg/TLE-family of transcriptional co-regulators. We now show that Oct-2 with Grg/TLE also can repress promoter activity through EBVs FR. Although all Grg/TLE variants could repress the Oct-2 induced activity, Grg-3 was most efficient. When cotransfected with Oct-2, Grg/TLE 3 also competetively repressed the EBNA1-induced FR-enhancer effect. Reversely, increasing amounts of EBNA1 efficiently counteracted the transcriptional repression evoked by Oct-2 and Grg/TLE-3. We also demonstrate that the levels of EBNA1 and Oct-2 differ considerably between latency I and III cells. Oct-2/Grg/TLE may compete with EBNA 1 as one key regulator of the on-off switch of the EBV C promoter. An in silico model of this switch based on EBNA1/Oct-2/Grg/TLE regulation will be presented. Together with the established autoregulation of Qp the model provides critical boundaries for switching. With this model we propose one mechanism for switching between EBV driven cell cycle entry or exit, as consequencies of the latency program switches.

**5.017**

**A Regulatory Loop Between Kaposi's Sarcoma-Associated Herpesvirus Replication and Transcription Activator (RTA) and Epstein-Barr Virus Latent Membrane Protein 1 (LMP-1)**

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The majority of AIDS-associated primary effusion lymphomas (PEL) are co-infected with both Kaposi sarcoma-associated herpesvirus [KSHV; also called human herpesvirus 8 (HHV-8)] and Epstein-Barr virus (EBV). The relation between the two viruses in the cancer cells is unknown. KSHV replication and transcription activator (K-RTA) is necessary and sufficient for the switch from KSHV latency to lytic replication. EBV latent membrane protein 1 (LMP-1) is required for EBV transformation and establishment of latency in primary B cells in vitro. We show that K-RTA induces LMP-1 in EBV latency cells. The induction is independent of EBV nuclear antigen 2 (EBNA-2) and is at the RNA level. K-RTA binds to interferon stimulated response element (ISRE) in LMP-1 promoter in vitro and activates LMP-1 promoter reporter constructs in transient transfection assays. LMP-1, in turn, inhibits KSHV lytic replication via reduction of K-RTA expression. Suppression of LMP-1 expression in dually infected PEL cells enhances the expression of K-RTA and lytic replication of KSHV upon chemical induction. Moreover, KSHV infection of EBV latency cells induces LMP-1. These results suggest that KSHV may potentiate EBV latency via the induction of LMP-1 and use LMP-1 to control KSHV lytic replication by inhibiting the expression of K-RTA. Thus, the coexistence of KSHV and EBV in PEL cells would benefit both viruses for maintenance of their latencies. The regulatory loop between K-RTA and LMP-1 may play a role in the pathogenesis of dually infected PELs.

**5.018**

**Raji EBV's Alternate Origin: A Paradigm for Mammalian ARSs?**

**C. Wang**, B. Sugden. University of Wisconsin Madison, McArdle Lab for Cancer Research, Madison, WI, USA

oriP of EBV was identified by an ARS-like assay as a DNA replication origin (Yates et al, 1984) at which ORC is recruited in an EBNA1-dependent manner (Gahn and Shildkraut, 1989; Schepers et al, 2001). A second class of origins which we term 'Raji ori' has been found in EBV which is akin to a cellular zone of DNA replication initiation (Norio and Schildkraut, 2004). Raji ori, not oriP, is the predominate origin used in Raji EBV (ibid). Our EMSA assays indicate that Raji ori, which is 14 kbp in length, does not bind EBNA1 detectably distinguishing it from the DS origin in oriP. A vector with Raji ori supports DNA synthesis transiently in Raji cells when the maintenance element of oriP, FR, is present in cis, at 1% of the level of oriP but is lost and fails to support long-term replication. How then does Raji ori support EBV DNA synthesis in Raji cells? When oriP is newly introduced into cells, it supports DNA synthesis but is lost precipitously until an epigenetic event supports 1 to 10% of the replicons becoming established (Leight and Sugden, 2001). We have hypothesized that Raji ori and, perhaps, cellular origins can not be established as can oriP. We have tested our hypothesis by introducing oriP plasmids, in which DS is flanked by loxP sites, plus or minus Raji ori or Raji middle, a 14 kbp DNA fragment from EBV genome that had been shown not to serve as a DNA replication origin by a single molecule replication assay (Norio and Schildkraut, 2004), into Raji cells. Cell clones selected by resistance to puromycin were verified as maintaining the vector DNAs as plasmids. The introduction of Cre into the clones led to the excision and loss of DS. Cells with vectors that lacked Raji ori, including that harbored Raji middle, died; those having Raji ori survived as did controls with a second copy of DS in place of Raji ori. Their plasmids are extra-chromosomal. Thus Raji ori, and we speculate cellular origins of DNA synthesis can maintain but not establish extra-chromosomal DNA synthesis.

**5.019**

**Intracellular Localization of Ribosomal Protein L22 in EBV-infected Cells May Be Determined by Competition Between RNA Ligands**

**J.L. Houmani**, I.K. Ruf. University of California, Irvine, Irvine, CA, USA

The Epstein-Barr virus encoded RNAs (EBERs) are two small, non-coding RNAs expressed at high levels in all forms of EBV latency. Of the limited EBV latent gene products expressed in Burkitt Lymphoma (BL) tumors, the EBER RNAs are key mediators of EBV-dependent tumorigenicity, as evidenced by their ability to enhance the tumorigenicity of EBV-negative BL cells. They have also been shown to enhance the growth potential of gastric and nasopharyngeal carcinoma-derived cell lines. The mechanisms through which the EBERs achieve this enhancement, however, as well as their role in viral latency and persistence, remain elusive. The function of the EBERs may be mediated through protein-RNA interactions or via disruption of protein complexes. The current study aims to characterize the interaction between EBER-1 and the ribosomal protein L22 (rpL22), a component of the 60S ribosomal subunit previously demonstrated to be relocalized from the nucleoli to the nucleoplasm of EBV-infected BL cells. Although the function of rpL22 *in vivo* has not been fully defined, it has been shown that rpL22 can bind to specific sequences of 28S rRNA *in vitro*. As such, we hypothesized that EBER-1 may compete with these cellular RNAs for binding to rpL22, resulting in the relocalization of the protein. To test this hypothesis, we designed a series of truncation and substitution mutants of rpL22 to identify the RNA-binding domain of the protein. *In vitro* RNA-binding assays, including magnetic bead assays and electrophoretic mobility shift assays, demonstrated that a cluster of basic amino acid residues is required for binding to EBER-1. Analogous experiments using 28S rRNA revealed a similar dependence on this region. Furthermore, competition assays using EBER-1 and 28S rRNA support our hypothesis that EBER-1 and cellular rRNA compete for a common binding site on rpL22. Parallel studies using fluorescence localization assays in 293T cells demonstrated that mutant rpL22 proteins unable to bind rRNA are relocalized relative to wild type rpL22, synonymous with the relocalization of rpL22 observed in EBV-infected B lymphocytes and consistent with our model. Ultimately, the relocalization of rpL22 by EBER-1 in EBV-infected cells may represent a key mechanism of action for EBER-1 which could lead to some functional gain or consequence with respect to rpL22 function within the cell and perhaps contribute significantly to the tumorigenic potential of EBER-1.

**5.020**

**Cell Contact Mediated Regulation of LMP-1 Expression in EBNA-2 Negative Cell Lines. Relevance to the EBV Encoded Protein Expression in Type II Tumors**

**E. Klein**, L.L. Kis, M. Takahara, N. Nagy, H. Ishii. Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden

**Background:** The Type III EBV 'growth program' is only expressed in B lymphocytes. In these cells, LMP-1 expression is under the control of EBNA-2. In Hodgkin's (HL)- and nasal T/NK lymphomas EBV expression is restricted to EBNA-1 and LMP-1 (Type II). These proteins are not sufficient to induce proliferation. We have obtained evidence that cytokines can induce LMP-1 in the absence of EBNA-2.
The Burkitt lymphoma (BL) derived Daudi line lacks EBNA-2. It carries the typical Ig-myc translocation, and is therefore not dependent on EBV for proliferation. We have shown earlier that contact with CD40L carrying L cells and IL-4 could induce LMP-1. Similarly to normal NK cells, the EBV positive type II NK lines require IL-2 or IL-15 for in vitro proliferation. They express LMP-1. We have found that proliferation and LMP-1 expression declined in absence of these cytokines. IL-10 or IFNgamma could maintain LMP-1 expression, but the cells did not proliferate.

**Methods:** 1. Daudi cells were cultured in presence of PHA with CD4 T lymphocytes separated from blood. LMP-1 expression was tested 24 hours later in immunoblots.
2. Cells of the NK tumor derived line, SNK6, were washed, cultured for 24 hours without IL-2, labelled with CSFE and cultured with blood derived monocytes. Proliferation and LMP-1 expression were compared with samples without monocytes, and with IL-2 (immunofluorescence and immunoblot).

**Results:** 1.The Daudi cells which were in contact with CD4 enriched blood lymphocytes in the presence of PHA for 24 hours expressed LMP-1.
2. Compared to the culture without IL-2 LMP-1 expression and proliferation of the SNK6 NK-line was higher in the cells that have been in contact with monocytes.

**Conclusions:** These results are relevant to the in vivo situation, since cytokines are abundantly produced in the granuloma tissue, a characteristic of NK/T nasal lymphomas and of HL. They substantiate the assumption that normal cells in the microenvironment contribute to the viral expression phenotype of the malignant cells.

**5.021**

## Epstein-Barr Virus Non-coding RNAs are Confined to the Nucleus and do not Undergo Nucleocytoplasmic Shuttling

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**Background:** EBER1 and EBER2 (EBV-encoded RNA) promote cellular transformation in various systems and inhibit apoptosis induced by IFN-alpha. These activities have been attributed to binding and inhibition of dsRNA-dependent protein kinase, PKR, despite multiple reports that EBERs are nucleoplasmic, while PKR and its well-documented effect on translation initiation are cytoplasmic. We explore a potential EBER-PKR interaction by studying the cellular trafficking of the EBERs.

**Methods:** We used heterokaryon (with EBV-infected cells) and Xenopus oocyte microinjection assays to ask whether EBERs are exported from the nucleus. Exportin5 mediates nuclear export of pre-microRNAs and adenovirus non-coding RNA VAI via binding to a terminal stem. Using an electrophoretic mobility shift assay, we tested whether Exportin5 binds EBER1.

**Results:** We find the EBERs do not undergo nucleocytoplasmic shuttling and are confined to the cell nucleus. We also find that EBER1 does not bind Exportin5 in vitro.

**Conclusion:** Although controversial, an inhibition of the cytoplasmic PKR has been described for the predominantly nuclear EBERs. Results from two recent papers indicate that EBERs indeed do not inhibit PKR activity in vivo when cells are challenged with various PKR stimuli. Our observation that EBERs do not shuttle between the nucleus and cytoplasm of EBV-infected cells provides a mechanism to explain the absence of a functional interaction between EBERs and PKR inside cells. In contrast to VAI, EBER1 does not bind Exportin5 and is therefore unlikely to act by interfering with microRNA biogenesis. The confinement of EBERs in the nucleus suggests that future studies on EBER function should be concentrated on identifying their interacting proteins in this compartment.


# Abstracts

**Sunday, July 9, 2006**
**Room: Poster Area/Level 2**

## Session 6: Poster Session I
## Oncogenesis and Cell Cycle
## 20:00-22:00

**6.001**

**Inhibitory Effects of Epstein-Barr Virus BARF1 Gene on the Growth of Macaque Cells at Preimmortalized Stage**

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We previously reported the oncogenic transformation of rodent fibroblasts and the immortalization of simian Patas epithelial cells by Epstein-Barr Virus-encoded BARF1 protein. Since simian adherent cells were recently shown able to spontaneously overcome the limits of proliferation, BARF1 oncogene functions were further analyzed by transfection of simian Macaque fibroblast-like cells with extended replicative lifespan due to loss of p53 function. Several BARF1-positive clones were isolated and examined for growth rate and replicative lifespan. They showed lower growth rates than mock transfected cells, and they terminated cell division after 9.15±2.02 population doublings (pd), earlier than mock transfected cells which stopped growing after13.92±1.79 pd at the limit of proliferation (crisis). In contrast, introduction of human telomerase catalytic subunit (hTERT) led Macaque cells to grow further than 50 pd, indicating that they were immortalized. However, introduction of BARF1 did not affect hTERT-tansfected cell immortalization, and transfection with hTERT could not lead BARF1-transfected cells to overcome the crisis. These data suggested that BARF1 is not able to immortalize Macaque fibroblast-like cells, but has inhibitory effects on the growth of these cells at preimmortalized stage.

**6.002**

## An Evaluation of the Contribution of EBNA-1 Expression to Transgenic Mouse Lymphomagenesis

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**Background:** There is growing evidence that EBNA-1 can influence B-cell survival, but whether this is sufficient to predispose transgenic mice to lymphomagenesis is controversial. We have previously shown that two out of 12 established transgenic mouse lines generated using an E$\mu$EBNA-1 transgene expressed EBNA-1 (Wilson et al., 1996, EMBO J., 15, p3117). First, this demonstrated that *in vivo* expression of full length EBNA-1 from a relatively simple transgene is not efficient. Second, mice of the two expressing lines succumb to B-cell lymphoma with identical pathology, but with dramatically different penetrance and latency to onset, essentially a fast tumour line (line 26) and a slow tumour line (line 59). A further development from the "fast line" was that a spontaneous partial transgene deletion arose, giving rise to a sub-line of mice (designated 26A), which no longer showed EBNA-1 expression or developed lymphoma.

**Methods:** In order to explore the contribution of EBNA-1 to the phenotype and to examine if there is any influence from cellular sequences at the sites of transgene insertion, we have taken three approaches: [1] To explore the phenotype of lymphocytes from both transgenic lines, prior to tumour development, where any characteristic in common between the two lines must result from EBNA-1 expression; [2] To use dominant negative EBNA-1 expression to examine the effect of EBNA-1 "withdrawal"; [3] To identify and characterise the transgene insertion sites.

**Results:** [1] Lymphocytes explanted and cultured from mice of both transgenic lines initially show enhanced proliferation and then prolonged survival compared to non-transgenic wild-type sibling controls. This property is only evident when the cells are cultured in the presence of interleukin-2 (IL-2). [2] Transfection and expression of dominant negative forms of EBNA-1 in a cell line derived from an EBNA-1 expressing line 59 tumour (co-expressing LMP1) is not compatible with the survival of these cells, while expression in cell lines derived from LMP1-only tumours is innocuous. [3] The transgene insertion site for line 59 has been precisely mapped to murine chromosome 4 band D3. No known oncogenes lie withing a Mb region encompassing the transgene. The transgene insertion site for sub-line 26A has been precisely mapped to murine chromosome 3 band H2. No known oncogenes lie within a Mb region encompassing the transgene. The transgene insertion site for line 26 has been mapped to murine chromosome 5 band B and fine mapping is ongoing.

**Conclusion:** EBNA-1 promotes lymphocyte survival in the transgenic system.

**6.003**

**EBNA-5 Binds to MDM2 and Decreases the Half-Life of p53**

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EBNA-5 (EBNA-LP) is expressed very early (8-12 hours) after EBV infection of primary B-cells, in parallel with EBNA-2. We have found previously that EBNA-5 binds to p14ARF, one of the main upstream regulators of the p53 pathway. In order to study the EBNA-5 effect on the p14ARF-induced growth arrest and apoptosis pathway, we used three different cell systems, MCF7 with wild type p53, DG75 with mutant p53, and Saos-2 with no p53. p14ARF could induce growth arrest and cell death in all three systems. It has been shown that p14ARF could arrest cells/induce apoptosis independently of p53 through the targeting of E2F [Qi, Y., et al., Nature, 2004, 431, 712-7] or p34 (cdc2) [Normand, G., 2005, JBC, 280, 7118-30]. We have shown that EBNA-5 reduces the apoptosis inducing effect of p14ARF significantly. In the MCF7 cells p14ARF accumulated in nuclear but extra-nucleolar inclusions where it co-localized with p53, MDM2 and Hsp70. Co-transfection of p14ARF and EBNA-5 led to the complete relocation of homogeneous nuclear EBNA-5 into the p14ARF inclusions. The p14ARF-induced accumulation of EBNA-5 in the nuclear inclusions was also observed in NIH-3T3, HeLa, SW480 and Saos-2 cells. The p14ARF containing nuclear inclusions also attracted the PML bodies and the 20S proteasome subunits. Our hypothesis was that EBNA-5 could bind p14ARF-p53 complex and promote it degradation. Most recently we have found that EBNA-5 could bind directly to MDM2 in lymphoblastoid cell lysates, as shown by GST pull down assays and SPR. To study the architecture of multimeric protein complexes we used our SPR based assay. We have shown the formation of a ternary complex that contained MDM2, p14ARF and EBNA-5 (p53). We compared the p53 half-life in the MCF7 cells, and MCF7 cell that expressed pBabe vector control or pBabe-EBNA-5 constitutively. We observed the decrease in the p53 half-life upon EBNA-5 expression. Based on our findings, we suggest that EBNA-5 may interact directly with p14ARF-MDM2-p53 tri-molecular complexes and thereby contribute to the downregulation of p14ARF and p53 protein 3-5 days after infection that we have found earlier. We are carrying out the experiments on the infected peripheral blood B-cells, infected with EBV and stimulated with anti-CD40 and IL-4 now. We shall also study the influence of EBNA-5 on the of MDM2 protein.

**6.004**

**EBV-encoded EBNA-6 Protein Binds to MRS18-2 and Controls pRb-E2F Association**

M. Yurchenko[1], B. Snopok[2], L. Szekely[3], G. Klein[3], **E. Kashuba[3]**. [1]Institute of Experimental Pathology, Oncology and Radiobiology NASU, Kyiv, Ukraine; [2]Institute of Semiconductor Physics NASU, Kyiv, Ukraine; [3]MTC, Karolinska Institute, Stockholm, Sweden

EBNA-6 is believed to play a role in cell cycle control. It was found to bind to cyclin A and to the E3 ubiquitine ligase SCF complex (Skp2 and Roc1 proteins). In association with cyclin A, EBNA-6 targets p27 for degradation [Knight, J., et al., 2005, Mol Cell Biol, 25, 1749-63]. EBNA-6 was reported to bind to Rb protein in vitro (GST pull down assay), but this could not be verified in vivo. However, the pRb level decreased upon transient EBNA-6 expression [Parker, G., et al, Oncogene, 1996, 13, 2541-9; Knight, J., et al, PNAS, 2005, 102, 18562-6]. We have found that EBNA-6 binds to MRS18-2, a pRb-binding protein, using the yeast two-hybrid system. Predominantly cytoplasmic MRS18-2 was targeted to the nucleus when EBNA-6 was expressed. These two proteins were co-localized in the nucleus. The MCF7 breast carcinoma that expressed c-myc-MRS18-2 constitutively showed a formation of multinucleated cells with enlarged nucleoli. The total pRb level was decreased significantly when MRS18-2 was expressed. We have shown that MRS18-2 could bind both - hypo- and hyper-phosphorylated - forms of retinoblastoma protein. By GST-pull down assay, we could show that the MRS18-2 - pRb binding was specific, because p107 was not detected as the binding partner for MRS18-2 under the same conditions. To further explore the details of the direct protein-protein interactions we have used Surface Plasmon Resonance (SPR), based on resonant coupling of light energy into a free-electron cloud on a metal surface. We have developed a convenient SPR based approach to study multimeric protein-protein complexes. Using this protocol we have mapped the binding site of the pRb protein (A box - spacer - B box) to MRS18-2. Using SPR and a GST-pull down assay we could show that EBNA-6 can complex with pRb through MRS18-2. MRS18-2 binds to pRb and disrupts the pRb-E2F complex formation: E2F could not be detected on GST-MRS18-2 beads together with pRb. Our hypothesis is that EBNA-6 can regulate the level of free E2F by enhancing the binding of MRS18-2 to pRb, and thereby inhibiting the pRb control of the cell cycle. We shall further reveal the influence of MRS18-2 overexpression on the pRb-E2F binding and the role of EBNA-6 in this multiprotein complex, using EMSA, modified CHIP-assay, and immunoprecipitation.

**6.005**

## hTERT Contributes to the Establishment of EBV Latency In B Lymphocytes by Inhibiting Viral Replication and Promoting Cell Proliferation

**R. Dolcetti**[1], L. Terrin[2], I. Corradini[2], S. Indraccolo[2], J. Dal Col[1], R. Bertorelle[2], L. Bonaldi[2], G. Esposito[2], A. De Rossi[2]. [1]CRO-National Cancer Institute, Aviano, Italy; [2]Dept. of Oncology and Surgical Sciences, Section of Oncology, University of Padova, Padova, Italy

**Background:** Transformation of primary B lymphocytes by EBV requires the establishment of a strictly latent infection, the expression of several latent viral protein and a sustained telomerase activity. Nevertheless, the dynamics of telomerase activation and its relationship with latent/lytic EBV gene expression remain to be elucidated.

**Methods:** We investigated the interplay between activation of hTERT, the catalytic rate-limiting component of the telomerase complex, and expression of latent/lytic EBV genes during establishment of a stably latent EBV infection in normal B lymphocytes.

**Results:** B cell cultures at early passages after EBV infection greatly differed for the timing of hTERT expression and telomerase activation. Induction of hTERT was dependent on the balance between latent and lytic EBV gene expression, being positively associated with a high ratio of latent/lytic isoforms of latent membrane protein 1 (LMP1), and negatively associated with the expression of BZLF1 gene, the main activator of the viral lytic cycle. In turn, hTERT expression and telomerase activation was followed by a decrease in EBV lytic gene expression, and viral production. Consistently, induction of EBV lytic cycle with TPA and butyrate caused a dramatic reduction in both hTERT transcripts and telomerase activity. Moreover, ectopic expression of hTERT in BZLF1-positive B cell cultures resulted in BZLF1 down-regulation, increased resistance to lytic cycle induction, and enhanced in vitro growth properties.

**Conclusions:** These findings indicate that hTERT contributes by multiple mechanisms to EBV-driven transformation of B lymphocytes. Furthermore, the finding that hTERT confers increased growth capacity to EBV-carrying B lymphocytes further supports the notion that hTERT may constitute a relevant therapeutic target also for EBV-associated B cell lymphomas.

**6.006**

## The Epstein-Barr Virus Nuclear Antigen (EBNA) -3C Interacts with Homeodomain Interacting Protein Kinase 2 (HIPK2)

**A.G. Burgess**[1], M. Buck[1], T. Hofmann[2], T.B. Sculley[1]. [1]Queensland Institute of Medical Research, Brisbane, Australia; [2]German Cancer Research Center, Heidelberg, Germany

**Background:** EBV transformed cells are able to overcome drug induced cell cycle check points and are resistant to apoptotic cell death. The mechanism by which this is achieved in unknown but several of the EBV Nuclear Antigens (EBNAs) are thought to be required including EBNA-3C.

**Methods:** We have used confocal microscopy, immunoprecipitation, cell cycle analysis and apoptotic assays to determine whether EBNA-3C may prevent apoptosis by binding to homeodomain interacting protein kinase 2 (HIPK2), a serine/threonine kinase involved in transcriptional regulation, growth control and apoptosis.

**Results:** We have identified a novel binding partner for EBNA-3C as HIPK2. HIPK2 is able to induce apoptosis via p53-dependent and independent pathways. Results show that when overexpressed HIPK2 and EBNA-3C co-localize to nuclear bodies while immunoprecipitation has been used to show that these proteins are able to interact, with EBNA-3C binding to both the SUMOylated and unSUMOylated forms of HIPK2. The region of interaction in HIPK2 has been localized to the N-terminus, while the region in EBNA-3C appears to be at the C-terminus.

**Conclusion:** The interaction of EBNA-3C and HIPK2 may be a mechanism by which EBNA-3C is able to interfere with the apoptotic pathways in cells.

**6.007**

## LMP1 induces an epithelial mesenchymal transition (EMT) in MDCK epithelial cells via activation of the ERK-MAPK and PI3-K pathways

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**Background:** Previous studies have shown that LMP1 can induce profound effects on the growth and differentiation of epithelial cells. LMP1 expression is associated with altered cell morphology, a loss of E-cadherin expression, and the acquisition of a more motile phenotype: all features of the epithelial mesenchymal transition (EMT). To better understand the mechanism(s) by which LMP1 induces these effects, we generated stable clones of MDCK cells (the reference cell line for the study of EMT) in which LMP1 and mutant derivatives were stably expressed and examined the cell signalling pathways responsible for this effect.

**Methods:** MDCK epithelial cells were transduced with recombinant retroviruses carrying LMP1, or CTAR1 and CTAR2 defective versions of LMP1. Polyclonal populations were expanded and analysed for the effects of LMP1 expression on the induction of EMT using RT-PCR, western blotting and indirect immunofluorescence. LMP1 expressing cells were analysed for (i) cell morphology, (ii) cell adhesion molecule expression, and (iii) signalling pathways implicated in the generation of EMT. Selective pharmacological inhibitors were used to investigate the contribution of a number of signalling pathways to the induction of the LMP1-induced EMT phenotype.

**Results:** We have found that expression of LMP1 in MDCK cells is associated with EMT induction as evidenced by characteristic morphological changes and by accompanying loss of E-cadherin, desmosomal cadherin and tight junction protein expression. RT-PCR analysis established that LMP1 expression was associated with the induction of SLUG, a transcriptional repressor that targets and negatively regulates the expression of E-cadherin and desmosomal cadherins. The induction of EMT phenotype required an intact CTAR1 domain, as an LMP1 mutant defective for CTAR1 but carrying a functional CTAR2 domain, was unable to induce this change. Chemical inhibition of signalling pathways implicated in the generation of EMT revealed contributions from ERK-MAPK, PI3-K but not tyrosine kinase, NF-kappa-B, JNK or p38.

**Conclusions:** LMP1 induces an EMT in MDCK cells through the CTAR1 domain. Activation of the ERK-MAPK and PI3-kinase pathways are essential for the induction of this phenotype.

**6.008**

**Epstein-Barr Virus Nuclear Protein EBNALP is Critical for Maintaining Lymphoblastoid Cell Line Growth**

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Epstein-Barr virus (EBV) is a ubiquitous human herpes virus and EBV infection causes latent infection and growth transformation of primary B-lymphocytes result in lymphoblastoid cell lines (LCLs). EBV-encoded nuclear antigen 2 (EBNA2) and EBNALP are the first viral protein products expressed after infection of primary B-lymphocytes and are essential for EBV-induced B-lymphocyte growth transformation. EBNA2 functions as a transcriptional activator of viral and cellular genes. EBNALP plays an important role as a coactivator in EBNA2-mediated transcriptional activation. Previously, we discovered that mutant EBNALP that contains a 10-amino acid-truncation at its C-terminus does not have the ability to coactivate and it preferentially interacts with the EBNA2 acidic transactivating domain. Furthermore, the mutant EBNALP has a dominant negative effect on the coactivation activity of the wild-type EBNALP. The functional relevance of EBNALP in maintaining LCL cell growth is yet to be characterized. Therefore, we established cell clones derived from an LCL in which a dominant negative form of EBNALP (DNLP) is conditionally expressed by the Cre-loxP system. This was required in order to analyze the effect of DNLP expression on EBV-induced cell proliferation. After drug addition, the generated LCL clones expressing DNLP showed retardation of cell proliferation and reduced cell viability. The results indicate that EBNALP plays a critical role in maintaining LCL growth and EBV-induced cell proliferation.

**6.009**

## Upregulation of the Novel Cell-cycle Regulatory Gene RGC-32 by EBNA 3C Disrupts Cell-cycle Checkpoints

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**Background:** EBNA 3C has been shown to deregulate the cell-cycle by disrupting checkpoints induced by a variety of stimuli and promoting inappropriate cell-cycle progression. Since the mechanisms through which EBNA 3C exerts its effects have not been fully defined, we carried out DNA microarray analysis to identify cellular genes differentially regulated as a result of EBNA 3C expression.

**Methods:** We used Affymetrix genechip arrays to identify genes differentially expressed in an EBV negative B-cell-line (BJAB) stably expressing EBNA 3C, compared to a control cell-line. Real-time pcr was used to confirm these results. Follow-up cell-cycle studies involved CDK1 immunoprecipitations, H1 kinase assays, the generation of stable and transiently expressing cell-lines, etoposide treatment and cell-cycle analysis using propidium iodide.

**Results:** We discovered that mRNA levels of a gene encoding a novel cell cycle regulatory protein, Response Gene to Complement-32 (RGC-32), were upregulated 6.63-fold in an EBNA 3C expressing cell-line. RGC-32 has been shown to bind and activate the cyclin-dependent kinase, CDK1 (p34cdc2), and to promote S-phase and M-phase entry in serum-starved aortic smooth muscle cells raising the possibility that the upregulation of RGC-32 directly contributes to cell-cycle disruption mediated by EBNA 3C. Real-time pcr analysis of a panel of BJAB cell-lines expressing each of the EBNAs individually confirmed that RGC-32 was significantly upregulated in all three EBNA 3C expressing lines examined and not in control cell-lines and cell-lines expressing EBNAs 1, 2, -LP, 3A or LMP 1. Strikingly, we found that in Raji cell-lines expressing EBNA 3C, RGC-32 expression was upregulated up to 120-fold. These EBNA 3C-expressing Raji cells did not arrest in G2/M in response to etoposide treatment consistent with the disruption of this checkpoint as a result of RGC-32 overexpression. In addition, we have generated cell-lines stably over-expressing RGC-32 and have demonstrated that these lines have increased CDK1 activity and override G2/M arrest induced by etoposide.

**Conclusion:** Since RGC-32 overexpression in B-cells upregulates CDK1 kinase activity and disrupts the G2/M checkpoint induced by etoposide, the upregulation of RGC-32 expression by EBNA 3C is likely to form part of the mechanism by which this viral protein deregulates the cell-cycle and contributes to the EBV-driven immortalisation process.

**6.010**

## Crystal Structure of the BARF1 Oncogene

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**Background:** The Epstein Barr virus is associated with numerous epithelial cancers, principally undifferentiated nasopharyngeal carcinoma and gastric carcinoma. The BARF1 gene is expressed in a high proportion of these cancers. An oncogenic, mitogenic and immortalizing activity of the BARF1 protein has already been shown. BARF1 is a secreted glycoprotein.

**Methods:** The BARF1 protein was expressed in HeLa cells using a recombinant adenovirus vector and purified from the culture media using concanavalin A and gel filtration chromatography. Crystals were grown using the vapour diffusion method and the structure was solved by single anomalous dispersion method using a platinum derivative. The structure was refined to 2.3 A resolution. Solution studies using dynamic light scattering and ultracentrifugation as well as electron microscopy were also performed.

**Results:** the BARF1 protein is composed of two immunoglobulin (Ig) domains that are assembled in hexameric rings and interact using unusual contacts between Ig domains. This hexamerisation is consistent with the solution studies and is also observed in electron microscopy. The structure of the monomer is closely related to the structure of CD80, a co-stimulating molecule found on antigen presenting cells.

**Conclusion:** BARF1 forms hexameric rings in solution as well as in the crystals. It may be evolutionary related to CD80 but still, domain orientation and oligomerisation differ between BARF1 and CD80. It had been shown that BARF1 binds to CSF1, the colony stimulating factor 1, but due to the unusual oligomerisation and contacts of BARF1, this interaction has to be principally different to the one between CSF1 and CSF1 receptor.

**6.011**

## The Placental Specific Gene, PLAC1, is Induced by the Epstein Barr Virus and is Expressed in Human Tumor Cells

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**Background:** Burkitt's lymphoma cell lines containing the Epstein Barr virus have been shown to form tumors in nude mice while clonal derivatives of such cell lines in which the viral genome has been lost do not. EBV contributes to tumor maintenance and survival through multiple mechanisms. In vivo, the interaction between EBV genes with cellular genes may contribute to EBV's tumorigenic properties. In this study, we have attempted to identify cellular genes regulated by EBV that may contribute to its tumorigenic properties.

**Methods:** We have enforced genome loss in the Burkitt's lymphoma (BL) line, MutuI, by introducing a dominant negative form of the episomal replication factor, EBNA1 and carried out gene array analysis followed by RT-real time PCR, western blot, luciferase Assay and immunohistochemistry.

**Results:** One of the genes identified by this analysis is PLAC1, a gene originally identified as being expressed exclusively in placental tissue. Real time RT-PCR analysis verified higher expression in EBV positive vs. EBV negative Mutu clones. Western Blot and immunohistochemistry show that PLAC1 is a membrane protein. Analysis of a panel of RNAs from 20 normal tissues demonstrated the highest level of expression in placenta but significant expression was also observed in testis and brain cerebellum. PLAC1 expression was also observed in non-BL tumor cell lines derived from breast, ovary, and prostate. Lastly, expression of PLAC1 was found to be higher in some primary breast tumors compared to normal adjacent tissues. Of interest, the identify PLAC1 promoter is active in EBV positive cells but not in EBV negative cells. Further experiments to identify which EBV gene involve in PLAC1 promoter activation is in process.

**Conclusion:** This data indicates that PLAC1 is induced by EBV and that the PLAC1 promoter is active in EBV positive cells in vitro. PLAC1 is a member of the cancer/testis group of tumor antigens.

**6.012**

**The Epstein-Barr Virus (EBV) Encoded Gene BDLF2 is an Early Gene Encoding a Membrane Associated Protein Essential for the Immortalization of B-lymphocytes**

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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.

We analyzed the expression kinetics of BDLF2 and, as a reference, of the immediate early gene BZLF1 in cells of the EBV genome negative cell line BJAB after infection with wildtype EBV. BDLF2 transcripts were detected one hour post infection (BZLF1: 30 min) and gained maximal values after four hours (BZLF1: 2 h). The transcript abundance of BDLF2 was significantly lower compared to BZLF1 transcripts. Immunofluorescence assays with a BDLF2-specific polyclonal antibody and cells of the BDLF2-transcript positive cell line B95-8 revealed a membrane associated signal as did the analysis of the localization of a BDLF2/GFP fusion protein by confocal laser microscopy. These results suggest that BDLF2 is an early gene coding for a membrane associated protein.

Using transposon mutagenesis we generated an EBV-BDLF2 knock out mutant. Compared to wildtype EBV the mutant lacked the capacity of triggering aggregation of infected B-lymphocytes. Expression of genes indicative of the viral lytic cycle as BZLF1, BMRF1, BHRF1, and BRLF1 can be detected in mutant-infected B-lymphocytes. The latent genes LMP2, EBNA3A, EBNA3B, EBNA3C, and EBER1 are expressed, while expression of LMP1, EBNA1, and EBNA2 is not detectable. These results were confirmed with BDLF2-specific RNAi experiments. The BDLF2-negative mutant lacks the capacity to immortalize B-lymphocytes. Infected cells show a self-limiting viral activity and die within weeks post infection.

Taken together, the EBV-BDLF2-mutant combines a lack of expression of important latency genes with failure in immortalization of B-lymphocytes suggesting BDLF2 to be essential for immortalization of B-lymphocytes.

**6.013**

## Analysis of EBV-mediated Resistance to Cytotoxic Agents in BI Cells Using Recombinant EBNA3-knockout Viruses

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**Background:** EBNA3A and 3C, but not EBNA3B, are essential for efficient EBV-driven B cell transformation and have been reported to modulate cell cycle regulation. Several EBV-negative Burkitt lymphoma (BL)-derived cell lines are extremely sensitive to cytotoxic drugs, rapidly undergoing cell cycle arrest and cell death. Latent infection of these cells with EBV protects them from both arrest and apoptosis (for example, Wade and Allday, MCB, 2001). The EBNA3s may be the latent genes involved.

**Methods:** In order to determine the role of each EBNA3 in the cellular response to cytotoxic drugs, EBNA3A, 3B and 3C-knockout viruses were made by homologous recombination between a bacterial artificial chromosome (BAC) harbouring the EBV genome and a shuttle vector containing a fragment of the EBV genome with an internal deletion of the appropriate EBNA3 gene. Each knockout virus was reverted by the same method, replacing the appropriate deleted gene. The resulting BACs were stably introduced into 293 cells and infectious virus generated. EBV-negative BL31 cells were infected with these recombinant viruses and EBV-converted lines were established after hygromycin selection. Other EBV-negative lines are being converted in a similar way. EBNA3-knockout and revertant lines have been validated by Western blotting for EBNAs and treated with a variety of cytotoxic agents, including the microtubule poison, nocodazole, and the cdk inhibitor, roscovitine.

**Results:** Preliminary data indicate that both EBNA3A and 3C contribute to the resistance of EBV-positive cells to cell cycle arrest and cell death. The presence of EBNA3A is associated with inhibition of caspase activity and therefore apoptosis, whereas EBNA3C appears to inhibit cell death in mitosis induced by nocodazole.

**Conclusions:** Both EBNA3A and 3C play roles in the resistance of EBV-positive BL cells to cytotoxic agents, whereas EBNA3B has no obvious effect on resistance to the drugs studied to date.

**6.014**

**Long-term Retention of Defective Het DNA with Concurrent Loss of Prototype EBV Genomes Upon Infection of Cultured Epithelial Cells**

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Previous reports have identified the presence of defective, rearranged (heterogeneous or het) EBV DNA in tumor biopsies from sporadic Burkitt lymphoma and Hodgkin lymphoma without evidence for co-existing prototypical viral genomes. Because transfection of the immediate early gene BZLF1 accelerates the loss of EBV episomes from cells, we questioned whether the constitutive expression of BZLF1 consequent to rearrangements in het DNA could be responsible for this unusual pattern of infection. To explore the dynamics of primary infection with inoculums containing mixtures of EBV variants, we exposed the keratinocyte cell line SVK, which had been transfected with CR2 and CIITA, to either a combination of P3HR1-derived prototype and het EBV or to prototype virus alone. By real-time quantitative RT-PCR, latency protein EBNA1, which is required for episomal maintenance, was initially expressed at equivalent levels in both infection groups. However, EBNA1 rapidly declined to undetectable levels in het-infected cells against a background of comparatively high BZLF1 expression. At 100 days post infection, het DNA remained detectable using PCR primers that spanned junctions of its four abnormally juxtaposed EBV DNA segments. By contrast, prototype P3HR1 sequences were no longer amplified from either cell group. At six months in continuous culture, het-positive cells still expressed BZLF1-encoded Zta, as shown by RT-PCR, immunoblotting, and immunofluorescence. Terminal repeat analysis revealed a banding pattern consistent with integration of the het genome as a concatameric trimer, a reiteration that may be required for packaging of the approximately 50kb het genome into infectious particles. Retention of het DNA alone in absence of prototype virus confirms in epithelial cell culture an observation first made in clinical materials, and may be useful in modeling the process by which het DNA impacts EBV latency, persistence, and disease.

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*M. Ding deceased 1/2006

**6.015**

**Proliferative Versus Cytostatic Effect of EBV LMP1 is Linked to Reiteration of Terminal Repeat Sequences**

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EBV is associated with an array of cancers, where it has been assigned a causal role based in part on a uniform number of viral terminal repeats (TRs) in each tumor cell. Acting as a viral marker of clonality, homogeneity of fused TRs supports the argument that EBV infection preceded clonal expansion and was therefore causal. We present evidence in a carcinoma cell line to show that EBV clonality does not exclude the possibility of de novo infection of an established malignancy, cell selection being driven by a TR number that permits optimal levels of LMP1 expression. To evaluate the effect of TR number on LMP1 levels, a recombinant EBV containing a selection cassette in exon 2 of LMP2A was used to infect a carcinoma cell line transfected with CR2 and CIITA. Cell clones were isolated with varying TRs (6TR, 8TR, 12TR) but equivalent episomal copy number. By real-time quantitative RT-PCR, LMP1 transcript levels were inversely related to TR number. Quantitative differences in LMP1 mRNA were mirrored at the protein level. Because LMP1 has been shown to have either proliferative or cytostatic effects at minor fold-differences in physiologic expression, the growth phenotype of these clones was analyzed by quantifying cell population doubling times. Compared to the 12TR clone, which expressed the least LMP1, the growth of the 8TR clone was enhanced. However, further augmentation of LMP1 expression in the 6TR clone resulted in impaired growth, reflecting LMP1-induced cytostasis. Such growth patterns are consistent with our notion of what might be a rapid transition from polyclonal to monoclonal TR in tumor tissue newly amenable to infection by EBV endogenous to the host. Thus, rather than acting as a tumor initiator in all cases of EBV-associated cancer in which clonality has been ascertained, virus may infect pre-existing lesions to contribute to tumor progression.

**6.016**

**A Technical Approach to the Reevaluation of Epstein-Barr Virus Clonality**

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The notion of a causal role for EBV in tumorigenesis is based in part on the analysis of terminal repeat sequences (TR). The reiterated 500 base-pair unit is numerically heterogeneous among infectious virions. Upon infection, circularization of the linear genome by random recombination of TRs generates a variable TR length in resultant episomes that provides a viral measure of clonality in infected cell populations. A uniform length of joined termini in every tumor cell implies infection preceded clonal expansion. Since EBV was present at the genesis of the tumor, it can be argued to have been factorial. Data from our laboratory now provide an alternate interpretation for EBV clonality that is consistent with infection as a sequel to malignant transformation. We predict fortuitous de novo infection of some tumors by virus endogenous to the host, with transition from polyclonal infection of a few tumor cells to monoclonal outgrowth of a subset driven by TR modulation of an optimal LMP2A and/or LMP1 expression. To demonstrate such a transition in vivo, presumed monoclonal TR sequences in 5 diagnostic biopsies of NPC were first visualized by Southern blotting. Subdominant clones were then sought by a PCR approach in which endonuclease-restricted DNA, extracted from agarose plugs obtained along the length of the electrophoretic gel, was amplified with primers specific to TRs. Two of five tumors contained subdominant TR bands. The reiteration of TRs in the minor bands located by PCR greatly exceeded the TR number in the predominant band (n) as well as that which might occur in predicted derivative molecules in the event of low level viral replication: i.e., linear concatenated replicative intermediates (n) or progeny molecules (2n-2). Instead, the MW of subdominant fused TRs was consistent with a representation of subdominant and unrelated episomal clones in cells exhibiting a comparatively reduced growth potential.

**6.017**

## A Proteomic Investigation Into the Role of LMP1Cao in the Early Stages of Carcinogenesis

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In order to model the consequences of latent membrane protein 1 (LMP1) expression in epithelia leading to carcinoma, we have developed transgenic mouse lines expressing LMP1 of the Cao strain of Epstein-Barr virus. LMP1Cao expression is directed to the epidermis using the EBV lytic promoter L2 (Stevenson D et al (2005), Cancer Res., 65, 8826-8835). The most severe phenotype is seen in the ears of the mice and progresses with time. In early stages (0-30 days old), the epidermis becomes more vascularised compared to transgene negative, age-matched controls. Over time this advances to hyperplasia, severe hyperplasia and the tissue becomes degenerative. Keratoacanthomas form and this can sometimes progress on to carcinoma.

We have investigated the global protein effects of LMP1Cao expression at two distinct stages of the phenotype, both the mildly hyperplastic and the necrotic ear stages using a proteomic approach, namely the DIGE (Difference Gel Electrophoresis) technology. 37 proteins have been found to be de-regulated; 16 up-regulated and 21 down-regulated in the L2.LMP1Cao transgenic tissue. Differences include cytoskeletal proteins, proteases, bio-energetic proteins, calcium binding proteins, immunological proteins and proteins of undefined function. Confirmation of a selection of these changes has been conducted by western blotting. These differences and their relation to LMP1 expression and signalling will be presented.

Furthermore, the contribution of LMP1 to carcinogenesis has been investigated in vitro. LMP1 function has been inhibited in various LMP1.B95-8 transgenic carcinoma and lymphoma cell lines by way of a dominant negative form of LMP1, LMP1AAAG (Brennan, et al (2001), JBC, 276, 1195-1203). Loss of LMP1 signalling leads to growth inhibition in both LMP1 positive carcinoma and lymphoma cell lines, but not in LMP1 negative lines. LMP1AAAG expression cannot be sustained in the lymphoma cell lines and apoptosis has been demonstrated as the means of growth inhibition in this setting.

**6.018**

**EBER1, Oncogenic in Vivo?**

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Transfection of the EBERs into Epstein-Barr virus negative Akata cells, which were shown to lose the malignant phenotype and resistance to apoptosis present in EBV positive Akata cells, restored these properties. Thus suggesting an oncogenic role of the EBERs in Burkitt's lymphoma cells and therefore indicating their possible contribution to the disease process of EBV-associated tumours (Komano et al., 1999). Furthermore, Yajima et al., (2005) have recently reported that the EBERs contribute to the transformation process of EBV in primary B cells.

In order to investigate the action of EBER1 as a potential oncogenic RNA, we have generated 13 lines of transgenic mice designed to express EBER1 in lymphoid cells. The transgenes incorporate a novel combination of tissue-specific RNA polymerase II and polymerase III elements and their expression was first confirmed in culture.

Mice of 10 of the transgenic lines have been shown to express EBER1 with specificity towards lymphoid tissues. Mice expressing EBER1 are viable. The phenotypic consequences of EBER1 expression in vivo is being examined and lymphoid expansion in mice of several lines has been observed at a young age as well as the development of B-cell lymphoma in one of the lines to date. The role of EBER1 in response to dsRNA stimulation in vivo is under study. Cross-breeding programmes were undertaken which revealed cooperation in B-cell lymphomagenesis between EBER1 and N-myc although this was not evident with the highly tumourigenic c-Myc line. This might suggest that the oncogenic predisposition in the EBER mice is elicited through a cell survival mechanism.

The results in this study support the hypothesis that EBER1 has oncogenic properties, the first polymerase III RNA described as such. Thus implicating the RNA in the pathogenesis of EBV associated lymphoma in addition to, or possibly as a consequence of, its role in immune evasion.

**6.019**

## Epstein-Barr Virus (EBV) Latent Membrane Protein-1 (LMP-1) Oncogenic Activity is Down-Regulated by Lytic LMP-1 (LyLMP-1)

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**Background:** The Epstein-Barr virus (EBV) is an oncogenic human herpesvirus. EBV latent membrane protein 1 (LMP-1) is a viral oncogene that manifests its oncogenic phenotype through activation of cellular signaling pathways involved in cell growth, survival, differentiation, and transformation. Lytic LMP-1 (lyLMP-1) is a related EBV gene without oncogenic properties. The lyLMP-1 gene is found in 60% of the EBV strains circulating in nature, but it is not found in EBV strains associated with nasopharyngeal carcinoma. We recently demonstrated that lyLMP-1 down-regulates the half-life of LMP-1 in epithelial cells. Therefore in this study, we tested the hypothesis that lyLMP-1 concomitantly down-regulates LMP-1 oncogenic activity.

**Methods:** LMP-1 signaling activation was measured for the NF-kB, JNK/AP-1 and JAK/STAT pathways using dual-luciferase reporter assays in transiently transfected RHEK-1 epithelial cells. LMP-1 effect on the oncogenic phenotype was examined by epithelial cell growth and survival assays and rodent fibroblast cell transformation assays. Western blot and flow cytometry were performed with fluorescent fusion proteins of LMP-1 and lyLMP-1 to evaluate the mechanism of the lyLMP-1 effect on LMP-1.

**Results:** Lytic LMP-1 inhibited LMP-1 oncogenic activity in a statistically highly significant and dose-dependent parallel manner across all functional assays, including signaling activation, epithelial cell growth and survival, and fibroblast cell transformation. Western blot and flow cytometry studies demonstrated that lyLMP-1 manifested this down-regulatory effect through the promotion of LMP-1 degradation and a reduction in the expressed quantity of LMP-1.

**Conclusion:** Lytic LMP-1 functions as a post-translational negative regulator of LMP-1 oncogenesis. These results support a model of EBV-associated epithelial oncogenesis in which lyLMP-1 may act in vivo to reduce the risk of LMP-1-mediated transformation and is therefore subjected to negative selection in nasopharyngeal carcinoma pathogenesis.

**6.020**

## EBNA3C Disrupts the G2 Phase of Cell Cycle by Interacting with Chk2 in the ATM/ATR Pathway

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**Background:** The Epstein-Barr virus (EBV) infects most of the human population and persists in B-lymphocytes for the lifetime of the host. During the establishment of latent infection a unique repertoire of genes are expressed. The EBV nuclear antigen EBNA3C is essential for growth transformation of primary B-lymphocytes in vitro and regulates the transcription of a number of viral and cellular genes important for the immortalization process. This study demonstrates another associated function of EBNA3C which involves disruption of the G2/M checkpoint.

**Methods:** This was tested in LCLs and BJAB cells. We use nocodazole, a drug used to block the cell at G2/M checkpoint of cell cycle. Cell cycle distributions were analyzed by flowcytometry. We also use western blot and immunofluoresce to prove our hypothesis.

**Results:** We show that lymphoblastoid cell lines (LCLs) treated with a G2/M inhibiting drug nocodazole, did not show a G2/M checkpoint response. However, these cells display an increase in cell death, a characteristic due to sensitivity of the cells to the cytotoxic effects of the drug. Cell cycle analysis demonstrated that expression of EBNA3C is capable of disrupting the G2/M checkpoint response induced by nocodazole resulting in increased toxicity. The G2 arrest in response to this drug was rescued by caffeine, suggesting an involvement of ATM/ATR signaling in this checkpoint. We show that direct interaction of EBNA3C with Chk2 is responsible for this G2 phase disruption.

**Conclusion:** Overall our data suggest a role for EBNA3C in regulating components of the host cell cycle machinery.

**6.021**

**Proteomic Analysis of B Lymphocytes Expressing Latent Membrane Protein-1**

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**Background:** Latent membrane protein-1 (LMP-1) expression is essential for transformation of B lymphocytes. The cellular factors involved in the process of transformation are incompletely defined.

**Methods:** EBV-negative Mutu cells were stably transfected with wild-type LMP-1 and control retroviral vectors. Cellular proteins were fractionated into cytoplasmic, membrane/organelle and nuclear/cytoskeletal fractions. Fractions were labeled with Cy3 (control cells) or Cy5 (LMP-1 expressing cells) and subsequently analyzed for protein expression by 2D difference gel electrophoresis (DIGE). Gel spot intensity was analyzed using DeCyder differential analysis software. In addition, total RNA was prepared and analyzed for expression of cellular miRNA transcripts by microarray chip containing probes specific for 328 miRNA species.

**Results:** Mutu cells expressing LMP-1 demonstrated increased expression of 21 proteins and decreased expression of 8 proteins isolated in the cytoplasmic fraction, when compared to control Mutu cells. No significant differences were observed in the nuclear/cytoskeletal and membrane/organelle fractions of Mutu cells with or without LMP-1. Fifteen species of miRNA were expressed at >2-fold higher levels in lymphocytes expressing LMP-1 compared to control cells. LMP-1 was associated with >2-fold reduced expression of six species of miRNA.

**Conclusion:** Complementary techniques such as proteomics and miRNA microarray analysis may help elucidate the cellular components involved in LMP-1 dependent transformation of B lymphocytes.

**6.022**

## Increase of Genomic Instability by EBV DNase in Human Epithelial Cells and B Lymphocytes

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Genomic instability is the hallmark of human malignancies. It has been considered either as a cause or result of carcinogenesis. Gene products of many human viruses have been demonstrated to induce genomic instability in respective associated tumors. We had demonstrated that EBV LMP1 induces genomic instability through repression of DNA repair in human epithelial cells. It was further elucidated that LMP1 represses p53 transcriptional transactivation and contributes to the nucleotide-excision repair-associated instability (NIN). In this study, we demonstrate that another EBV gene product, DNase, is able to induce genomic instability both in human epithelial cells and B lymphocytes.

HEp2 cells were transfected with EBV DNase expressing plasmid, pEGFP/BG9. DNA strand breaks and micronucleus formation were increased using staining of r-H2AX and DAPI. A microsatellite instability assay was carried out using pBsd4-puro containing CA repeats out of frame and downstream of initiation codon of blasticidin resistance gene. Blasticidin resistant clones were significantly increased in HEp2 cells expressing EBV DNase. The results suggest EBV DNase might contribute to the genomic instability of human epithelial cells through induction of microsatellite instability (MIN).

Raji cells were transfected with an EBV DNase expression-inducible plasmid pIN-BG9 to obtain RB68 cells. Induction of EBV DNase resulted in DNA strand breaks as detected by comet assay. HPRT mutation clones were significantly increased under HAT medium selection in RB68 cells than the control Raji cells which did not express active EBV DNase. The results indicated EBV DNase might also contribute to genomic instability and carcinogenesis in human B lymphocytes.

We conclude that, in addition to LMP1, EBV may contribute to genomic instability and carcinogenesis by EBV DNase.

**6.023 (cancelled)**

**LMP1, LMP2A and CD40 Are All Essential for EBV-associated Lymphoma Cell Survival**

I. Guasparri, **E. Cesarman**. Weill Medical College of Cornell University, New York, NY, USA

LMP1, LMP2A and CD40 induce signals that lead to activation of the transcription factor NF-kB. Since inhibition of NF-kB leads to the apoptosis of EBV-infected lymphoblastoid and lymphoma cell lines, we sought to determine whether elimination of one or more of these signals is sufficient to induce apoptosis of lymphoma cells expressing this proteins.

We developed and tested siRNA targeting LMP1 and LMP2A, which were transfected into two EBV positive (Type III)-lymphoma cell lines (IBL-1 and BCKN1) and two lymphoblastoid cell lines (RPMI-8402 and LCL-9001). LMP1 or LMP2A suppression with siRNA resulted in inhibition of basal NF-kB by approximately 75% in the Type III cell lines tested, indicating that a significant proportion of the constitutive NF-kB activity is being induced by both LMP1 and LMP2A. Simultaneous elimination of both LMP1 and LMP2A showed no significant additive or synergistic effect. Elimination of LMP1 and or LMP2A was sufficient to induce apoptosis of EBV infected cells, as evaluated by flow cytometry for Annexin V after suppression of these proteins. No effect was seen in LMP1 negative controls. Simultaneous suppression of both proteins resulted in similar levels of apoptosis, with no additive or synergistic effect. As EBV+ lymphoma cell lines express CD40, as well as CD154 (CD40 ligand) causing an autocrine loop, we hypothesized that inhibition of CD40 signaling may enhance the apoptotic effect induced by suppression of LMP1 and LMP2A. Treatment with blocking antibody to CD154 (CD40 ligand) in conjunction with LMP1 or LMP2A suppression increased apoptosis by approximately 20%. These data indicate that CD40, LMP-1 and LMP2A are critical for survival of EBV-infected lymphoma cells expressing these proteins. Thus CD40 represents a potential cellular target, and LMP1 and LMP2A may be useful viral targets for the treatment of EBV-associated lymphomas.


# Abstracts

**Sunday, July 9, 2006**
**Room: Poster Area/Level 3**

## Session 7: Poster Session I
## Signal Transduction
## 20:00-22:00

**7.001**

## BS69, a Specific Adaptor in the Latent Membrane Protein 1-Mediated c-Jun N-Terminal Kinase Pathway

**W. Zhang**. HKUST, Hongkong, China

We previously demonstrated that the Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) potently activates the cellular c-Jun N-terminal kinase (JNK) pathway by sequentially engaging an unknown adaptor, TRAF6, TAB1/TAK1, and JNKKs. We now show that BS69, a MYND domain-containing cellular protein, is the missing adaptor that bridges LMP1 and TRAF6, as the MYND domain and a separate region of BS69 bind to the carboxyl termini of LMP1 and TRAF6, respectively. While LMP1 promotes the interaction between BS69 and TRAF6, the complex formation between LMP1 and TRAF6 is BS69 dependent. A fraction of LMP1 and BS69 is constitutively colocalized in the membrane lipid rafts. Importantly, knockdown of BS69 by small interfering RNAs specifically inhibits JNK activation by LMP1 but not tumor necrosis factor alpha. Although overexpression of either BS69 or a mutant LMP1 without the cytoplasmic carboxyl tail is not sufficient to activate JNK, interestingly, when BS69 is covalently linked to the mutant LMP1, the chimeric protein restores the ability to activate JNK. This indicates that the recruitment and aggregation of BS69 is a prerequisite for JNK activation by LMP1.

**7.002**

**The Putative Leucine Heptad Motif in the First Membrane-spanning Domain of LMP1 is Important for LMP1's Trafficking and Signaling**

**J. Lee**, B. Sugden. University of Wisconsin-Madison, Madison, WI, USA

The latent membrane protein 1(LMP1) of Epstein Barr virus (EBV) is important for maintaining proliferation of EBV-infected B cells (Dirmeier et al.,Oncogene, 2005). LMP1 shares features with CD40 and other TNFR-1 receptor family members in that it signals by interacting with TRAF and TRADD molecules to activate NF-kB, AP-1, and JAK-STAT pathways. LMP1 needs both to aggregate and to traffick to lipid rafts to signal efficiently, and both of these functions are mediated by its transmembrane domains (Gires et al., EMBO J, 1997, Higuchi et al., Proc Natl Acad Sci USA, 2001, Kaykas et al., EMBO J, 2001).The first membrane-spanning domain of LMP1 encodes a putative leucine heptad motif, a motif reported to be important for protein-protein interactions. A derivative of LMP1 encoding substitution mutations of this putative leucine heptad motif (LZ1) fails to signal through NF-kB (Kaykas et al., J Virol, 2002).

We have further examined the role of the putative leucine heptad motif in LMP1's trafficking and signaling using both biochemical and genetical approaches. First, LZ1's raft localization was studied by a flotation assay using sucrose gradients. The percentage of total LZ1 found in raft fractions was reduced to 25% of that of wild-type LMP1. LZ1's defect in both homing to lipid rafts and signaling through NF-kB could be complemented by a mutant of LMP1 with wild-type six membrane-spanning domains but no carboxy-terminal signaling domain. Second we made a recombinant EBV that encodes LZ1 instead of LMP1(LZ1-EBV) by homologous recombination (Dirmeier et al.,Cancer Res,2003) to test if LZ1 can support proliferation of B cells in the presence of the other EBV genes normally expressed. LZ1-EBV induces and/or maintains proliferation of B cells only 60% as efficiently as wt EBV. LZ1 accumulates more than wild-type LMP1 in order to compensate its inefficient signaling in B cell clones harboring LZ1-EBV. However, there was no difference in the average virus copy numbers per clone or the percentage of cells that formed cap-like structures of LMP1 when compared to clones harboring wild-type EBV.

Together, these findings show that the putative leucine heptad motif in the first membrane-spanning domain of LMP1 is important for both LMP1's trafficking to lipid rafts and signaling to induce and/or maintain proliferation of B cells.

**7.003**

**Phosphorylation and Nuclear Translocation of STAT3 Regulated by EBV-LMP1**

Y.P. Liu, **Y. Cao**. Cancer Research Institute, Xiang-Ya School of Medicine, Central South University, Chang Sha, China

Latent membrane protein 1 (LMP1), an important protein encoded by Epstein Barr virus (EBV), has been implied to link with the pathogenesis of nasopharyngeal carcinoma (NPC). Our findings suggest that in NPC cell lines, EBV-LMP1 can up-regulate tyrosine705 phosphorylated STAT3 and serine727 phosphorylated STAT3 and promote their nuclear accumulation through different signaling pathways. Further LMP1 up-regulates tyrosine705 phosphorylated STAT3 via JAK3 signaling pathway and up-regulates serine 727 phosphorylated STAT3 through MAPK/ERK other than MAPK/JNK signaling pathway. These results make STAT3 a key molecule to combine JAK/STAT and MAPK/ERK signaling pathways and hence to extend the function of EBV LMP1 to develop the pathogenesis of EBV related tumors from a new view.

**7.004**

**Blockade of AP-1 Activity by Dominant-negative TAM67 Can Abrogate the Oncogenic Phenotype in Latent Membrane Protein 1-positive Human Nasopharyngeal Carcinoma**

X. Jin, **Y. Cao**. Cancer Research Institute, Xiang-Ya School of Medicine, Central South University, Chang Sha, China

Although AP-1 transcription factors play an important role in mediating metastasis for nasopharyngeal carcinoma, the biological and physiological functions of AP-1, in relation to the oncogenic phenotype of nasopharyngeal carcinoma, are not fully understood. Our previous study showed that the latent membrane protein 1 induced high AP-1 activity and mediated a primary dimer form of c-Jun and JunB. In this study we used HNE2-LMP1 cells that express a specific inhibitor of AP-1, a dominant-negative c-Jun mutant (TAM67), to disrupt the interaction of c-Jun and JunB to investigate the role of AP-1 in regulating the nasopharyngeal carcinoma oncogenic phenotype. First, we observed that TAM67 inhibited cell growth in vitro and in vivo and caused a block in the G1 phase of the cell cycle. Next, using Western blotting and immunohistochemistry, we discovered that TAM67 impaired the cyclin D1/cdk4 complex but had little effect on the cyclin E/cdk2 complex in vitro and in vivo, concomitantly with inhibiting Rb phosphorylation and reducing E2F activity. In addition, RT-PCR and luciferase assay results demonstrated that the levels of cyclin D1 mRNA and the promoter activity in TAM67 transfectants were reduced as compared with parental cells. The electrophoretic mobility shift assay and supershift results suggest that c-Jun and JunB bind directly to the cyclin D1 promoter. Thereby, we show that c-Jun and JunB are involved in the transcriptional regulation of the cyclin D1 gene. They also represent an important step toward the design of a new generation of compontents, like TAM67, as inhibitors of c-Jun and JunB interaction.

**7.005**

**Identification of Novel Phosphoproteins in Signaling Pathways Triggered by Latent Membrane Protein 1 Using Functional Proteomics Technology**

G.R. Yan, **Y. Cao**. Cancer Research Institute, Xiang-Ya School of Medicine, Central South University, Chang Sha, China

Previous studies have shown that the Epstein-Barr virus (EBV)-encoded latent membrane protein1 (LMP1) could activate NF kappa B, AP-1 and JAK/STAT pathways. However, many signaling molecules and downstream target proteins triggered by LMP1 have not been identified. To determine the functional components in signaling pathways triggered by LMP1, we combined the novel strategy of phosphoprotein enrichment with proteomics technology to elucidate the signaling cascade activated by LMP1. We found that LMP1 could increase the quantity of total phosphoproteins by 18.03% and 43 proteins showed significant changes in the degree of phosphorylation when LMP1 was expressed. Twenty-five signaling molecules or downstream targets of signaling pathways triggered by LMP1 were identified, several of which had previously been implicated in LMP1 signal pathways. The other proteins, including annexin A2, Hsp27, stathmin, annexin I, basic transcription factor 3 (BTF3) and porin, were novel signaling molecules or targets with no previously known function in LMP1 signal transduction. The method used here has proven to be suitable for the identification of molecules involved in various signaling pathways.

**7.006**

## Induction of EBV Lytic Cycle Alters the Program of Cellular Gene Regulation Stimulated Following Ligation of Surface Immunoglobulin

P. Broderick[1], M. Hubank[2], **A.J. Sinclair**[1]. [1]University of Sussex, Brighton, United Kingdom; [2]Insitute of Child Health, London, United Kingdom

**Background:** Stimulation through immunoglobulin receptors on the surface of B-lymphocytes induces latent EBV to enter the lytic or replicative cycle. The stimulation results in the expression of two viral transcription factors which activate the expression of further EBV genes and promote lytic cycle entry. However, stimulation through immunoglobulin receptors is also known to profoundly alter the expression of cellular genes and it is not known whether this further influences the EBV lytic cycle or whether EBV modulates the response in order to optimise the cellular environment for lytic replication. We address these questions here.

**Methods:** Host gene expression in EBV positive and EBV negative BL cells and their expression four hours post-stimulation through the immunoglobulin receptor were identified and compared by screening Affymetrix chips in triplicate and analysing the resulting data using Genespring software.

**Results:** A direct comparison of unstimulated EBV positive and negative cells revealed that EBV down regulates the expression of components of the TLR signal transduction pathway. One example is TLR9, which is responsible for sensing cytoplasmic DNA, which may be formed during EBV replication. We observed that myd88, a component of the TLR9 signal transduction pathway, is regulated at both the RNA and protein levels and this correlates with reduced signal transduction through TLR9. Furthermore, we observed that a sub-set of interferon response genes, including OAS1 and OAS2 were down regulated by EBV. The effect of this would be to reduce the synthesis of 2' 5'-oligoadenylate (2-5A) and so contribute to the previously observed down regulation of the normal interferon response to viral infection.

The most surprising result was the observation that EBV appeared to inhibit the normal global changes in gene expression observed following immunoglobulin stimulation. Of particular interest were a set of genes normally involved in up regulating interferon induced gene expression in response to immunoglobulin stimulation; this was blocked in EBV positive cells.

**Conclusion:** EBV mounts a concerted attack on the anti viral signal transduction during both latency and the viral lytic cycle. This will increase the potential for cells harbouring EBV to survive in vivo.

**7.007**

## Zta Physically Interacts with the Cellular DNA-Damage Response Protein 53BP1 In Vivo

**S.G. Yarranton**[1], M. Meyer[1], A. Doherty[2], A.J. Sinclair[1]. [1]University of Sussex, Brighton, United Kingdom; [2]Genome Damage and Stability Centre, University of Sussex, United Kingdom

**Background:** Zta (BZLF1) is a key regulator of the EBV lytic cycle. Zta is multifunctional in its mode of action: it is a transcription factor; a replication factor; it interacts with cell signalling pathways and it can perturb cell cycle control. These functions depend on the C-terminal half of Zta, which includes the basic region (DNA-binding), the ZIP region (coiled-coil dimerisation interface) and the adjacent CT region (aids multimerisation). We undertook a proteomics approach to identify cellular proteins that interact with these key regions of Zta.

**Methods:** A vector was designed to express the bZIP and CT regions of Zta in fusion with the dual tags of protein A and calmodulin binding protein. HEK-293 cells were engineered to express this construct and Zta and associated proteins were purified using Tandem affinity purification and identified by Nano-LC coupled mass spectrometry. Association assays were undertaken in vitro using labelled Zta and GST-53BP1 and in vivo using his-tagged Zta and endogenous 53BP1.

**Results:** Ten cellular proteins that interact with Zta were identified. The association with one of these, 53BP1, has been followed up to date. 53BP1 interacts with full length Zta in vivo. Domain mapping of 53BP1 and Zta revealed that the bZIP-CT region (aa 112-245) of Zta interacts with a specific region of 53BP1 (aa1052-1709), which includes the Tudor and myb domains. We are currently mapping the specific requirements of Zta for the interaction with 53BP1, using a series of Zta mutants in the bZIP CT region with defined functional defects. This will allow us to identify the potential involvement of 53BP1 for transactivation, replication, cell signalling and cell cycle modulation.

**Conclusions:** 53BP1 is associated with the cellular DNA damage response specifically following double strand DNA breaks and it signals to the ATM pathway resulting in cell cycle arrest and either DNA repair or apoptosis. The onset of the EBV lytic cycle has recently been shown to elicit activation of the ATM DNA-damage response pathway, although no signalling is observed beyond p53. Therefore, the interaction of Zta with 53BP1 may act to modulate signal transduction through the ATM pathway, perhaps by inhibiting the apoptotic response.

**7.008**

## The Effect of MAP Kinase Pathway on p53 Phosphorylation Triggered by Epstein-Barr Virus Latent Membrane Protein 1

**L.L. Li**[1], Y.G. Tao[1], G.R. Yan[1], M. Ye[1], H. Zheng[1], M. Tang[1], S.W. Tsao[2], Y. Cao[1]. [1]Cancer Research Institute,Central South University, Changsha,Hunan, China; [2]Department of Anatomy, Faculty of Medicine, University of Hong Kong,, Hong Kong, China

**Background:** EBV encoded latent memebrane protein 1 (LMP1), an oncogenic protein, plays an important role in the carcinogenesis of NPC. p53 is an important tumor-suppressor gene, phoshporylation of p53 protein is likely to play the key role in regulating its activity. Mechanisms other than mutation can cause p53 inactivation in human malignancies. In NPC, p53 protein accumulates but mutation of p53 gene is not common. It is also reported that the expression of p53 correlated with malignant stage. The molecular mechanisms leading to stabilization of p53 have not been completely elucidated. Here, we determined the role of MAP kinase in LMP1 induced phosphorylation of p53.

**Methods:** In this study, we chose immortalized nasopharyngeal carcinoma cells NP69, and respectively stable transfected control vector (NP69-pLNSX), LMP1 plasmid (NP69-LMP1) as an experimental model, to examine the potential role of MAP kinase activation in regulation of the p53 response to LMP1.

**Results:** We presented data to show that p53 was not mutated in the carcinogenesis of NPC. LMP1 could upregulate the expression of p53 protein and promote the accumulation of p53 protein in the nucleus. Ionizing radiation (5Gy) and reporter gene assay indicated that p53 was functional in LMP1-expressing cells. More interestingly, p53 could be activated and phosphorylated clearly at serine 15, serine 20, serine 392 and threonine 81 induced by LMP1. Transient transfection assay further confirmed that LMP1 could regulate the phosphorylation of p53. Kinase activity assays showed that LMP1 could regulate MAP kinase activity obviously. We also provided evidence that LMP1-induced phosphorylation of p53 at serine 15 was directly by ERKs kinase; at serine 20 and threonine 81 by JNK kinase, at serine 392 by p38 kinase. We found that LMP1 could promote ERK, JNK and p38 to form the complex with p53 following the initiation of LMP1 expression. By pretreatment of cells with PD98059, SB202190 or SP600125, our data further showed that LMP1-induced p53-dependent transcription activity was blocked. The amount of MDM2 in the p53 immunoprecipitates triggered by LMP1 remained at a low level and increased by kinase inhibitors.

**Conclusion:** These results strongly suggest that ERKs, JNK and p38 kinase have a direct role in LMP1-induced phsophorylation of p53 at multiple sites, which provide a novel view for us to understand the mechanism of the accumulation of p53 in the carcinogenesis of nasopharyngeal carcinoma.

7.009

## Transforming Growth Factor-Beta 1 Stimulates Epstein-Barr Virus Reactivation by Mechanisms which Require ERK1/2 MAPK and NF-kB Pathways

**V. Ramirez**[1], W. Zhang[1], C. Cochet[1], H. Arbach[1], A. Mauviel[2], R. Sierra[3], I. Joab[1].
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**Background:** Higher risk of Non-Hodgkin's lymphoma (NHL) occurs in immunocompromised individuals with Epstein - Barr virus (EBV) reactivation. EBV, an ubiquitous human herpes virus, can infect cells in either a latent or lytic state. Entry into the lytic cycle is triggered by expression of the EBV immediate-early gene, BZLF1, which codes for ZEBRA protein. This protein functions as transcriptional factor; it also activates expression of cellular genes that are thought to assist the virus in modulating the host immune response. A role for lytic infection in the development of lymphoproliferation in nuce mice was recently demonstrated. The treatment of different EBV-positive Burkitt's lymphoma cell lines (BLL) with Transforming Growth Factor-beta 1 (TGF-b1) induces a time-dependent activation of BZLF1 transcription with a corresponding increase in the production of the ZEBRA protein. This study was undertaken to identify the pathways involved in TGF-b1 signalling leading to ZEBRA expression.

**Methods:** The effects of the TGF-b1 on the viral reactivation were studied in different BLL: MutuI, SavI and KemI. The expression of ZEBRA was estimated by Western blot. The MAPK kinase, Smad1 and Smad2 activation was estimated by Western blot. The NF-kB activity was estimated by EMSA using a probe corresponding to NF-kB binding site on the Ig promoter; the NF-kB subunits involved were identified by supershift experiments.

**Results:** The use of inhibitors of intracellular routes MAPK (U0126) and NF-kB (BAY11-7082, Triptolide and IKK2 Inhibitor V), showed that these pathways are involved in the induction of the lytic cycle by TGF-b1. Moreover as early as 5 minutes after stimulation, TGF-b1 induces Smad1 and Smad2 phosphorylation. TGF-b1 could then transduce signals through ALK1 (Smad1) and ALK5 (Smad2). Smad1 and Smad2 phosphorylation was followed by transient activation of Rel A (p65), ERK1/2 was then phosphorylated and finally this activation is followed by NF-kB p52 unit activation suggesting the use of the non canonical NF-kB signaling pathway.

**Conclusion:** All these processes occurring during the early events of EBV reactivation are compatible with anti-apoptotic signals leading to increase of survival, migration and proliferation.

The characterization of the signaling pathways involved in the induction of the cycle lytic by TGF-b1 will allow us to target elements allowing a pharmacological action appropriate to modulate this action.

**7.010**

**LMP2A and LMP2B Modulate Interferon Signalling in Human Epithelial Cells Through Selective Targeting of p48, a Component of the ISGF3 Complex**

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**Background:** Whilst the association of Epstein-Barr virus (EBV) with the development of certain carcinomas is well documented, the precise role that individual EBV gene products play in the carcinogenic process remains unknown. The EBV-encoded latent membrane 2A (LMP2A) and LMP2B proteins are frequently expressed in EBV-positive carcinomas (NPC and gastric adenocarcinoma), suggesting that they play an essential role in epithelial cell growth transformation and/or in the maintenance of the virus-transformed state. Our recent findings have uncovered a novel function for these proteins in epithelial cells, namely, the ability to modulate signalling from type I and type II interferon receptors. These findings suggest that LMP2A and, to a lesser extent, LMP2B, may exert more 'global' effects on signal transduction pathways in EBV-infected epithelial cells than previously thought.

**Methods:** Epithelial cells stably expressing LMP2A/2B were generated by retroviral transduction. The impact of LMP2A/2B expression on interferon signalling was analysed by ISRE/GAS luciferase reporter assay. Western blotting, RT-PCR, immunofluorescence staining, and FACS analysis was used to analyse the effects of LMP2A/2B expression on the induction of interferon stimulated genes.

**Results:** We found that LMP2A/2B-expressing epithelial cells showed decreased responsiveness to interferon (IFN-$\alpha$ and IFN-$\beta$) as assessed by ISRE and GAS promoter activation. Furthermore, LMP2A/2B-expressing cells displayed weaker induction of 'classical' interferon targets (2'5'-OAS, IRF-7, PKR, and MHC). Although the levels of interferon receptors and receptor-associated signalling components were similar, the levels of p48/IRF9 were significantly lower in LMP2A/2B-expressing cells. Preliminary data suggest that both LMP2A and LMP2B modulate interferon signalling by promoting the degradation of p48.

**Conclusion:** Here we describe novel findings showing that LMP2A/2B functions to attenuate interferon signalling in EBV-infected epithelial cells through a mechanism which involves the selective targeting of p48. Taken together, these findings suggest that LMP2A/2B may dampen 'anti-viral' responses elicited by EBV and serve to protect virus-infected epithelial cells from immune recognition.

**7.011**

## EBV Latency III immortalization Program Sensitizes B-Cells to Induction of CD95 Mediated Apoptosis via LMP1: Role of NF-κ, STAT1 and p53

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EBV induces CD95 expression and the CD95 gene is regulated by NF-κB, STAT1 and/or p53. To understand the contribution of these factors in the regulation of CD95 by EBV in lymphoblastoid cells (LCL), we cloned dominant active I-κB, active (STAT1 β and inactive (STAT1 α) forms of STAT1, p53, a dominant negative mutant of LMP1 and wild type LMP1 into a novel double inducible episomal vector, pRT-1. These plasmids were stably transfected either into wild type LCLs or EREB2-5 cells, an LCL with an estrogen-regulatable EBNA2 protein. Inhibition of LMP1 signaling decreased expression of CD95, whereas over-expression of LMP1 markedly increased it. Induction of the latency III program in EREB 2-5 cells correlated with activation of NF-κB, STAT1 and p53. CD95 expression was regulated by these 3 transcriptional systems. STAT1 and p53 activation were secondary to NF-κB, activation. CD95 surface expression sensitized EBV-infected B-cells to the induction of CD95-mediated apoptosis. In vitro inhibition of CD95-CD95 ligand interaction was found to reverse T-cell killing of EBV infected B-cells. Therefore, LMP1 activation of NF-κB, sensitizes infected B-cells to CD95-mediated apoptosis and renders EBV-latency III immortalised B-cells susceptible to elimination by the immune system, contributing to the establishment a host/virus equilibrium.

**7.012**

**Myc Impairs Interferon Induction in EBV Infected B Cells by Downregulating IRF7**
**G.W. Bornkamm**. GSF - Nat. Research Center Inst. Molec. Biol. and Tumour Genetics, Munich, Germany

Deregulation of the proto-oncogene c-myc is a key event in the pathogenesis of many human tumors. A paradigm is the activation of the c-myc gene by chromosomal translocations in Burkitt lymphoma (BL). Despite the expression of Epstein-Barr viral (EBV) antigens, BL cells are not recognized by antigen-specific cytotoxic T cells (CTLs) because of their inability to process and present HLA class I-restricted antigens. In contrast, cells of EBV-driven posttransplant lymphoproliferative disease (PTLD) are readily recognized and rejected by EBV-specific CTLs. To better understand the molecular mechanisms determining the immunogenicity of EBV-associated B cell malignancies, we have compared the mRNA expression profiles of BL and EBV-immortalized cells (taking the latter as model for PTLD). Among the genes downregulated in BL cells we have identified many genes involved in the NF-κB and interferon response that play a pivotal role in antigen presentation and immune recognition. We show that c-myc activation directly antagonizes the interferon system by downregulation of STAT1, the central player linking the type I and type II interferon response, and IRF7. These findings imply that immune escape of tumor cells is not only a matter of in vivo selection but may be directly promoted by activation of a cellular oncogene.

**7.013**

## EBV Induces a Unique Form of DNA Bound STAT1

**J.E. McLaren**[1], M. Rowe[2], P. Brennan[1]. [1]Cardiff University, Cardiff, United Kingdom; [2]University of Birmingham, Birmingham, United Kingdom

**Background:** Ever since 'constitutive activation' of STAT1 was first described in Epstein-Barr virus (EBV)-associated malignancies (1), there has been controversy regarding the molecular identity of the STAT1-DNA binding complex. Although it is known that latent-membrane protein-1 (LMP-1) is responsible for inducing constitutive STAT1 expression (2, 3), it is disputed whether this form of STAT1 is tyrosine phosphorylated (3, 4). In this study, we analyzed STAT1 post-translational modifications in EBV-transformed lymphoblastoid cell lines (LCL) in order to define their impact on the ability of STAT1 to bind DNA and thus be transcriptionally active.

**Methods:** STAT1 post-translational modifications were analyzed from cell lysates and STAT1 immunoprecipitates of EBV-transformed LCLs by Western blotting using phospho-specific STAT1 and acetyl-lysine antibodies. STAT1 DNA binding was measured using DNA-Affinity precipitation (DNA-AP) and Electrophoretic Mobility Shift Assay (EMSA).

**Results:** Constitutive STAT1 DNA binding was observed in the absence of tyrosine phosphorylation in EBV-transformed LCLs. This constitutive binding was confirmed by both DNA-AP and EMSA. We could only detect STAT1 tyrosine phosphorylation following interferon stimulation. We have also observed STAT1 serine phosphorylation in EBV-transformed LCLs but no detectable lysine acetylation. This serine phosphorylation of STAT1 was inhibited by a combination of both PI3K and MEK inhibitors, implicating a role for these pathways. We could not detect serine phosphorylation on DNA bound STAT1 and incubation of these inhibitors seemed to increase the ability of interferon-stimulated STAT1 to bind DNA.

**Conclusion:** We have observed a unique, EBV-induced, STAT1 DNA binding complex, distinct from that stimulated by interferon-alpha. STAT1 serine phosphorylation is easily detectable in EBV-transformed LCLs and occurs downstream of PI3K and MEK. This event appears to restrict interferon-stimulated STAT1 DNA binding and suggests that EBV reprograms STAT1 for its own ends.

**References:**

(1) Weber-Nordt et al. (1996) Blood 88:809–16
(2) Richardson et al. (2003) J. Virol 77:4439–43.
(3) Zhang et al. (2004) Virology 323:141–52.
(4) Najjar et al. (2005) J. Virol 79:4936–43.

**7.014**

**High Physiological Levels of LMP1 Activate PERK to Induce Phosphorylation of eIF2alpha: Is this how LMP1 Regulates Itself?**

**D. Lee**, B. Sugden. University of Wisconsin, Madison, WI, USA

Epstein-Barr Virus (EBV) requires signaling by the viral membrane protein Latent Membrane Protein 1 (LMP1) to drive proliferation of infected B-cells (Dirmeier *et al.*, 2005). Individual cells within a clone of EBV-infected B-cells express LMP1 at levels that range over 100-fold (Lam *et al.*, 2004). However, in those cells in which LMP1 is expressed at high, but physiological levels, the cells do not proliferate, eukaryotic initiation factor 2 alpha (eIF2alpha) is phosphorylated, and protein synthesis is inhibited. We examined the pathway by which LMP1 mediates the phosphorylation of eIF2alpha in order to illuminate its seemingly contradictory roles. The membrane spanning domains of LMP1 are needed for this phosphorylation (Kaykas and Sugden 2000; Lam *et al.*, 2004). Four kinases are known to phosphorylate eIF2alpha: GCN2, PKR, HRI, and PERK (Dever *et al.*, 1993; Berry *et al.*, 1985; Chen and London, 1995; Harding *et al.*, 1999). We found that a dominant negative derivative of PERK inhibits the phosphorylation of eIF2alpha induced upon conditional expression of LMP1's six membrane-spanning domains but a dominant negative derivative of PKR does not. In addition, high levels of LMP1 in PERK-/- mouse embryo fibroblasts, MEF, do not induce phosphorylation of eIF2alpha. However, when wild type PERK is reconstituted in PERK -/- MEFs, these cells do induce phosphorylation of eIF2alpha in the presence of LMP1. We conclude that high levels of LMP1 activate PERK to induce phosphorylation of eIF2alpha and inhibit protein synthesis. We hypothesize that LMP1 has developed this pathway to regulate its ligand-independent signaling. We are testing the hypothesis using a recombinant Maxi-EBV that expresses a LMP1-RFP fusion protein to identify cells in a clone infected with this recombinant that express high levels of LMP1. If our hypothesis is correct such cells should survive and after several generations, regenerate a daughter population with a broad distribution of levels of expression of LMP1.

**7.015**

**LMP1 Strain Variants: Biologic and Molecular Properties**

**B. Mainou**, N. Raab-Traub. University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

**Background:** Latent membrane protein 1 (LMP1) is necessary for EBV-induced transformation of B-lymphocytes and is able to transform Rat-1 fibroblasts. LMP1 can activate a wide array of signaling pathways including PI3K-Akt and NFkB. Seven sequence variants of LMP1; Alaskan, B95.8, China 1, China 2, Med+, Med-, and NC; have been identified and individuals are infected with multiple variants. The frequency of detection of these variants differs in various EBV-associated malignancies from different geographic regions although all variants have been identified in malignancies associated with EBV. The biological and signaling properties of the LMP1 variants have been characterized.

**Methods:** The biological properties of the LMP1 strain variants were analyzed through focus formation, colony formation in soft agar, cell migration and homotypic adhesion assays. The molecular properties of the variants were assessed through western blot analysis, immunofluorescence microscopy, and NFkB reporter assays.

**Results:** All of the LMP1 variants transformed Rat-1 fibroblasts as measured by contact-independent growth and anchorage-independent growth. All LMP1 variants induced increased motility of HFK cells as well as increased homotypic adhesion of BJAB cells. The PI3K-Akt signaling pathway was activated by all variants and this activation targeted the Akt-target GSK3b but not the Akt-target mTOR. The protein levels of various cellular markers that are associated with G1/S progression were equivalently affected by the LMP1 variants. While all LMP1 variants robustly activated NFkB signaling, the Alaskan, China 1 and Med+ variants had increased NFkB signaling activity, which correlated with decreased binding to the E3 ubiquitin ligase component HOS.

**Conclusion:** The findings of this study indicate that the signature amino acid changes of the LMP1 variants do not hinder or enhance their in vitro transforming potential or signaling properties in a significant manner. All LMP1 variants activated the PI3K-Akt and NFkB signaling pathways and all LMP1 variants induced a variety of markers associated with G1/S cell cycle progression. All LMP1 variants successfully transformed Rat-1 fibroblasts and induced increased motility of HFK cells. These similarities in the biologic and molecular properties and the detection of all variants in EBV-malignancies suggest that they may have equivalent pathogenic potential.

**7.016**

**The Deubiquitinating Enzyme UCH L1 Mediates Activation of Beta-catenin by EBV**

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**Background:** Beta-catenin is a target for the ubiquitin-proteasome pathway, stabilization of which is a critical step for its function in cells. We reported previously that beta-catenin is stabilized and activated in EBV type III latency, and that the cytoplasmic beta-catenin is associated with one or more active deubiquitinating enzymes (DUBs). Among other DUBs, EBV infection increases deubiquitinating activity of Ubiquitin-C-terminus Hydrolase L1 (UCH L1). We hypothesized that UCH L1 is one of the DUBs associated with beta-catenin and started examining its role in beta-catenin signaling.

**Methods:** We used DUB-specific ubiquitin probe and co-immunoprecipitation technique to determine physical association between beta-catenin and enzymatically active UCH L1 in type III EBV-infected B-cells. Further, we established UCH L1 siRNA and control siRNA stable lines to study beta-catenin signaling upon knockdown of UCH L1 expression. Western blotting and TCF reporter assays were used to study whether UCH L1 affects beta-catenin protein levels and the ability to transactivate TCF promoter.

**Results:** Our data indicate that endogenous beta-catenin and UCH L1 are physically associated in both EBV-immortalized B-cells (LCL cells) and epithelial cells (293 cells). Knockdown of endogenous UCH L1 expression by siRNA leads to decreased beta-catenin protein levels, which indicates that UCH L1 is involved in accumulation of beta-catenin. TCF reporter assay showed that UCH L1 increases beta-catenin/TCF transcriptional activity.

**Conclusion:** The results indicate that the deubiquitinating enzyme UCH L1 directly or indirectly is involved in beta-catenin regulation by EBV. We suggest that EBV-dependent dysregulation of the host deubiquitinating system is a critical step during viral transformation.

**7.017**

## Phosphatidylinositol 3-kinase/Akt Pathway Cell Growth and Lytic Cycle of Epstein-Barr Virus in the Burkitt's Lymphoma Cell Line, P3HR-1

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**Background:** The serine/threonine kinase Akt is a crucial regulator of cell growth, survival, and apoptosis. Akt activation is mediated through a phosphatidylinositol 3-kinase (PI3-K)-dependent mechanism. The PI3-K/Akt pathway is involved in various malignancies. In EBV immortalized B-cells, the effect of PI3-K/Akt pathway is cooperative with EBV proteins. LMP2A activates the PI3-K/Akt pathway to inhibit TGF-β-induced apoptosis in Burkitt's lymphoma (BL) cell lines. LMP1 also activates the PI3-K/Akt pathway to promote cell survival. BRLF1, but not BZLF1, requires PI3-K/Akt activation for lytic EBV infection. PI3-K is a determinant of responsiveness to B cell antigen receptor -mediated EBV activation. However, the contribution of PI3-K/Akt pathway to cell growth and to EBV reactivation in BL cells is not well elucidated. This study specifically addressed the characterization of PI3-K/Akt pathway on cell growth and EBV reactivation in P3HR-1 cells, which are spontaneously EBV-reactivated.

**Methods:** Cells were grown in RPMI 1640 supplemented with 10 % heat-inactivated fetal bovine serum (FBS). Cell growth was assayed by the incorporation of MTT dye. Expression and phosphorylotion of Akt, and expression of ZEBRA and EAD were analyzed by Western Blot. Expression of RNA was analyzed by RT-PCR. VCA positive cells were detected by immunofluorescence.

**Results:** Akt was constitutively phosphorylated in BJAB, P3HR-1, Akata, and Daudi but not in Ramos and Raji cells. We characterized Akt phosphorylation on cell growth and EBV lytic cycle in P3HR-1 cells, which were phosphorylated most intensively. The rates of Akt phosphorylation and cell growth were similar in P3HR-1 cells cultured with and without FBS for a few days. PI3-K specific inhibitor LY294002 inhibited Akt phosphorylation and cell growth in dose- and time-dependent manners. LY294002 markedly down regulated expression of EBV lytic gene BRLF1 protein Rta, BMRF1 protein EA-D, but not BZLF1 protein ZEBRA. Down regulation of Rta by LY294002 occurred at the transcriptional level. The LY294002 reduced VCA positive cells. The results demonstrate that LY294002 specifically inhibits the lytic cycle on downstream of ZEBRA in P3HR-1 cells and suggests that the PI3-k/Akt pathway continuously stimulates the spontaneous EBV reactivation.

**Conclusions:** PI3-K/Akt pathway is activated constitutively in P3HR-1 cells; it promotes cell growth and the lytic cycle cascade.

**7.018**

## Overexpression of Epstein-Barr Virus Latent Membrane Protein 1 (LMP1) in EBV Latency III B-cells Induces Caspase 8 Mediated Apoptosis: Role of CD95

**C. Le Clorennec**[1], T.S. Ouk[1], C. Jayat-Vignolles[1], I. Youlyouz-Marfak[1], E. Adriaenssens[2], J. Coll[2], G.W. Bornkamm[3], J. Feuillard[1]. [1]UMR CNRS 6101, Limoges, France; [2]CNRS UMR 8527, Lille, France; [3]Institute of Clinical Molecular Biology and Tumor Genetics,1 GSF-National Research Center for Environment Health, Munich, Germany

Epstein-Barr virus (EBV) contributes to oncogenesis by transforming normal B lymphocytes into lymphoblastoid cell lines (LCLs) in latency III. LMP1 has been described as the major transforming protein of EBV latency III program. In different cellular type, LMP1 could, by diverting TNF-R family signaling pathway, induce apoptosis. Thus, recent studies show that LMP1 overexpression would be toxic for cell. This process of positive regulation of apoptosis by LMP1 is not well-known. In precedent studies, we demonstrated that LMP1 is responsible of CD95 regulation via principally NF-κB and secondary STAT1 and p53, in EBV-latency III B-cells. This work aims to study the regulation by LMP1 of cellular pathways implied in the apoptotic response in EBV latency III B-cells lymphocyte immortalized.

To understand the toxic effect of LMP1 in LCLs, we cloned a dominant negative mutant of LMP1 (LMP1CT) and wild type LMP1 into a novel double inducible episomal vector, pRT-1, who expressed the gene of interest under control of a bidirectional doxycycline regulatable promoter that allowing simultaneous expression of truncated NGF receptor, used as a surrogate marker of inducibility. These plasmids were stably transfected either into LCLs. Induction of the cDNA of interest was performed with doxycycline for 24h to 120h. Positive cells for NGFR were purified using magnetic beads.

In order to describe the process of apoptosis by LMP1 in LCLs, we tested effect of LMP1 overexpression. We observed markedly increased CD95 expression in cell surface detected by FACS and confocal microscopy and rapidly CD95 aggregation, DISC formation and CD95 internalization were observed. Then, caspase 8 activity was detected and causing activation of caspase 9 and caspase 3. Finally, late stages of apoptosis were observed thereafter, like cleavage of PARP, as well as DNA's fragmentation. We performed immunoprecipitation experiments of CD95 and caspase 8 and we demonstrated that caspase 8 activity was due exclusively to formation of CD95 DISC. In addition, induction of LMP1CT does not cause CD95 aggregation in cell surface.

Consequently, we demonstrated, in EBV Latency III B-cells, that CD95 aggregation and apoptosis was dependent of LMP1 overexpression, via LMP1 signalling. Finally, overexpression of LMP1 potentiates CD95 mediated apoptosis.

Therefore, our results suggest that LMP1 overexpression potentiates EBV-latency III immortalised B-cells to CD95 mediated apoptosis.

**7.019**

**LMP2a Induction of Epithelial Cell Invasion by Recruitment of Syk**

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Latent Membrane Protein 2A (LMP2A) can be detected in the EBV-positive epithelial malignancy nasopharyngeal carcinoma (NPC). LMP2A is also tumorigenic in human epithelial cells transplanted to SCID mice. To investigate the function of LMP2A in these epithelial cell tumors we have used synthetic peptides that mimic the ITAM and PPPPY ligand binding sites within LMP2A as probes to identify associating proteins in an LMP2A-transfected epithelial bladder carcinoma cell line 5637 and in the NPC cell line TWO3. An unanticipated observation was the identification of the spleen tyrosine kinase (Syk), commonly considered restricted to hematopoietic cell lineages, interacting with the LMP2A ITAM-motif peptide. Mass spectrometric data, Western-blot analysis and shRNA silencing were used to confirm the presence of Syk in these cell lines. Functionally, the endogenous Syk in these cell lines was not activated, however the reintroduction of LMP2A resulted in detectible levels of tyrosine-phosphorylated Syk associated with LMP2a. Phosphotyrosine-specific antibodies were used to demonstrate activation of Syk. Expression of LMP2a enhanced the invasive capacity of epithelial cells, in a fashion that depends on Syk recruitment. These data posit a role for Syk in epithelial cell biology that is co-opted by the EBV LMP2A.

**7.020**

## Epstein-Barr Virus Latent Membrane Protein 1 C-Terminal Sites 1 and 2 Activate NF-kB through TRAF2/NIK/IKKa/p52 and TRAF6/TAK1/Another Kinase Respectively

**V. Soni**, Y.J. Song, E. Cahir-McFarland, M.S. Kang, E. Kieff. Brigham, Boston, MA, USA

Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) NF-kB activation through C-terminal cytosolic domains 1 and 2 (S1 and S2) is critical for B-cell growth transformation and survival. We have continued to use mouse embryo fibroblasts knocked out for the expression of proteins implicated in NF-kB activation to identify factors essential for LMP1 S1 and S2 signaling. We now find that: (i) LMP1 S1 requires TRAF2, NF-kB inducing kinase (NIK), IkB kinase alpha (IKKa) and p52, but not TRAF6 for NF-kB activation. (ii) In contrast, LMP1 S2 does not require NIK, IKKa, p52, or TRAF2. (iii) LMP1 S2 critically requires TRAF6 for NF-kB activation. (iv) NF-kB activation by S2 is surprisingly only partially TAK1 dependent. LMP1 S2 was also found to induce TRAF6 K48 and K63 polyubiquitination. These data are consistent with a model wherein LMP1 S1 activates NF-kB primarily through TRAF2, NIK, IKKa and p52, whereas LMP1 S2 activates NF-kB through TRAF6, TAK1, and another kinase.

**7.021**

**Role of TRAF3 in EBV LMP1 Signaling and B Lymphocyte Development**

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EBV contributes to the development of several human malignant lymphomas. EBV LMP1 is critical for the growth transformation of B cells and is expressed in the EBV-associated lymphomas such as Hodgkin's lymphomas. LMP1 interacts with tumor necrosis factor receptor-associated factors (TRAF) and mimics CD40 signal as a constitutively activated receptor in B cells. In the TRAF family, TRAF3 has been identified as an interacting partner for the LMP1 C-terminus cytoplasmic region in which NF-κB, JNK and p38 MAP kinase are induced. However, little is known about the involvement of TRAF3 in the LMP1 signaling and the physiological role in the B lymphocyte development. To elucidate the TRAF3 function, we generated TRAF3-deficient (T3 ko) mice. Luciferase assay revealed that LMP1 normally induced NF-κB activation in the mouse embryonic fibroblasts (MEFs) from T3 ko mice. Increased amount of CCL4 and CXCL9 mRNAs both of which were NF-κB-dependent genes was observed in the T3ko MEFs. LMP1 expression in T3ko MEFs induced no longer enhanced expression of those chemokine mRNAs. These results indicate that TRAF3 is dispensable for the LMP1-induced NF-κB activation and also that TRAF3 is a negative regulator for NF-κB activation. To analyze the B cell development in the TRAF3-deficient condition we also generated the bone marrow chimera of RAG2-/- mice which lacked mature lymphocytes completely with T3ko fetal liver cells because T3ko mice showed embryonic lethality. Flow cytometric analysis exhibited decreased number of immature and mature B cell populations in the TRAF3-null background. T3 ko B cells was also susceptible to cell death compared to wild-type B cells suggesting the importance of TRAF3 for the B cell survival. LMP1 would serve survival signal for the B lymphocytes through the interaction of TRAF3 like as CD40 and TRAF3-mediated pathway in the LMP1 signaling might play a non-redundant role in EBV-induced lymphoid malignancies.

**7.022**

**The EBV Oncoprotein Latent Membrane Protein 1 Affects the Expression of Suppressors of Cytokine Signaling in Transformed B Lymphocytes**

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The latent membrane protein 1 (LMP1) of Epstein-Barr Virus (EBV) is a protein with known oncogenic properties. Growth and survival of EBV-infected cells is regulated by certain signaling pathways involving LMP1 as their main activator. Among these pathways, the STAT signaling is of interest, because of its central role in regulating proliferative and apoptotic processes. In normal signaling, STAT molecules are only transiently activated mainly by cytokines, whereas in specific tumors a permanent activation has been observed. The expression of full length LMP1 is responsible for an enhanced STAT1 protein expression in Burkitt lymphoma cells (BL). STAT3 is tyrosine phosphorylated without changes in STAT3 protein levels. A specific pattern of Janus kinases is activated by LMP1. Furthermore, members of the family of suppressors of cytokine signaling (SOCS) are upregulated in EBV positive BLs. LMP1 expression induces SOCS3 involving p38 mediated mRNA stabilisation. Using an inducible LMP1 and respective MEFs, the mechanism of SOCS3 regulation is further analysed in parallel to cytokine induced SOCS3 expression in B cells. Our results support the role of LMP1 in modulating cytokine signaling and responsiveness (supported by DFG Ku 954/7-2 and the Graduiertenkolleg 1034).

**7.023**

## RIP Mediates LMP1 Ubiquitination and Activation of IRF7

L. Huye, **S. Ning**, J. Pagano. Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA

**Background:** As a key mediator of type-I interferon (IFNalpha/beta) responses, Interferon Regulatory Factor 7 (IRF7) is essential for host immune defenses. Activation of IRF7 generally requires virus-induced C-terminal phosphorylation. Lys63-linked ubiquitination regulates functions of target proteins rather than their proteasomal degradation through Lys48-linked ubiquitination.

**Methods:** For luciferase assays, 293 or 3T3 cells were transfected by use of Fugene 6 or Lipofectamine reagent. ISRE-Luc was used as reporter, and Renilla as internal control. For immunoprecipitation, 3T3 cells were transfected with FlagIRF7, LMP1, RIP and HA-Ub by use of Lipofectamine reagent. Cell lysates were first immunoprecipitated with Flag M2 antibody. After washes, protein complexes on the beads were dissociated in 1% SDS, diluted 1:10, and a second immunoprecipitation was performed with IRF7 antibody. Western Blots were performed with HA antibody. For RNA interference, siRIP was transfected by use of Lipofectamine 2000 reagent, and cells were cultured for 36 h before transfection for promoter-reporter assay.

**Results:** Transient expression of LMP1 enhances IRF7 transcriptional activity. Moreover, LMP1 promotes ubiquitination of IRF7. At least a portion of this ubiquitination involves Lys63-linked ubiquitin, which has protein regulatory functions. Overexpression of ubiquitin or Lys63-only ubiquitin, which can only participate in Lys63-linked ubiquitination, activates IRF7 in a dose-dependent manner and increases LMP1-stimulated IRF7 transcriptional activity. It suggests that LMP1-promoted ubiquitination enhances IRF7 activity. In addition, LMP1 promotes the interaction of IRF7 with RIP. Finally, repression of expression of RIP in 293 cells by RNA interference results in significant decrease in LMP1-stimulated IRF7 activity, and in RIP knockout (RIP-/-) 3T3 cells, LMP1 can neither promote ubiquitination of IRF7 nor increase its transcriptional activity.

**Conclusions:** IRF7 can be activated by the EBV oncoprotein, LMP1, through the Lys63-linked ubiquitination pathway, and RIP is required for this activation. These findings may also have more general significance for LMP1 signalling.

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The first two authors contributed equally

**7.024**

**EBV LMP1 Upregulates HIF1alpha through Siah1 E3 Ligase-mediated Down-regulation of Prolyl Hydroxylases 1 and 3**

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**Background:** Invasion into surrounding tissues is characteristic of EBV-associated cancers such as NPC and B-cell lymphomas. Recently, we reported that LMP1 up-regulates the expression of HIF1alpha the principal oxygen-sensing molecule important in neoangiogenesis. Siah E3 ubiquitin ligase proteins degrade prolyl HIF-hydroxylases PHD 1 and 3, which results in stabilization of HIF1alpha in mouse cells. We have reported that LMP1 modulates the expression of Siah1 in B lymphoma cells, which results in up-regulation of beta-catenin. Here we examine whether Siah family proteins regulate another target, HIF1alpha, in epithelial cells.

**Methods and Results:** 1. LMP1 induces expression of Siah1. Endogenous levels of Siah1 protein are higher in Type II latently infected cells. Transfection of LMP1-expressing plasmid into EBV-negative nasopharyngeal epithelial cells up-regulates expression of Siah1.

2. Siah1 is responsible for activation of HIF1alpha by LMP1. The level of HIF1alpha in the presence of LMP1 was increased further by exogenous Siah1, but dramatically decreased by Siah1 DN.

3. LMP1 stabilizes Siah1 protein. In the absence of protein synthesis, Siah1 degrades more slowly in cells expressing LMP1. A proteasome inhibitor had little if any effect on Siah1 in LMP1-expressing cells.

4. Degradation of PHD1 and PHD3 by Siah1 is responsible for activation of HIF1alpha by LMP1. PHD 1/3 levels were down-regulated by LMP1 and decreased further in the presence of exogenous WT Siah1, but were restored almost to control levels when Siah1 DN was introduced.

5. LMP1 disrupts interaction between HIF1alpha and VHL. In co-immunoprecipitations strong interaction between VHL and HIF1alpha proteins was detected in cell lysates. Expression of LMP1 greatly decreased this interaction.

**Conclusion:** LMP1 up-regulates the level of Siah1 ligase by enhancing its stability, which then induces proteasomal degradation of prolyl HIF-hydroxylases 1 and 3 that regulate HIF1alpha stability. As a result, LMP1 prevents formation of VHL/HIF1 complex, which results in HIF1 stabilization. Thus Siah1 is implicated in the regulation of HIF1 and involved in a recently appreciated aspect of EBV-mediated tumorigenesis, namely, the angiogenesis process triggered by LMP1.

**7.025**

**LMP2B Modulates LMP2A Signaling**

**M. Rovedo**, R. Longnecker. Northwestern University, Chicago, IL, USA

Latent membrane protein 2A (LMP2A) and LMP2B are expressed during Epstein-Barr virus (EBV) latency in EBV immortalized B-cells grown both in culture and in humans. LMP2A and LMP2B contain 12 putative hydrophobic transmembrane domains and a cytoplasmic 27 amino-acid C-terminal tail. In addition, LMP2A contains a cytoplasmic 119 amino acid N-terminal tail. LMP2A has important roles in modulating B cell receptor (BCR) signal transduction by associating with the cellular tyrosine kinases Lyn and Syk via specific phosphotyrosine motifs found within the LMP2A N-terminal tail domain. LMP2A has been shown to alter normal BCR signal transduction in the EBV-negative B-cell line BJAB by reducing levels of the Lyn protein tyrosine kinase and by blocking tyrosine phosphorylation and calcium mobilization following BCR crosslinking. Although little is currently known about the function of LMP2B in B-cells, the similarity in structure between LMP2A and LMP2B suggests that they may localize to the same cellular compartments. We hypothesized that LMP2B plays a role in modulating the effects of LMP2A. Four BJAB cell lines were constructed to examine the role of LMP2B in modulating LMP2A signaling: cells that express LMP2A, LMP2B, neither of the proteins or both proteins. Co-expression of LMP2B with LMP2A in BJAB cells prevents the constitutive phosphorylation of LMP2A, which in turn restores Lyn levels as well as calcium mobilization and tyrosine phosphorylation upon BCR crosslinking. LMP2B may be expressed by EBV to down regulate the effects of LMP2A, rapidly shuttle B-cells out of the latency program and allow lytic replication of the virus.

# Abstracts

**Sunday, July 9, 2006**
**Room: Poster Area/Level 3**

## Session 8: Poster Session I
## Viral Replication
## 20:00-22:00

**8.001**

## The Epstein-Barr Virus BNRF1 Protein Allows Efficient Transfer from the Endosomal Compartment to the Nucleus of Primary B Lymphocytes

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All herpesviruses possess an envelope wrapped around the nucleocapsid that contains the double stranded viral DNA. The virtual space between the nucleocapsid and the envelope, the tegument, contains several proteins of viral and cellular origin. After crossing the B cell membrane, the virus enters cytoplasmic vesicles where decapsidation takes place to allow transfer of the viral DNA to the cell nucleus. BNRF1 has been characterised as the EBV major tegument protein but its precise function is unknown. We have constructed a viral mutant that lacks the BNRF1 gene and report here its *in vitro* phenotype. A recombinant virus devoid of BNRF1 (ΔBNRF1) showed efficient DNA replication and production of mature viral particles as determined by Gardella gel analysis and electron microscopy. B cells infected with the ΔBNRF1 mutant presented viral lytic antigens on MHC class II molecules as efficiently as B cells infected with wild type or BNRF1 trans-complemented ΔBNRF1 viruses. Antigen presentation in B cells infected with either wild type or ΔBNRF1 viruses was blocked by leupeptin, an inhibitor of lysosomal proteases, showing that both viruses reach the endosome/lysosome compartment. These data were confirmed by direct observation of the mutant virus in endosomes of infected B cells by electron microscopy. However, we observed a 40-fold reduction in the number of B cells expressing the nuclear protein EBNA2 after infection with a ΔBNRF1 mutant virus as compared to wild type infection. Likewise, ΔBNRF1 viruses transformed primary B cells much less efficiently than EBV-wt or BNRF1 trans-complemented ΔBNRF1 viruses. We conclude from these findings that BNRF1 is dispensable for viral DNA replication and production of mature viral particles but does play an important role for viral transport from the endosomes to the nucleus.

**8.002**

**Inter-Regulation of Zta, Rta, and Egr-1 as a Positive-Feedback Network in Spontaneous Reactivation of Epstein-Barr Virus**

**Y. Chang**[1], Y.T. Chen[2], H.H. Lee[2], S.Y. Wu[1], C.W. Chen[1], C.H. Tsai[2]. [1]Division of Clinical Research, National Health Research Institutes, Tainan, Taiwan; [2]Graduate Institute of Microbiology, National Taiwan University, Taipei, Taiwan

Reactivation of Epstein-Barr virus (EBV) into the lytic cycle is initiated from activation of two immediate-early viral promoters, Zp and Rp, which are driven to express two lytic transactivators, Zta and Rta proteins. Expression of Zta and Rta is augmented since they autostimulate their own expression and reciprocally induce each other. We propose that the positive-feedback regulation may include a cellular transcription factor, early growth response-1 (Egr-1). Egr-1 has been reported to activate Rp in previous studies, but it remains unknown whether any EBV protein regulates Egr-1 expression. In this study we first show that Egr-1 can be induced by Zta. The Egr-1 promoter contains at least two Zta-responsive regions; one covers two adjacent copies of Zta-binding elements, while the other represents a SRE-Ets element targeted by the SRF-Elk-1 ternary complex. Zta not only contributes to DNA binding of Zta itself to the Egr-1 promoter, but also activates the ERK signaling pathway which induces the Elk-1-containing complex binding to the Egr-1 promoter. In a model of spontaneous EBV reactivation in EBV-infected 293 cells, inhibition of Zta by Zta-targeted siRNA diminishes Egr-1 expression, and siRNA-directed knockdown of Egr-1 significantly reduced the spontaneous expression of Zta, Rta and the downstream lytic genes. These results suggest that inter-regulation of Zta, Rta and Egr-1 as a positive-feedback network is required to sustain the spontaneous EBV reactivation.

**8.003 (cancelled)**

**Down-Regulation of Cellular Proteolytic Systems Following Epstein Barr Virus Activation in Burkitt's Lymphoma Cells**

G. Matusali, A. De Leo, L. Di Renzo, F. Cuozzo, M. D'Erme, **E. Mattia**. University "La Sapienza", Rome, Italy

In Burkitt's lymphoma cells, Epstein Barr virus (EBV) by interacting with the ubiquitin-proteasome system through latency products promotes episomal maintenance and eludes host immune response while the tripeptidylpeptidase II (TPPII) functions as an alternative protease. In the present study, we have examined in Raji and in Akata cells the activities of the proteasome and that of the TPPII complex after induction of the early phases or the complete pattern of productive infection, respectively. We performed also Western blot analysis to examine the protein levels of the two proteolytic systems. The results showed that chimotrypsin-like and caspase-like activities of the proteasome were substantially reduced in Raji and Akata cells. Similarly, TPPII activity diminished in both cell lines but was recovered in Akata cells at longer time after induction. Protein levels of alfa/beta subunits of the 20S proteasome and TPPII concentration decreased to different extent after EBV activation, whereas S6' subunit of the 19S regulatory complex increased three to four fold along with the levels of ubiquitin-conjugates. Collectively, these observations demonstrate impairment of two major cellular proteolytic systems at the onset of EBV lytic infection.

**8.004**

## An Essential Role for Zta Cysteine 171 in C/EBP Binding Site Recognition and Lytic Cycle Replication

**P. Wang**, L. Day, P.M. Lieberman. The Wistar Institute, Philadelphia, PA, USA

**Background:** Zta is the immediate early DNA-binding protein of EBV that is required for lytic cycle gene activation and DNA replication. Zta can bind multiple sequence elements, including AP1 and C/EBP-like recognition sites, found in the viral and cellular genome. Mechanisms regulating Zta DNA binding may have significant impact on viral reactivation and pathogenesis, but little is known with regard to this regulation. In this work, we investigate aspects of Zta sequence specific DNA binding that may be subject to post-translational regulation. We have previously found that a redox sensitive cysteine C189 was important for DNA replication function, but had little effect on DNA binding and transcription activation. We now show that a second cysteine, C171, is essential for lytic cycle reactivation and that mutations in C171 disrupt binding to C/EBP-sites, but not AP-I site.

**Methods:** Site directed mutagenesis of Zta was used to substitute C171 and C132 to serine or alanine. Zta mutants were assayed for DNA binding to multiple Zta Recognition Sites (ZREs), as well as canonical AP1 and C/EBP sites. Zta mutants were also assayed for transcription activation, stimulation of lytic cycle DNA replication, and co-immunoprecipitation with Rta.

**Results:** We found that serine or alanine substitution of C171, but not C131 abrogated Zta function in DNA replication and reactivation from latency. C171S was defective for transcription activation of EA-D, and was further found to be incapable of binding to a subset of ZREs. Further analysis revealed that C171S was defective at binding C/EBP sites, but was only modestly reduced at binding AP1 sites.

**Conclusions:** Substitution mutation of C171 to serine or alanine disrupted the DNA binding functions of Zta. These findings are surprising since C171 is thought to be amino-terminal to the DNA binding domain defined in crystallographic and other structural studies. These findings suggest that C171 may induce conformational changes in the DNA binding domain to allow recognition of C/EBP sites, in addition to AP1 sites. These findings also raise the possibility that cysteine C171 is subject to post-translational modifications that can readily modulate lytic cycle reactivation, and may provide novel mechanisms for inhibition of EBV lytic replication.

**8.005**

**EBV BGLF4 Is a Virion Tegument Protein that Dissociates from Virions in a Phosphorylation Dependent Process and Phosphorylates BZLF1**

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Epstein-Barr virus (EBV) BGLF4 is a viral protein kinase that is expressed in the lytic phase of infection and is packaged in virions. We report here that BGLF4 is a tegument protein that dissociates from the virion in a phosphorylation dependent process. We also present data that BGLF4 interacts with and phosphorylates BZLF1, a key viral regulator of lytic infection. These conclusions are based on the following observations. (i) In in vitro tegument release assays, a significant fraction of BGLF4 was released from virions in the presence of physiological NaCl concentrations. (ii) Addition of physiological concentrations of ATP and MgCl2 to virions enhanced BGLF4 release, but phosphatase treatment of virions significantly reduced BGLF4 release. (iii) A recombinant protein containing a domain of BZLF1 was specifically phosphorylated by purified recombinant BGLF4 in vitro and BGLF4 altered BZLF1 post-translational modification in vivo. (iv) BZLF1 was specifically co-immunoprecipitated with BGLF4 in TPA-treated B95-8 cells and in COS-1 cells transiently expressing both of these viral proteins. (v) BGLF4 and BZLF1 were co-localized in intranuclear globular structures, resembling the viral replication compartment, in Akata cells treated with anti-human IgG. Our results suggest that BGLF4 functions, not only in lytically infected cells by phosphorylating viral and cellular targets, but also immediately after viral penetration like other herpesvirus tegument proteins.

**8.006**

**The KSHV Lytic Gene ORF57 is Essential for Infectious Virion Production**

Z. Han, **S. Swaminathan**. University of Florida Shands Cancer Center, Gainesville, FL, USA

The ORF57 gene of KSHV is expressed early during lytic KSHV replication. ORF57 has a regulatory function, post-transcriptionally activating expression of intronless genes. In addition, ORF57 displays gene specificity in its activation function, preferentially enhancing expression of some cellular and viral target genes. The ORF57 protein, like its homologs in other herpesviruses, is thought to bind mRNA and enhance mRNA stability and nuclear export. ORF57 may also affect transcription by enhancing KSHV ORF50 (Rta) activity. To determine whether ORF57 is essential for lytic replication of KSHV, we constructed recombinant KSHV genomes in which the ORF57 coding region was specifically interrupted.

ORF57 was insertionally inactivated by generating a recombinant KSHV using the BAC36 KSHV BACmid (gift of SJ Gao). Briefly, an ORF57 targeting cassette was generated by PCR amplification of a kanamycin resistance gene with primers encoding 60 bp of ORF57 at the 5' termini. The cassette was electroporated into bacteria carrying BAC36 and a plasmid with an inducible recombinase. After recombination was induced, recombinant bacmids were selected and verified. Vero and 293 cells were transfected with the recombinant BACmids and selected with hygromycin.

Inactivation of ORF57 led to an inability of the mutant KSHV to express several early and late replicative genes after induction of the lytic cycle by ORF50 transfection. Lytic gene expression could be rescued if ORF57 was provided in trans. Similarly, ORF57-null virus infected cells were unable to produce infectious virus unless transfected with ORF57. In order to determine whether SM, the EBV homolog of ORF57, was able to rescue ORF57-null virus, the cells were transfected with EBV SM. Despite the fact that SM efficiently enhanced expression of several KSHV replicative genes, including DNA polymerase, it was unable to rescue infectious virus production. Conversely, ORF57 was unable to rescue replication of an SM-null virus.

These experiments demonstrate that ORF57, like its homologs HSV ICP27, EBV SM and VZV ORF4, is essential for lytic replication. In addition, ORF57 has enhancing effects on several lytic genes in the context of viral replication. However, it is likely that ORF57 has other essential KSHV-specific functions since EBV SM was unable to functionally replace ORF57 to permit virus production. This appears to reflect a general property of the ICP27-related herpesvirus proteins, since no member of this family has demonstrated significant functional complementation of mutants in another virus.

**8.007**

**Postreplicative Mismatch Repair Factors are Recruited to Epstein-Barr Virus Replication Compartments**

**T. Tsurumi**, A. Kudoh, T. Daikoku. Division of Virology, Aichi Cancer Center Research Institute, Nagoya, Japan

The mismatch repair (MMR) system, highly conserved throughout evolution, corrects nucleotide mispairing that arises during cellular DNA replication. We report here that proliferating cell nuclear antigen (PCNA), the clamp loader complex (RF-C), and a series of MMR proteins like MSH-2, MSH-6, MLH1 and hPSM2 can be assembled to Epstein-Barr virus replication compartments, the sites of viral DNA synthesis. Levels of the DNA-bound form of PCNA increased with progression of viral productive replication. BrdUrd-labeled chromatin immunodepletion analyses confirmed that PCNA is loaded onto newly synthesized viral DNA as well as BALF2 and BMRF1 viral proteins during lytic replication. Furthermore, the anti-PCNA, -MSH2, -MSH3, or -MSH6 antibody could immunoprecipitate BMRF1 replication protein probably via viral DNA genome. PCNA loading might trigger transfer of a series of host MMR proteins to the sites of viral DNA synthesis. The MMR factors might function for the repair of mismatches that arise during viral replication or act to inhibit recombination between moderately divergent (homeologous) sequences.

**8.008**

## BZLF1 Induces Retinoic Acid Production in Epithelial Cells by Enhancing the Conversion of Retinol Into Retinoic Acid

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**Introduction:** Lytic EBV replication in oral hairy leukoplakia is confined to differentiated epithelial cells. Retinoic acid (RA) induces epithelial differentiation. The conversion of retinol into its active form, retinoic acid, requires retinol dehydrogenase enzymes. Here we show that BZLF1 induces expression of the enzyme DHRS9/RDHL, which mediates conversion of retinol into RA.

**Methods:** EBV-positive AGS gastric carcinoma cells were generated using wild-type B95-8 virus, or viruses deleted in the BZLF1 or BMRF1 genes. Cellular gene expression was compared in EBV-negative versus EBV-positive AGS cells using microarray analysis. Retinol conversion into the RA metabolite, 4-Hydroxy RA, was measured by HPLC in AGS cells transfected with BZLF1 or a control vector.

**Results:** Lytically-infected, but not latently-infected, AGS cells expressed 10-fold more DHRS9 message than EBV-negative AGS cells. BZLF1 transfection was sufficient to induce DHRS9 expression in AGS, HeLa and Telomerase immortalized keratinocytes. BZLF1 activated a DHRS9 promoter construct 300 fold, and this effect was mediated through several BZLF1-binding (ZRE) sites. BZLF1-transfected AGS cells converted retinol into retinoic acid much more efficiently than vector control cells.

**Conclusion:** BZLF1 enhances retinol metabolism, thereby increasing the production of retinoic acid. Retinoic-acid induced differentiation of primary EBV-infected keratinocytes likely promotes lytic viral replication.

**8.009**

**Phosphorylation of MCM4 at Sites Inactivating DNA Helicase Activity of the MCM4-6-7 Complex during Epstein-Barr Virus Productive Replication**

**A. Kudoh**, T. Tsurumi. Aichi Cancer Center Research Institute, Nagoya, Japan

Induction of Epstein-Barr virus (EBV) lytic replication blocks chromosomal DNA replication notwithstanding an S-phase like cellular environment with high CDK2 activity. We report here that the phosphorylated form of MCM4, a subunit of the MCM complex essential for chromosomal DNA replication, increases with progression of lytic replication, Thr-19 and Thr-110 being CDK2 targets whose phosphorylation inactivates MCM4-6-7 complex-associated DNA helicase. Expression of EBV-encoded protein kinase (EBV-PK) in HeLa cells caused phosphorylation of these sites on MCM4, leading to cell growth arrest. In vitro, the sites of MCM4 of the MCM4-6-7 hexamer was confirmed to be phosphorylated with EBV-PK with the same loss of helicase activity as with CDK2/cyclin A. Introducing mutations in the N-terminal six Ser and Thr residues of MCM4 reduced the inhibition by CDK2/cyclin A, while EBV-PK inhibited the helicase activity of both wild type and mutant MCM4-6-7 hexamer, probably due to that EBV-PK can further phosphorylate MCM6 and other site(s) of MCM4 in addition to the N-terminal residues. Therefore, phosphorylation of the MCM complex by redundant actions of CDK2 kinase and EBV-PK during lytic replication might provide one mechanism to block chromosomal DNA replication in the infected cells, through inactivation of DNA unwinding by the MCM4-6-7 complex.

**8.010**

## Phosphorylation of the EEBV ZEBRA Protein at S173 in the Regulatory Domain is Required for Viral Lytic DNA Replication but not for Transcriptional Activation of Viral Early Genes

**A. El-Guindy**[1], L. Heston[2], H.J. Delecluse[3], G. Miller[4]. [1]Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, CT, USA; [2]Department of Pediatrics, Yale University School of Medicine, New Haven, CT, USA; [3]Department of Tumor Virology, German Cancer Research Center, Heidelberg, Germany; [4]Departments of Pediatrics, Molecular Biophysics, New Haven, CT, USA

ZEBRA, a multi-functional protein, plays a key role in the initiation of lytic DNA replication of Epstein-Barr virus. The protein activates transcription of viral genes required for replication, binds to DNA encompassing the origin of lytic replication and interacts with several components of the replication machinery. The mechanistic basis of ZEBRA's contribution to viral replication has been difficult to assess due to the lack of a ZEBRA mutant that can distinguish between the protein's role as an activator of transcription and its role as an essential viral replication protein. Here, we describe such a mutant and we provide evidence for the importance of phosphorylation at S173 in the regulatory domain of ZEBRA in the activation of viral replication. Phosphorylation of this site is not mandatory for the transcriptional activation function of ZEBRA since an alanine substitution mutant at this site is fully competent to induce expression of early genes. However, lack of phosphorylation at S173 markedly reduced the DNA binding activity of ZEBRA in vitro and diminished its association with oriLyt in vivo by 3-fold, as assessed by chromatin immunoprecipitation. The S173A mutation caused 10- fold more ZEBRA protein to be released from a DNA-protein complex in vivo. A mutant Z(S173D) with a phosphomimetic substitution maintained the ability of ZEBRA to activate viral replication and to associate tightly with DNA in vitro and in vivo at levels comparable with wild type protein. These novel experiments emphasize the importance of phosphorylation in the replication function of the origin binding protein, ZEBRA. In addition, our findings support the hypothesis that the ability of ZEBRA to initiate viral DNA replication requires stronger DNA binding affinity relative to its capacity to activate transcription of early genes.

**8.011**

**Characterization of Binding of Soluble gH to Epithelial Cells**

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EBV entry into epithelial cells requires glycoproteins gB and a complex of gH and gL. If cells express CR2 then attachment is mediated by gp350/220. In its absence virus can use gHgL for attachment instead. Truncated gH expressed in recombinant baculovirus together with full length gL binds specifically to CR2-negative epithelial cells and gH null virus fails to bind. gHgL binding is reduced by a monoclonal antibody that blocks virus binding to CR2-negative cells and blocks entry into CR2-positive cells. The identity of the gHgL receptor is unknown but reduction of binding by proteases is consistent with it being a protein. Binding is thought to trigger membrane fusion required for entry. To evaluate the affinity and number of binding sites per cell we purified truncated gH from the supernatant of baculovirus-infected cells. Protein was purified over 500 fold in a two-step procedure using Lentil Lectin Sepharose chromatography and anion-exchange chromatography. Densitometry indicated that the protein was more than 85% pure. Biological activity of the gH during purification was evaluated by flow cytometric analysis of protein bound to cells on ice and stained with a monoclonal antibody. Binding was saturable and fitted well to a hyperbolic curve. Biologic activity was confirmed by its ability to block virus infection. Initial estimates indicated approximately 450,000 binding sites per cell with a $K_D$ of $20x10^{-9}$M. This compares with previous estimates of 60,000 binding sites on high-expressing, CR2-positive Raji cells with a $K_D$ of $1x10^{-8}$M. We could detect no gL in the purified gH, suggesting that the binding site is on gH itself. The results are consistent with previous work indicating that virus made in B cells can bind almost as well to CR2-negative cells via gH as to CR2-positive cells via gp350/220 and that inefficiency of infection is related to a subsequent block.

**8.012**

**Characterisation of a Docking Site for Protein Kinase Ii (CK2) in the EBV mRNA Export Factor, EB2**

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The Epstein-Barr Virus (EBV) early nuclear protein, EB2 (also called Mta, SM or BMLF1), has been shown to be an export factor for some intronless and unspliced viral late mRNAs. By using an EBV recombinant deleted of the EB2 gene, we showed that EB2 is essential for the production of EBV infectious virions. EB2 is a phosphoprotein with orthologues in other herpesviruses such as ICP27 in HSV-1, ORF57 in KSHV and IE2 in HHV-6. We now show in vivo by transient transfection of epithelial cells and co-immunoprecipitation, and in vitro by the use of EB2 peptides fused to GST, that there is a docking site in EB2 for Protein Kinase II, previously called Casein Kinase 2. These experiments showed specific binding of both CK2 subunits, and phosphorylation at the N-terminal part of EB2. The interaction was further corroborated by immunofluorescence showing co-localisation of both CK2 subunits with EB2. Importantly, we also demonstrated that phosphorylation of EB2 by CK2 had an effect on its function as an mRNA export factor.

**8.013**

## Characterization of Two New EBV Producing Cell Lines

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**Background:** Two novel EBV transformed cell lines were generated by spontaneous immortalization of peripheral blood mononuclear cells (PBMCs) from two hairy cell leukemia (HCL) patients.
This study aims to characterize these two new EBV producing lines, HCL-B and HCL-P.

**Methods:** To determine whether each cell line was unique, 7 microsatellite regions in the major histo-compatibility complex (MHC) were analyzed using multiplex PCR. Fluorescence-activated cell sorting (FACS) analysis was used to characterize the expression of cell differentiation (CD) markers on the cells. Immunofluorescence analysis (IFA) was used to detect EBV viral proteins. Taqman® PCR to EBV polymerase was used to determine if treatment with tetradecanoyl phorbol acetate (TPA) induced viral replication in the cell lines. Naïve B cells were incubated with concentrated virus to determine if the EBV produced by the cell lines was capable of transforming cells. We assessed EBV viral gene expression in each cell line during viral reactivation.

**Results:** Comparison of data from 7 MHC microsatellite regions demonstrated that each cell line was unique. Both cell lines were positive for common B-cell cell differentiation (CD) markers and negative for T cell, NK cell and monocyte CD markers. IFA showed the cells were positive for the EBV viral proteins EBNA-1 and LMP-1. EBV DNA was detectable at levels 10-fold higher in TPA treated (induced) cells than in untreated cells. The virus produced by both cell lines was capable of transforming B-cells into immortalized cell lines. The results of EBV whole genome viral gene expression profiling allowed us to classify the cell lines into two different viral gene programs. In uninduced HCL-P cells, the expression of lytic viral genes is 5-8 fold higher than the level in uninduced HCL-B cells. TPA induced viral reactivation in HCL-B cells shows a classic latent to lytic progression in viral gene transcription while in HCL-P, the levels of lytic gene expression increase only slightly.

**Conclusion:** Two unique EBV expressing cell lines have been generated from two different HCL patients. The difference in viral programs elucidated by viral gene transcript profiling of the two cell lines may lead to further insights into differential viral gene regulation and expression.

**8.014**

**Accumulation of EBV BMRF1 Protein EA-D in EBV Reactivated Raji Cells**

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**Background:** EBV early gene BMRF1 protein (EA-D) is a major phospho-protein in EBV reactivation, but the mode of intra-cellular expression has not yet been fully elucidated. To study the early events of EBV reactivation, we tried to establish an inducible EBV reactivation system in EBV-positive Raji cells using the Tet-On system. The Tet-On system did not work; however, we found that the EBV latency was spontaneously disrupted and a large amount of EA-D molecules were accumulated in the cells.

**Methods:** Raji cells were transfected with the pTet-On and pTRE2hyg-BZLF1-oriP plasmids. EBV reactivation was analyzed by Northern blot, immunofluorescence, and Western blot. Cell apoptosis was analyzed by double staining of PI and Annexin V-GFP, and mapping of TUNEL and immunofluorescence of EA-D. Cells were separated by percoll gradient methods. Dephosphorylation of EA-D was analyzed by using protein phosphatase and okadaic acid.

**Results:** The EBV latency was disrupted spontaneously in the cells transfected with the plasmids. The strong EA-D fluorescence positive-small size cells, -intra-cellular vesicles, and -extra-cellular particles gradually accumulated in the culture from 1 week to 3 weeks after the transfection. The EA-D molecules with 58-, 50-, 48-, and 44-KDa were expressed in the cells. The 58- and 50-KDa, and 48- and 44-KDa molecules appeared at 1 week and 2 weeks, and 2 weeks to 3 weeks, respectively. The low molecules of EA-D were accumulated in the cells with the appearance of the EA-D positive-small size cells, -intra-cellular vesicles, and -extra-cellular particles. The EA-D positive cells were positive for TUNEL. The cultured cells at 3 weeks were fractioned by the percoll gradient-method. The EA-D positive-small cells, -intra-cellular vesicle containing cells, and -extra-cellular particles were mainly detected in the bottom fraction and they contained 50-, 48-, and 44-KDa EA-D molecules. The scanning electron microscopy indicated the extra-cellular particles to be covered with a thin membrane structure. The appearance of EA-D molecules was similarly observed in P3HR-1 cells at 33°C and anti-human IgG treated Akata cells. EA-D molecules of 48- and 44-KDa were brought from 58- and 50-KDa EA-D molecules by phosphatase treatment.

**Conclusion:** Our result demonstrates the sequential events of polymorphic EA-D molecules associated with phosphorylation to the intra-cellular expression, processing, accumulation, and exocytosis in EBV reactivated in Raji cells.

**8.015**

**Characterization of EBV BKRF3 Product**

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Uracil-DNA glycosylase (UDG or UNG) is a key DNA repair enzyme that is responsible for the removal of uracil from DNA. Recent studies further suggest that human UNG2 or UDG of large DNA viruses may coordinate with DNA polymerase accessory factor to enhance DNA replication. The putative UDG of EBV, BKRF3, was demonstrated to enhance oriLyt-dependent DNA replication in a co-transfection assay previously. However, the expression and enzyme activity of EBV BKRF3 have not yet been characterized. In this study, His-BKRF3 was bacterially expressed and purified for biochemical analysis. Similar to E. coli UDG, His-BKRF3 preferentially excised uracil from single-stranded DNA than from double-stranded DNA. UDG activity of BKRF3 is also inhibited by the purified phage inhibitior UGI. Additionally, BKRF3 is able to complement the the E. coli udg mutant in a rifampicin resistance mutator assay. The expression kinetics and subcellular localization of BKRF3 products were detected in EBV positive lymphoid and epithelial cells using BKRF3 specific mouse serum. We further demonstrated that expression of BKRF3 is predominantly regulated by EBV immediate early transactivator Rta. Taken together, we demonstrated that BKRF3 encoded UDG is expressed within EBV replicating cells and the BKRF3 UDG activity is able to complement the E.coli udg mutant. Our data thus suggest BKRF3 might participate in DNA repair system to ensure the viral DNA replication fidelity.

**8.016**

**Identification of EBV-Lytic-Cycle-Associated MicroRNAs**

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**Background:** MicroRNAs (miRNAs) are 19-25 nucleotide RNA molecules that post-transcriptionally regulate gene expression. Accumulating evidence indicates that miRNAs play a potential role in cell growth, differentiation and apoptosis, including human development and disease. Recently, in EBV-latently-infected cells, a group of EBV-encoded-miRNAs have been discovered, suggesting EBV has evolved to exploit RNA silencing for regulation of both host and viral genes.

**Methods:** miRNAs were isolated from different EBV-infected/non-infected cell lines. miRNA expression profilings were determined by miRNA microarrays. miRNA functional analysis was also performed by either enforced expression or inhibition of targeted miRNAs.

**Results:** Here, we reported that EBV lytic replication alters the expression profile of cellular miRNAs. A class of miRNAs, that have been shown previously to be involved in the induction of growth arrest and differentiation, are significantly elevated during EBV reactivation, which may contribute to the induction and progression of EBV reactivation.

**Conclusion:** This finding suggests a new mechanism through which cellular DNA synthesis is inhibited to allow EBV to achieve a robust lytic replication.

**8.017**

**Bortezomib: Most Potent Inducer of Lytic Infection in FDA Drug Library**

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**Background:** Induction of EBV lytic infection may be important in the pathogenesis or treatment of malignancies.

**Methods:** A library of 2720 agents including most FDA approved drugs was screened. The screen involved a Zta-luciferase reporter assay and a BX1 Akata GFP assay.

**Results:** The Zta luciferase assay identified 195 agents, the BX1 GFP assay identified 146 agents. The overlap was 54 agents. Bortezomib, a proteasome inhibitor that has recently been approved for the treatment of cancer, proved to be the most potent. Followup studies confirmed that Bortezomib is a potent viral lytic activator as assessed in Burkitt's cell lines (Akata, Rael) and a gastric carcinoma cell lines (SNU-719). Bortezomib's therapeutic activity as an anticancer agent has been attributed to inhibition of NFKB resulting from stabilization of IkB. Other active agents included anti-tubulin drugs, DNA damaging agents, and nucleotide analogues.

**Conclusion:** Many approved agents are upregulators of EBV lytic infection. Bortezomib is the most potent approved drug.


# Abstracts

**Monday, July 10, 2006**
**Room: Grand Ballroom (Level 2)**

## Session 9: Replication II
## 09:00-10:00

**9.001**

## Amino Acids in the Basic Domain of Epstein-Barr Virus ZEBRA Protein Play Distinct Roles in Binding DNA, Activating Early Lytic Gene Expression and Promoting Viral DNA Replication

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The ZEBRA protein of Epstein-Barr virus binds DNA, activates transcription, and promotes viral DNA replication. These activities allow the protein to initiate the viral lytic cycle cascade. The capacity of ZEBRA to recognize specific DNA sequences resides in amino acids 178 to 194, a region of the protein in which 9 of 17 residues are basic, either lysine or arginine. In order to learn which residues in the basic domain were essential for activity of the protein a series of 46 single amino acid substitution mutants were examined for their ability to bind DNA containing ZIIIB, a high affinity ZEBRA binding site, and for their capacity to activate early and late EBV lytic cycle gene expression. Nineteen mutants that failed to bind DNA were unable to disrupt latency. A single substitution of an acidic for a basic amino acid destroyed DNA binding and biologic activity of the protein. Three mutants that bound weakly to DNA were defective at stimulating expression of Rta, the essential first target of ZEBRA in lytic cycle activation. Thus, DNA binding was required for the protein to activate the lytic cascade. Four amino acids, R183, A185, C189 and R190, are likely to contact ZIIIB DNA specifically, since alanine or valine substitutions at these position weakened or eliminated DNA binding. Twenty-four mutants that were proficient in binding to ZIIIB DNA could be separated into four groups: wild type (8 mutants), defective at activating Rta (6 mutants, including 5 S186 site mutants and K181A), defective at activating EA-D (3 mutants, R179A, S186T and K192A), and specifically defective at activating late gene expression (7 mutants). Three of the late mutants, Y180A, Y180E and K188A, were shown to be defective at stimulating EBV DNA replication. This catalogue of point mutants reveals that individual basic domain amino acids play distinct functions in binding to DNA, in activating Rta, in stimulating early lytic gene expression, and in promoting viral DNA replication.

**9.002**

## Region Adjacent to Zta bZIP (CT Region) Contributes to the Specificity of DNA Binding in Addition to Extending Dimerisation Interface

**C. Schelcher**, S. Al Mehairi, E. Verrall, M.J. West, A.J. Sinclair. Univeristy of Sussex, Brighton, United Kingdom

**Background:** Zta (BZLF1) is a key regulator of the EBV lytic cycle. Zta has homology with the bZIP family of transcription factors that form dimers through a coiled-coil domain (ZIP) and bind DNA through a DNA-contact region. Previous biophysical analyses of Zta structure reveal that the ZIP region only partly conforms to the bZIP model: a coiled coil dimer is formed but is less stable than for canonical bZIP proteins. Adjacent to the ZIP region is a unique sequence (CT region). The crystal structure of the bZIP, with part of the CT region (Petosa et al, 2006), revealed that the CT region extends the dimerisation interface. We undertook a comprehensive structural and functional analysis of the entire CT region of Zta to probe its contribution to multiple functions of Zta.

**Methods:** DNA-binding assays were undertaken using EMSA with a series of methylated and non-methylated sites. Dimerisation assays were undertaken using an in vitro association assay. The biophysical properties of synthetic peptides spanning the Zta ZIP and CT region were analysed by Circular Dichroism and Analytical Ultra Centrifugation. Cross-linking and mass spectrometry studies revealed close contacts within the structure. The ability of Zta to transactivate viral genes and to induce viral replication was quantitated using real-time PCR. The ability of Zta to promote cell cycle arrest was determined by FACs analysis.

**Results and Conclusions:** The amino terminal half of the CT region contributes to the ability of Zta to promote cell cycle arrest and to transactivate viral genes whereas the entire CT region is required to induce viral replication. Biophysical analyses revealed that the CT region increases the stability of the coiled-coil and forms intimate contacts with the ZIP region. Fine mapping of mutants within the CT region revealed the relevant contacts between the ZIP and CT regions. Unexpectedly, some mutants that were capable of forming dimers were unable to bind DNA and further analysis revealed that the CT region strongly influences the specificity of DNA binding.

**9.003**

**Structural Basis of the Preferential Recognition of Methylated DNA Target Sites by the EBV Lytic Switch Protein ZEBRA**

**C. Petosa**[1], P. Morand[2], P. Pagniez[2], M. Perrissin[2], F. Baudin[2], C. Mueller[1]. [1]European Molecular Biology Laboratory, Grenoble Outstation, Grenoble, France; [2]Institut de Virologie Moleculaire et Structurale, Grenoble, France

**Background:** The immediate-early protein ZEBRA (BZLF1, Zta, Z, EB1) is a basic-region leucine zipper (bZIP) transcription factor which activates the promoters of EBV lytic genes by binding to target sites termed ZEBRA response elements (ZREs). ZEBRA has broader target site specificity than most bZIP proteins and is unusual because it preferentially binds certain ZREs in their methylated state (Bhende et al., 2004; Nat Gen 36:1099). The latter activity is critical for lytic cycle activation, as the EBV genome is highly methylated during latency.

**Methods:** We determined the crystal structure of a C-terminal fragment encompassing ZEBRA's dimerization and DNA-binding domain in complex with an AP-1-like ZRE from the promoter of the EBV early lytic gene *BSLF2/BMLF1* (Petosa et al., 2006; Mol Cell 21:565). We have recently crystallized the same fragment in complex with a methylated ZRE (site ZRE-2, TGAG$C^{me}$GA) from the promoter of ZEBRA's most proximal target, the EBV immediate-early gene *BRLF1*.

**Results:** Structural analysis, together with modeling and mutagenesis data, indicates that methylation of ZRE-2 favors a specific conformational change of amino acid residues Asn182 and Ser186. This stabilizes the protein:DNA complex by allowing ZEBRA to engage in a pattern of hydrogen bonds with the ZRE-2 site which differs from that observed with the AP-1 site.

**Conclusion:** The results suggest a detailed mechanism that accounts for ZEBRA's ability to recognize a wide variety of ZREs and to preferentially bind specific ZREs in their methylated state.

**9.004**

**Binding of ZEB1 to ZV of the BZLF1 Promoter Plays Central Roles in Maintenance of Latency and Reactivation of EBV**

**X. Yu**, Z. Wang, J.E. Mertz. Dept. of Oncology, University of Wisconsin, Madison, WI, USA

Epstein-Barr virus (EBV), a human gamma herpes virus, can establish latent infection in B lymphocytes. Its immediate-early gene, BZLFl, encodes a protein, Zta, that plays a central role in regulating the switch from latency to lytic replication. Previously, we identified a sequence element, ZV, located at nt -17 to -12 relative to the transcription initiation site of the BZLF1 promoter, Zp. We showed that a zinc finger E box-binding protein, ZEB1, binds to ZV, repressing transcription from Zp. Here, we report the effects of a 2-bp substitution mutation in the ZV element in the context of the whole EBV genome on infection of human 293 cells. We found that latently infected cell lines could be established. However, these cells contained, on average, at least 20-fold more immediate-early Zta and Rta mRNA and 30-fold more Rta and early BMRF1 protein than did cells latently infected in parallel with the parental wild-type virus. Immunofluorescence staining indicated approximately 3% of the ZV mutant-infected cells contained Zta, Rta, BMRF1 and late gp350 proteins not observed in the wild-type-infected cells. Strikingly, the mutant-, but not the wild-type-infected cells also spontaneously produced infectious virus, with virus production even greater upon addition of the inducers TPA and sodium butyrate. Furthermore, analysis of expression microarray data indicated that ZEB1's abundance in cells is dramatically affected by both cell type and inducers of reactivation. Thus, we conclude that binding of ZEB1 to Zp's ZV element plays central roles in maintenance of EBV latency and regulation of its switch to lytic replication.

## Notes


# Abstracts

**Monday, July 10, 2006**
**Room: Grand Ballroom (Level 2)**

## Session 10: Latent Infection III
## 11:00-12:00

**10.001**

## LMP1 Transgenic Mice Develop Germinal Center Lymphomas that are IL4-Independent

**K.H.Y. Shair**, K.M. Bendt, E.C. Bedford, N. Raab-Traub. University of North Carolina, Lineberger Comprehensive Cancer Center, Chapel Hill, NC, USA

**Background:** LMP1 transgenic mice (IgLMP1) develop lymphomas at a four fold higher incidence than wild-type mice. Primary B cells, including germinal center (GC) B cells, require both IL4 and CD40 ligation to survive. Additionally, IL10 is a potent growth factor for activated B cells and EBV-transformed B cells. To further characterize the LMP1 transgenic B lymphocytes and the LMP1-induced lymphomas, their growth properties in response to IL4 treatment, cytokine profiles, and activated signaling pathways were determined.

**Methods:** Expression of LMP1 and GC B cell markers were identified by immunohistochemistry. LMP1 transgenic lymphomas were passaged by intraperitoneal injection of SCID mice. Splenocytes were cultured in the presence or absence of IL4 and pulsed with BrdU to assay for proliferation or assayed by MTS for cell viability. CD19-positive B cells were assayed for BrdU incorporation, annexinV, and PI staining by flow cytometry. The expression of cytokines was defined by an Rnase protection assay.

**Results:** LMP1 was expressed at low levels in a subset of cells in normal spleens but was detected homogeneously in lymphoma. The lymphomas were positive for peanut agglutinin staining, indicative of GC origin. As indicated by BrdU incorporation and MTS assays, splenocytes from IgLMP1 mice survived better than wild-type mice and proliferated in response to IL4. Upon progression to lymphoma, both LMP1-positive and negative lymphomas proliferated independently of IL4 co-stimulation. LMP1 transgenic B-cells had elevated phospho-STAT6. In contrast, the lymphoma cells did not express IL4 mRNA and phospho-Stat6 could not be detected. Interestingly IL10 mRNA was upregulated with concomitant activation of Stat3. Cell cycle proteins were also dysregulated such that lymphomas had decreased levels of p27, and increased levels of Cdk2 and phospho-Rb.

**Conclusions:** LMP1 transgenic B-cells proliferate in response to IL4 indicating that LMP1 bypasses the requirement for CD40 ligation. The GC-derived lymphomas that develop are IL4-independent and lack activated Stat6. The lymphomas express elevated levels of IL10 with high levels of activated STAT3. GC B-cells require IL4 and CD40 ligation to escape apoptosis while LMP1 transgenic lymphocytes respond to IL4 alone. In contrast, the LMP1 lymphomas do not require IL4 or activation of its signaling pathway. These characteristics may relate to EBV-associated B cell malignancies.

**10.002**

**LMP2A Confers Resistance to Anoikis Through ERK Activation in Epithelial Cells**

**D. Iwakiri**, M. Samanta, S. Maruo, H. Yoshiyama, K. Takada. Institute for Genetic Medicine Hokkaido University, Sapporo, Japan

**Background:** EBV has been reported to be associated with epithelial malignancies including nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC). To assess the effect of EBV infection on epithelial cells, we generated EBV-converted human intestinal epithelial cell lines using in vitro recombinant EBV infection system of epithelial cells.

**Methods:** Human fetal intestinal epithelial cell line Intestine407 was infected with recombinant EBV carrying the neomycin-resistant gene, and EBV-infected cells were cloned by G418 selection. RT-PCR and immunoblot analyses were performed to check the viral gene expression in EBV-infected cells. Cells were maintained in suspension using low-binding culture plate, and induction of detachment-induced apoptosis (anoikis) was evaluated by cell death assay and caspase activity assay. Immunoprecipitation and immunoblot analyses were carried out to investigate intracellular signal transduction and protein expression.

**Results:** RT-PCR and Immunoblot analysis demonstrated that EBV-infected Intestine407 cell clones expressed EBNA1, EBERs, LMP2A, but not LMP1. Anoikis assays revealed that EBV-infected Intestine407 cells were resistant to anoikis compared to uninfected cells. Western blot analysis demonstrated that MEK/ERK pathway was remarkably activated in EBV-infected cells compared to uninfected cells, whereas MEK inhibitor treatment resulted in reduction of resistance to anoikis in EBV-infected cells. Moreover, studies using expression plasmids showed that LMP2A expression in Intestine407 cells cause resistance to anoikis through ERK activation. Consequently, we generated Intestine407 cells infected with LMP2A-knock out (KO) EBV and used for further studies. LMP2A-KO EBV-infected cells showed reduced ERK activity and were susceptible to anoikis compared to wild type (WT) EBV-infected cells. Further analysis revealed that LMP2A-mediated ERK activation caused downregulation of Bim protein, which contributes to induction of anoikis.

**Conclusions:** Our results demonstrated that LMP2A confers resistance to anoikis in Intestine407 cells through activation of ERK pathway. LMP2A may play a role in epithelial cancer development.

**10.003**

**Epstein-Barr Virus Protein Interaction Networks**

**M.A. Calderwood**[1], K. Venkatesan[2], L. Xing[1], M.R. Chase[1], A. Holthaus[1], S. Ewence[1], N. Li[2], T. Hirozane-Kishikawa[2], D.E. Hill[2], M. Vidal[2], E. Kieff[1], E.C. Johannsen[1]. [1]Channing Laboratory, Harvard Medical School, Boston, MA, USA; [2]CCSB, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA

To identify and characterize key protein-protein interactions in the pathogenesis of Epstein-Barr virus (EBV) infections, high throughput yeast two-hybrid (Y2H) analysis was undertaken. Comprehensive Y2H matrix screening of ≈8000 potential EBV intraviral protein interactions identified 44 distinct interactions. These included 4 of the 15 known interacting pairs of proteins (27%) and 40 novel interacting pairs. To confirm the quality of the Y2H results, all the putative interactions were tested for β-galactosidase activity and by co-affinity purification resulting in a high-confidence dataset comprising approximately 60% of the original interactions. Seven of the interactions identified were homodimer/multimers including two previously identified protein pairs. Within the remaining interacting pairs the most robust interactions appeared to exist between proteins of similar functional groups, latent-latent and structural-structural. Moreover ≈60% of the interactions were between pairs of proteins from the same conservation class (ie core herpesvirus proteins interacted with other core proteins and gamma specific with other gamma specific). In addition to investigate the interactions of EBV with the host cell, a screen of a human cDNA library using 122 baits representing 85 EBV ORFs was undertaken and identified a high confidence set of 173 interactions. By merging the preliminary EBV-host interactome onto the existing human interactome a global view of how EBV interacts with the host cell could be obtained. Bioinformatical analyses of the EBV targets showed that they formed a highly interconnected sub-network within the host interactome suggesting high functional relatedness. Furthermore, the nodes of the host network targeted by EBV had a higher average degree of connectivity compared to the host interactome and this suggests that EBV may be specifically targeting highly connected proteins, or hubs. Overall this data identified a large number of novel protein interactions and provides a further insight into the pathways targeted by EBV within the host cell.

**10.004**

## Epstein-Barr Virus EBER2 Can Prevent a Stress Response in Transfected Human Cells

S. Pattle, **G. MacArthur**, P.J. Farrell. Imperial College Faculty of Medicine, London, United Kingdom

**Background:** The EBER RNAs have been proposed by other labs to play roles in the interferon response, transformation of B cells by EBV and the tumourigenicity of Burkitt's lymphoma cell lines but their biochemical mechanisms of action remain uncertain.

**Methods:** We devised a system to express EBER RNAs in transfected cells at levels comparable to those in EBV infected cell lines using the human H1 promoter in the pSUPER vector. Using Northern blotting and a flow cytometry assay to test EBER expression, the normal level of EBER expression was achieved in about 50% of transiently transfected 293 cells. RNA from these transfected cells was analysed on microarrays for changes in gene expression in response to EBER transfection, comparing EBER1 or EBER2 with an empty vector control. RNA from five separate transfection experiments was analysed using up to 6 replica arrays for each transfection.

**Results:** EBER1 did not significantly affect cellular gene expression, whereas EBER2 altered the levels of RNA from several NF-kB-regulated genes. It appears that a cell stress response activating NF-kB that occurs in response to DNA transfection is suppressed by expression of EBER2 from the transfected plasmid. No changes in PKR level or activity were observed in response to the expression of EBER1 or EBER2 in the transient transfection assays. Additional studies using a conditional (Tet-R regulated) expression system for EBER RNAs suggest that it will also be possible to study EBER function in stably transfected cell lines using these vectors.

**Conclusions:** The results indicate that EBER1 and EBER2 can have different biological functions. The ability of EBER2 to prevent a stress response in transient transfection may be a manifestation of a biological function of EBER2 in EBV infected cells.

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## Notes


# Abstracts

**Monday, July 10, 2006**
**Room: Grand Ballroom (Level 2)**

## Session 11: Therapy
## 14:30-15:30

**11.001**

**Targeting Dendritic Cells for Vaccination Against EBV-Associated Tumors**
**C. Gurer**, C. Munz. Rockefeller University, New York, NY, USA

**Background:** In most EBV infected individuals, EBV is controlled by strong T cell immunity. After immunosuppression, EBV associated B cell lymphomas occur, and these can be cured by adoptive transfer of EBV specific T cell lines. Furthermore, in immunocompetent virus carriers, EBV associated tumors of B and epithelial cell origin can arise spontaneously. Most of these tumors express only a subset of EBV antigens and neither immunotherapy nor vaccination exists against them. We and others have found that $CD4^+$ T cell responses consistently recognize EBNA1, which is expressed in all EBV associated malignancies, and that EBV-specific $CD4^+$ T cells can target most EBV associated B cell lymphomas. Since dendritic cells (DCs) seem to be capable of priming protective EBV specific T cell responses, we decided to target EBNA1 to dendritic cells for enhanced EBNA1 specific T cell expansion, and develop this strategy as a vaccine approach against EBV associated malignancies.

**Methods:** Our approach is to deliver the immunogenic C-terminal domain of EBNA1 to maturing DCs by fusing it to a monoclonal antibody that targets DEC-205, an endocytic receptor for antigen presentation on DCs. Because most individuals have protective immunity to EBV, we evaluated presentation of antibody-fused EBNA1 by monocyte-derived DCs to human EBNA1 specific $CD4^+$ and $CD8^+$ T cells by proliferation and IFN$\gamma$ secretion.

**Results:** We started to compare EBNA1 specific $CD4^+$ and $CD8^+$ T cell expansion in PBMCs after stimulation with antiDEC-205:EBNA1 and isotype control:EBNA1 loaded autologous monocyte-derived DCs. For this purpose we pulsed DCs with 1$\mu$g of each antibody and stimulated at a DC:PBMC ratio of 1:100. Afterwards, we evaluated IFN$\gamma$ production and proliferation of the expanded T cell cultures in response to an overlapping peptide library of $EBNA1_{400\text{-}641}$. We found antiDEC-205:EBNA1 loaded DCs to consistently be 3-5 times more efficient in expanding IFN$\gamma$ secreting EBNA1 specific $CD4^+$ as well as $CD8^+$T cells than isotype control:EBNA1 loaded DCs. Moreover, 4 times more EBNA1 specific $CD4^+$ and $CD8^+$ T cells proliferated after antiDEC205:EBNA1 mediated expansion.

**Conclusions:** Loading of DCs by EBNA1 targeting to the DEC-205 endocytosis receptor leads to efficient antigen processing for MHC class I and II presentation and should be further explored for vaccine development against EBV associated malignancies.

**11.002**

**Treatment of Epstein Barr Virus Positive Nasopharyngeal Carcinoma with Adoptively Transferred Cytotoxic T cells**

C.U. Louis, K. Straathof, V. Torrano, C.M. Bollard, A.M. Leen, M.H. Huls, M.V. Gresik, H. Weiss, A. Gee, M.K. Brenner, C.M. Rooney, H.E. Heslop, **S. Gottschalk**. Baylor College of Medicine, Houston, TX, USA

**Background:** The majority of undifferentiated nasopharyngeal carcinomas (NPC) are EBV positive, and EBV antigens expressed in NPC are attractive targets for the immunotherapy with antigen-specific cytotoxic T cells (CTL). We are evaluating the safety and efficacy of EBV-specific CTL (EBV-CTL) in two Phase I clinical trials. In the first clinical trial EBV-CTL are given alone (Straathof et al.; Blood 2005; 105:1898) and in the second clinical trial we are aiming to enhance the in vivo expansion of infused EBV-CTL by lymphodepletion prior to cell infusion. This should result in homeostatic proliferation of the infused T cells to restore the lymphoid compartment. For lymphodepletion prior to EBV-CTL infusion we are using a pair of monoclonal antibodies, targeted to the CD45 antigen (CD45Mabs).

**Study Design:** The primary objective of these phase I clinical trials is to determine the safety of escalating doses of EBV-specific CTL with or without CD45Mabs infusion in EBV positive NPC patients. The secondary objective is to determine the expansion, persistence and anti-tumor effects of infused EBV-specific CTL.

**Results:** Eighteen patients with advanced NPC have been treated with autologous EBV-CTL (2x10e7-2x10e8 cells/m2 per infusion). Patients received between 1 and 5 CTL infusions, which were well tolerated, although one patient developed transient swelling at the site of pre-existing disease. Prior to CTL infusion 6 patients were in remission (5CR/1CRu) and 12 had refractory/relapsed disease. All 6 patients treated in remission remain in remission 1 to 50 months post CTL infusion. Ten of 13 patients with disease have been followed for more than 3 months post CTL infusion; 3 had complete responses and remain in remission for 48 months after CTL infusion; 3 had stable disease for 7 - 27 months; 1 had a partial response for 12 months; 4 had progressive disease. Five of 12 patients with relapsed/refractory disease received anti-CD45Mabs followed by EBV-CTL. Infusion of anti-CD45Mabs resulted in a transient decrease in the absolute lymphocyte count (3.5 fold) that recovered to baseline within 14 days. In 2 patients with low precursor frequency of EBV-specific CTL in peripheral blood, EBV-CTL expanded 21- and 2.5 fold within 8 weeks post CTL infusion as judged by IFN-g Elispot assays.

**Conclusion:** Treatment of EBV-positive NPC with EBV-CTL appears to be safe and can be associated with significant anti-tumor activity. Infusion of anti-CD45Mabs prior EBV-CTL infusion resulted in transient lymhodepletion and appears to result in better expansion of adoptively transferred CTLs.

**11.003**

**Lytic Cycle Gene Activation for Targeted Radiotherapy**

**D. Fu**, J. Chen, M. Pomper, R. Ambinder. Johns Hopkins, Baltimore, MD, USA

**Background:** Radiation therapy is active in most EBV-associated malignancies but is limited by toxicities to normal tissues and an inability to focus on tumor tissue at a cellular level. Reactivation of viral lytic genes so as to kill cancer cells chemotherapeutically such as with ganciclovir has been limited because often only percentage of cells express lytic proteins. I 131 beta particles have a path length of 1-2 mm (approximately 100 cell diameters) resulting in killing of bystander cells.

**Methods:** We used a potent inducer of viral lytic infection (bortezomib) in combination with I-125 or I 131 FIAU (a nucleoside analogue that is selectively phosphorylated by the EBV TK) in cell lines and murine xenograft experiments to assess targeted delivery of radioisotope to tumor tissue and therapeutic response. Assessment involved scintillation counting, planar spect imaging and direct tumor measurement

**Results:** Labeled FIAU is concentrated in EBV(+) Burkitt's lymphoma and gastric carcinoma(Rael,Akata, SNU) treated with bortezomib but not in the absence of bortezomib or in EBV (-) Akata or other tumor lines. I-125 FIAU is concentrated in tumor xenografts (EBV(+) Rael, Akata and PEL cell lines) that are treated with bortezomib but not in controls as shown in Fig. 1. Bortezomib and I-131 FIAU induce tumor regression in murine xenograft models (Fig. 2). Bortezomib alone is much less active and I-131 FIAU alone is inactive.

**Conclusion:** Induction of the EBV TK in a percentage of cells allows targeted delivery of I-131 to tumor tissue and results in tumor responses.

**11.004**

**Epstein Barr Virus-Specific Cytotoxic T Lymphocytes Expressing an Anti-CD30 Chimeric T Cell Receptor (cTcR) for the Treatment of Hodgkin's Disease.**

**B. Savoldo**[1], C. Rooney[1], H. Heslop[1], H. Abken[2], A. Hombach[2], L. Zhang[1], M. Pule[1], M. Brenner[1], G. Dotti[1]. [1]Baylor College of Medicine, Houston, TX, USA; [2]Tumor Genetics, University of Cologne, Cologne, Germany

HD may be a suitable target for immunotherapy, and in patients with EBV+ HD, adoptive transfer of EBV-CTL has produced disease responses. An alternative target is the CD30 molecule, present on the malignant cells of almost all patients with HD. Recently, T lymphocytes have been engineered to express a chimeric TcR targeting the CD30 molecule. However, these chimeric molecules connect the antigen-recognition properties of CD30 antibodies with the endodomain of the CD3-zeta chain, which is insufficient to fully activate resting T cells to proliferate and release cytokines. EBV-CTL can fulfill this need, since the co-stimulatory signals delivered by EBV-infected B cells after native receptor engagement ensure full functionality when the CTLs subsequently bind to tumor cells through their cTcR. We have therefore transduced with CD30-cTcR EBV-CTL from 8 healthy EBV+ donors and showed that they retained killing of their autologous LCL targets through their native receptor (64.4±16% at 20:1 E:T ratio), and became able to lyse CD30+ malignant lymphoma targets through their cTcR (e.g. HDLM-2=45.4±16%). Killing of CD30+ tumor cells was significantly inhibited by preincubation with an anti-CD30 blocking antibody (16.5±12%). Of potential concern, however, is that CD30 is expressed by activated normal T lymphocytes: expression, undetectable on resting T cells, increased to 3-32% on day 4-7 after stimulation with LCL. Fortunately, expression dwindles to 3-6% by two weeks as an EBV-specific line emerges. Therefore, expression of a CD30 cTcR did not impair the antigenic repertoire of the EBV-CTL, which retained the same pattern of immunodominant MHC class I epitopes (detected by tetramer) as control cells. We also performed co-culture experiments to evaluate whether infusion of CTL-CD30 cTcR could cross-compromise the primary reactivation of other virus-specific CTL. Autologous EBV-CTLs engineered to express the CD30-cTcR were added to cultures of PBMC stimulated to reactivate CMV- or adenovirus-specific CTL. In 4/4 donors, the percentage of CMV pp65+ T cells did not change, while generation of adenovirus-specific T cells (Hexon-tetramer+) was significantly reduced in only 1/3 donor. Finally, in a xenograft SCID murine model, using an in vivo luminescence detection system (IVIS), we have confirmed that redirected EBV-CTLs retained their ability to migrate to the LCL and expand at the tumor site and were able to control the growth of implanted CD30+ tumor cells. These data support the feasibility of using EBV-CTL bearing a cTcR for CD30 to treat both EBV+ and EBV- HD.

## Notes

# Abstracts

**Monday, July 10, 2006**
**Room: Grand Ballroom (Level 2)**

## Session 12: Human Infections
## 16:30-17:30

**12.001**

## Expression of LAG-3 by Tumor-infiltrating Lymphocytes is Co-incident with the Suppression of Latent Membrane Antigen-specific CD8+ T-Cell Function in Hodgkin Lymphoma Patients

**M.K. Gandhi**[1], E. Lambley[1], J. Duraiswamy[1], U. Dua[1], C. Smith[1], S. Elliott[1], D. Gill[2], P. Marlton[2], J.F. Seymour[3], R. Khanna[1]. [1]QIMR, Brisbane, Australia; [2]Princess Alexandra Hospital, Brisbane, Australia; [3]Peter MacCallum, Brisbane, Australia

**Background:** In Hodgkins Lymphoma (HL) the malignant Hodgkin Reed-Sternberg (HRS) cells comprise only 0.5-10% of the diseased tissue. The surrounding cellular infiltrate is enriched with T-cells which are hypothesized to modulate anti-tumor immunity.

**Methods:** Immunohistochemistry, ex-vivo immune profiling and in-vitro culture experiments were performed. 94 patients with HL were prospectively evaluated, and results compared with healthy controls.

**Results:** We show that a marker of regulatory T-cells, LAG-3 is strongly expressed on infiltrating lymphocytes present in proximity to HRS cells. Circulating regulatory T-cells (CD4+CD25hiCD45ROhi, CD4+CTLA4hi and CD4+LAG-3hi) were elevated in HL patients with active disease when compared to remission. Longitudinal profiling of EBV-specific CD8+ T-cell responses revealed a selective loss of interferon-gamma expression by CD8+ T-cells specific for latent membrane proteins (LMP) 1 and 2, irrespective of EBV tissue status. Intra-tumoral LAG-3 expression was associated with EBV tissue positivity, whereas FOXP3 was linked with neither LAG-3 nor EBV tissue status. The level of LAG-3 and FOXP3 expression on the tumor-infiltrating lymphocytes was co-incident with impairment of LMP1/2-specific T-cell function. In vitro pre-exposure of peripheral blood mononuclear cells to HRS cell-line supernatant significantly increased the expansion of regulatory T-cells and suppressed LMP-specific T-cell responses. Deletion of CD4+ LAG-3+ T-cells enhanced LMP-specific reactivity. These findings indicate a pivotal role for regulatory T-cells and LAG-3 in the suppression of EBV-specific cell-mediated immunity in HL.

**12.002**

**BMI-1 is Induced by LMP1 and Regulates Cellular Gene Expression and Survival in Hodgkin's Lymphoma Cells**

**A. Dutton**, M.B. Chukwuma, J.I.K. Last, W. Wei, M. Vockerodt, S. Morgan, C.B. Woodman, L.S. Young, P.G. Murray. University of Birmingham, Birmingham, United Kingdom

**Background:** Polycomb Group (PcG) genes are necessary for the maintenance and renewal of embryonic and adult stem cells, embryogenesis and cell cycle regulation. Several PcG genes, including BMI-1/PCGF4, are also implicated in oncogenesis. In transgenic mice, BMI-1 induces down-regulation of p16INK4a and p19ARF, lymphoid proliferation, and the development of lymphomas. Although previous studies have reported the high level expression of BMI-1 by HRS cells, its regulation and contribution to the pathogenesis of Hodgkin's lymphoma (HL) is unknown.

**Results:** We have found that BMI-1 is upregulated in HL cells by LMP1. Furthermore, we show that this upregulation is mediated through the ability of LMP1 to induce constitutive NF-κB signalling in these cells. Importantly, downregulation of BMI-1 by siRNA decreased the survival of HL cells, suggesting that BMI-1 may mediate the pro-survival effects of LMP1-induced NF-κB signalling in HL cells. BMI-1 target genes, identified by microarray analysis following its knockdown in L428 HL cells, showed overlap with an HRS cell gene expression 'signature'. These included a number of known LMP1 targets and HL associated oncogenes and tumour suppressor genes, including hexokinase II and ataxia telangiectasia mutated (ATM) gene.

**Conclusion:** Our data suggest that BMI-1 contributes to LMP1-induced oncogenesis in HL via regulation of genes with known roles in cell growth and survival. As BMI-1 is widely overexpressed in many cancers, our data provides a more general insight into the role of this PcG protein in the oncogenic process.

**12.003**

**Epstein-Barr Virus Shed in Saliva is High in the B Cell Tropic Glycoprotein gp42**
**R. Jiang**, R.S. Scott, L.M. Hutt-Fletcher. Louisiana State University Health Sciences Center, Shreveport, LA, USA

EBV virions carry complexes of gHgLgp42 for B cell entry and complexes of gHgL for epithelial cell entry. In HLA class II-positive B cells, but not HLA class II-negative epithelial cells some three part complexes are lost to the class II processing pathway. Epithelial virus is thereby better able to infect B cells whereas B cell virus is more infectious for epithelial cells. gp42 blocks virus binding to a gHgL receptor (gHgLR) on epithelial cells. B cell virus binds almost as well to gHgLR as to CR2. Epithelial virus binds very poorly to gHgLR. Thus the relative ability of virus to bind to each can be used to identify its source. Lymphoblastoid cell lines (LCL) were made from healthy donors using virus in saliva from each. The relative ability of virus made by these LCL and virus shed in saliva to bind to CR2 and gHgLR-positive cells was determined by real-time QPCR. On average, slightly more LCL virus bound to CR2 than to gHgLR, although the ratio of binding was never higher than 3 and the variability in repeated assays was low. There was greater variability in virus binding from repeat collections of saliva. However, on average, for each donor, virus in saliva was significantly better able to bind to CR2 than to gHgLR. Flow cytometric analysis of saliva virus bound to CR2 and stained with antibody to gH or gp42, indicated a higher ratio of gp42 to gH than in LCL-virus from the same individual. These results indicate that on aggregate virus in saliva is shed from an HLA class II-negative cell. They are consistent with observations of EBV in tonsil epithelium of healthy carriers and with a model in which amplification of virus in an HLA class II-negative cell is part of the normal cycle of persistence.

**12.004**

## EBV Infection of Oral Mucosal Epithelium is Mediated by Monocytes and is Spread Unidirectionally Toward the Mucosal Surface

**S. Tugizov**[1], R. Herrera[1], P. Veluppillai[2], J. Greenspan[2], D. Greenspan[2], J. Palefsky[1].
[1]University of California, San Francisco, Department of Medicine, San Francisco, CA, USA; [2]University of California, San Francisco, Department of Orofacial Sciences, San Francisco, CA, USA

**Background:** Epstein-Barr virus causes hairy leukoplakia (HL), a benign lesion of oral epithelium. EBV replication in HL was restricted to its spinosum and granulosum layers. Mechanisms of EBV infection of oral epithelial cells and its partial dissemination within the terminally differentiated mucosal epithelium are not well understood. The objective of this study was to investigate the mechanisms of EBV dissemination within the oral mucosal epithelium.

**Methods:** Fresh biopsies of tongue and buccal mucosa containing epithelium and connective tissue were obtained using 4-mm diameter biopsy punches from healthy volunteers who had no inflammation in the oral cavity. Tissue explants were infected with EBV B95-8 and cultured for 1, 2, 3, 4 and 7 days. Then, tissue explants were fixed, sectioned and immunostained for EBV proteins, and immune and epithelial cell markers.

**Results:** Immunofluorescence, electron microscopy and barrier function analysis of oral epithelium showed it to be highly organized, multistratified and polarized. Immunostaining analysis of HL sections showed that oral mucosal epithelium contains EBV-infected intraepithelial macrophages and Langerhans cells. Tongue and buccal explants infected ex vivo with EBV showed that the virus first infects submucosal CD14+/CD68+ immune cells, which migrate into the epithelium and differentiate into macrophages and/or dendritic/Langerhans cells. Virus entry from EBV-infected intraepithelial macrophages/Langerhans cells into polarized spinosum and granulosum keratinocytes initiates productive viral infection. EBV enters the keratinocytes at their basolateral membranes, where the EBV BMRF-2 protein binds to b1 or a3b1 integrins. Unidirectional dissemination of EBV from keratinocyte apical surfaces toward the mucosal surface may lead to shedding of virus into saliva.

**Conclusions:** This study demonstrates the role of intraepithelial macrophages/Langerhans cells in initiating epithelial EBV infection and the importance of epithelial cell polarization in determining directional spread of EBV within the epithelium.

## Notes

# Abstracts

**Monday, July 10, 2006**
**Room: Poster Area/Level 2**

## Session 13: Poster Session II
## PTLD and Other Malignancies
## 17:30-19:00

13.001

## Persistently Elevated Cell-Associated EBV DNA Loads, Restricted EBV Transcription and Presence of Anti-EBV IgA in the Circulation of Asymptomatic HIV-Carriers

S.J.C. Stevens[1], P.H.M. Smits[2], S.A.M.W. Verkuijlen[1], D. Rockx[1], J.W. Mulder[3], **J.M. Middeldorp[1]**. [1]VU University medical center, Dept. Pathology, Amsterdam, Netherlands; [2]Slootervaart Hospital, Dept. Microbiology, Amsterdam, Netherlands; [3]Slootervaart hospital, Dept. Internal Medicine, Amsterdam, Netherlands

**Background:** In contrast to healthy EBV-seropositive donors, approx. 20% of asymptomatic HIV-carriers has elevated EBV DNA loads in whole blood (WB), i.e. >2000 copies/ml. We aimed to determine whether these high loads are persistent, to define EBV transcription in blood and to analyze anti-EBV antibody responses.

**Methods:** WB, PBMC and plasma samples were collected from 205 asymptomatic HIV-carriers in 2004 (approx. 2% of the Dutch HIV-population), and included 34 follow-up samples from patients already previously sampled in 1999. B-cells were isolated from PBMC by CD19 Dynabeads. EBV DNA load was measured by EBNA1-based LightCycler PCR. EBV RNA expression was determined in PBMCs by RT-PCR and NASBA. Anti-VCA-p18 and EBNA1 IgG/IgA were assessed by synthetic peptide-based ELISAs.

**Results:** Of 205 WB samples, 123 had detectable EBV DNA in blood, with 39/205 (19%) above 2000 c/ml. Unlike HIV RNA load, EBV DNA load did not correlate with CD4+ T-cell count. Of 10 HIV-carriers with elevated EBV DNA loads in 1999, 8 also had elevated loads in 2004, while 24 EBV DNA-negative HIV-carriers from 1999 remained negative in 2004. EBV-DNA was >90% associated with the B-cell fraction in all cases. EBV RNA analyses on PBMCs could be performed for 22 high EBV DNA load-carriers and showed positive BARTs in 68% and LMP2 in 55%. EBNA1 and LMP1 mRNA were never detected, despite positive human U1A mRNA in all samples. All tested HIV-carriers had anti-VCA-p18 IgG and IgG-VCA levels positively correlated with EBV DNA load. Anti-EBNA1 IgG was detected in 94% and inversely correlated to EBV DNA load. IgA to VCA-p18 and EBNA1 was found in 69% and 33% respectively but was unrelated to EBV DNA levels.

**Conclusion:** High B-cell associated EBV DNA loads are persistent in a subset of asymptomatic HIV-carriers for more than 5 years. EBV transcription in these cells is silent or restricted to LMP2 and BARTs, similar to HIV-negative EBV-carriers. High EBV DNA loads correlate with high anti-VCA-p18 IgG and low anti-EBNA1 IgG levels, indicating increased lytic replication and/or decreased (humoral) latency control. Unexpectedly, almost 70% of HIV-carriers had anti-VCA-p18 IgA, which was absent in healthy EBV-carriers. Triggering of humoral immune responses by EBV antigens shows clear difference for the IgA and IgG response, which possibly relates to source of antigen (cell-associated vs. virion-associated) or localisation (systemic vs. mucosal).

**13.002**

## Differential Internal Translation Efficiency of Epstein-Barr Virus Present in Solid Organ Recipients

**A. Isaksson**, M. Berggren, A. Ricksten. Institution of Biomedicine, Gothenburg, Sweden

**Background:** We have previously identified a functional internal ribosome entry site, the EBNA IRES, in the U leader exon of the 5'untranslated region of the EBNA1 gene. The EBNA IRES promotes cap-independent translation and increases the expression level of EBNA1 protein. Sequence analysis of the EBNA IRES was undertaken in samples from eight immunosuppressed organ transplant patients to investigate the genetic variability of the EBNA IRES. The EBNA IRES derived from organ transplant patients, as well as from the cell lines Rael and B95-8, was further analysed in a bicistronic vector system in transient transfection experiments. Secondary structure models from the IRES sequences are presented and discussed.

**Methods:** RT-PCR with total RNA prepared from patients or cell lines was carried out, and the cDNA was used as template for PCR amplification. The PCR products were sequenced and subsequently cloned into a bicistronic vector. Transient transfection experiments with five different human cell lines; DG75, P3HR-1, Rael, B95-8 and Molt-4 were performed. Secondary structure models for the IRES sequences were predicted by using the software mFold.

**Results:** We found a novel organ transplant specific mutation (G to A) at position 67 531 in the EBV genome. We have previously described a base substitution at position 67 585 (T to C) when comparing the EBNA IRES sequence from Rael with the sequence derived from B95-8. Neither the substitution at position 67 531 nor the substitution at position 67 585 appeared to have a significant impact on the IRES activity in B-cells. In contrast, the patient-specific change resulted in a 4-fold decrease of IRES activity in the T-cell line Molt-4, while the substitution at position 67 585 only slightly decreased the EBNA IRES activity in the same cell line. The secondary structure models showed high similarities despite the variations in IRES-activity.

**Conclusions:** The patient specific mutation found within the EBNA IRES sequence was present in samples from all eight organ transplant patients. The IRES sequences derived from transplant patients, Rael and B95-8 cell lines, result in differential internal translation efficiencies in a bicistronic reporter gene system. In specific, the various sequences give rice to a cell specific IRES activity with comparable activities in B-cells, but significantly lower activity from the patient specific sequence in T-cells.

**13.003**

## Expression of Interleukin 9 in The EBV-positive Nasal Natural Killer (NK)/T-Cell Lymphoma Cell Lines and Patients

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**Purpose:** Nasal natural killer (NK)/T-cell lymphoma is associated with Epstein-Barr virus and has peculiar clinical and histological features. However, little is known about its genetic features. In this study, we examined the genes expressed by SNK-6 and SNT-8 cells, which were established from the EBV-positive nasal NK/T-cell lymphomas, and found that interleukin 9 (IL-9) was specifically expressed in these two cell lines.

**Experimental Design:** Complementary DNA (cDNA) array was used to examine the genes expressed by SNK-6 and SNT-8 cells. Expression of IL-9 and IL-9 receptor (IL-9R) was investigated by reverse transcription-polymerase chain reaction, enzyme-linked immunosorbent assay (ELISA), and flow cytometry. Cell growth was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Immunohistological staining and ELISA were used to examine IL-9 expression in biopsies and sera from patients, respectively.

**Results:** In cDNA array, expression of IL-9 mRNA was much higher in SNK-6 and SNT-8 cells than in NK-92 cells from non-nasal NK-cell lymphoma and peripheral blood mononuclear cells from healthy volunteers. Furthermore, IL-9 was specifically expressed by SNK-6 and SNT-8 cells that were EBV-positive cell lines but not by other NK-, NK-like T-, and T-cell lymphoma/leukemia cell lines that were EBV-negative cell lines. IL-9R was also expressed on the surfaces of SNK-6 and SNT-8 cells. An IL-9-neutralizing antibody inhibited the growth of these two cell lines, whereas recombinant human IL-9 enhanced their growth. Most significantly, IL-9 was present in biopsies and sera from patients with this lymphoma.

**Conclusions:** We found here that IL-9 was expressed and produced only in SNK-6 and SNT-8 cells, which are EBV-positive, but that it is not expressed by EBV-negative NK- and NK-like T-cell lines, including NK-92, KHYG-1, DERL-2, and MTA cells. These findings suggest that EBV may be associated with IL-9 expression and production in nasal NK/T-cell lymphomas. These results suggest that IL-9 plays an important role in the EBV-positive nasal NK/T-cell lymphoma, possibly via an autocrine mechanism.

**13.004**

**EBV Status in an Organ Transplant Patient with Ulterior Autoimmune Disease**

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**Background:** We describe a transplant recipient with unusual EBV-status. The patient was bilaterally lung transplanted as a consequence of hypocomplementemic urticarial vasculitis syndrome (HUVS), which progressed to pulmonary obstructive lung disease. Organ transplant recipients are heavily immunosuppressed and at risk of developing post transplantational lymphoprolipherative disorder (PTLD) that is most commonly linked to activation of EBV. The patient was therefore closely monitored for EBV-expression at the time of transplantation and during 91 days post transplantation. The aim of this study was to relate EBV-status to other clinical findings and investigate the potential role of EBV in association with HUVS.

**Methods:** To fully evaluate the viral status we screened whole blood for expression of viral genes representing different latencies and lytic cycle. RT-PCR on EBNA1, EBNA2 and LMP1-expression served as markers for latency I, II and III. We also screened for promoter usage in the EBNA1 gene signifying different stages of infection e.g. early infection, maintaining latency and lytic phase. Sera were screened for EBV-DNA by PCR and EBV-IgG was quantified. EBER in situ hybridization was used on sections from graft lung tissue.

**Results:** The patient expressed EBNA1 in serial blood samples, which is common for PTLD patients, yet the patient had no signs PTLD even after a seven-year follow up. The EBNA1 transcripts originated mostly from the Q-promoter. EBNA2 and LMP1 expression was not detected. EBV-DNA in serum samples was only detectable twice. There was a high variation in EBV-IgG titers with inverted correlation to lymphocyte concentration. EBER In situ hybridization of graft lung tissue sections showed no sign of EBV infection.

**Conclusion:** EBV-status in this patient is categorized as latency I with EBNA1 Q-promoter usage, which indicates that the virus is set to maintain latency. The unusual high and persistent expression of EBNA1 in blood is probably due to the autoimmune nature of HUVS since the patient shows no signs of PTLD. Although need for further investigation, it is possible that EBNA1 expression in the serial blood samples is generated as cause or response to HUVS, by analogy with increased EBV expression seen in other autoimmune diseases.

**13.005**

## Variable EBV DNA Load Distribution and EBV RNA Expression in the Circulation of Stem Cell (SCT) and Solid Organ Transplant (SOT) Recipients: Consistent Expression of Barts

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**Background:** EBV DNA load monitoring in the circulation is a powerful tool for diagnosis, prognosis and preemptive treatment of posttransplant lymphoproliferative disease (PTLD). Aim of this study was to determine the distribution of EBV DNA load over the blood compartments and analyse the transcriptional phenotype of EBV in blood of SCT and SOT patients.

**Methods:** Simultaneous whole blood (WB), plasma and PBMC samples were obtained in follow-up from SCT and SOT recipients. EBV DNA load was determined by LightCycler real-time PCR. EBV RNA expression in PBMC specimes was defined by NASBA for EBNA1, LMP1, LMP2 and the human housekeeping gene U1A snRNP and RT-PCR for BARTs and U1A snRNP.

**Results:** The SCT patients (N=5), showed heterogeneous distribution of EBV DNA load, with 1 patient having EBV DNA positivity in plasma only (376,000 copies/ml), 1 patient showing EBV DNA in PBMCs only (1,426,300 copies/10E6 cells) and 3 patients with viral DNA in both specimens. The 4 SCT patients with cell-associated EBV DNA in the circulation showed consistent expression of BARTs and EBNA1, while LMP1 and LMP2 mRNA was found in 1 and 3 patients respectively. No EBV RNA was detected in the patient that only had EBV DNA load elevation in the plasma compartment. In SOT recipients, circulating EBV DNA load was always cell-associated, and plasma load was low or negative. Expression of BARTs was seen in all PBMC samples tested (N=15). LMP2 and EBNA1 mRNA was found in 5/15 and 2/15 samples, while LMP1 mRNA was only seen in 1 sample, coinciding with severe PTLD and high immunosuppression and extreme high EBV DNA loads (300,000 copies/ml WB).

**Conclusion:** SOT and SCT recipients differ in circulating EBV DNA distribution over the plasma and mononuclear cell compartments. They also have different EBV mRNA expression profiles in circulating EBV DNA+ cells. In both SCT recipients with cell-associated EBV DNA loads and in SOT recipients, BARTs are consistently detected in the circulation. In this pilot study, the SCT population shows more frequent EBNA1 and LMP2 mRNA expression. LMP1 mRNA is only found during severe immunosuppression and may directly represent circulating PTLD cells. Study of the diagnostic and prognostic value of circulating EBV RNA profiling after SOT and SCT is currently ongoing.

**13.006**

**Concomitant Increase of LMP1 and CD25 Expression Induced by IL-10 in the EBV-positive NK Lines SNK6 and KAI3**

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**Background:** Extranodal, nasal NK/T-cell lymphomas are regularly Epstein-Barr virus (EBV)-positive, with a type II latency pattern, expressing thus EBNA-1 and LMP1. The lymphoma cells are reported to produce IL-10 in immunohistochemical analysis, and the IL-10 is thought to make advantage for the tumor development by suppressing the attack of immunocompetent cells. But the direct effect of IL-10 to the tumor cells is unknown.

**Method:** SNK6 cell, which is EBV positive nasal NK/T-cell lymphoma cell line, were stimulated by IL-10. The proliferation and anti-apoptosis effect were measured by thymidine uptake, MTT assay, and cell-count method by Erythrosin B. LMP1 and Bcl-2 expression were checked by Western Blotting with anti-LMP1 antibody (DAKO) and anti-Bcl2 antibody (DAKO). CD25 expression on cell surface was examined by flow cytometry with anti-CD25 antibody (DAKO).

**Results:** IL-10 did not enhance the proliferation and anti-apoptosis effect, but enhanced LMP1 expression in dose dependent manner. In spite of enhancement of LMP1, IL-10 did not increase the Bcl2 expression. On the other hand, IL-10 could increase CD25 expression on SNK6 cells, but not on NKL cells (EBV negative NK cell line). IL-10 treated cells required lower amount of IL-2 for proliferation compared to the untreated cells. This effect was seen only with the EBV-positive NK lines in which LMP1 and CD25 were concomitantly upregulated.

**Conclusions:** It is known that the many inflammatory cells mixed with the lymphoma cells are seen in the lesion. In this situation, many cytokines including IL-2 must be produced. IL-10 is not related to proliferation directly, but related to indirectly by enhancing IL-2 induced proliferation through CD25 upregulation. We believe that LMP1 is working between IL-10 and cell surface CD25.

**13.007**

**Association of Serum Epstein-Barr Virus DNA with Clinical Course of Nasal NK/T-cell Lymphoma Patients**

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**Background:** Nasal NK/T-cell lymphoma is the lymphoproliferative disorder with necrotic granulomatous legions in the central part of head and neck region such as nasal cavity and hard palate. It is known EBV associates with etiology of the disease and is detectable in the tumor cells. To investigate the clinical significance of EBV in the patients, we investigated the link between serum EBV DNA levels and clinicopathological characteristics and the correlation with clinical course of the patients. As far as EBV DNA quantification, BamHI W fragment which has 11 genome repeats were used in many reports. In this time, we measured both LMP1 DNA and BamHI W copy numbers and compared each others. In order to investigate the association EBV DNA increase and EBV reactivation, we analyzed ZEBRA-expression of the patients' tissue.

**Methods:** Sera of 20 nasal NK/T-cell lymphoma patients were tested at pre-treatment, during treatment and at post-treatment. The quantitative real-time PCR assay was utilized to measure EBV DNA levels of the patients. ZEBRA-expression of the patients' tissues was analyzed by immunohistochemistry.

**Results:** EBV DNA was detected in all patients (median: 1550 copies/ml) but do not detected in 5 control subjects (P = 0.0017). Patients who had high EBV DNA level before treatment showed significant reduced survival compared with those who had low serum level (P = 0.023). Patients who had high EBV DNA level after treatment showed significant reduced survival compared with those who had low level (P = 0.0063). The ZEBRA-expression was significantly associated with EBV DNA levels. B-symptoms, high LDH level, ZEBRA-expression in tissue, high EBV DNA levels at pre-treatment and high BamHI W DNA level at post-treatment were associated with short disease-free survival and overall survival ($p<0.05$). Although the DNA levels of BamHI W and LMP1 significantly correlated, their dynamics were not always parallel. Patients with low pre-treatment both EBV DNA levels showed a favorable course, contrasting patients with high pre-treatment both DNA levels who showed aggressive course ($p=0.0085$).

**Conclusion:** The Serum EBV DNA levels were correlated to clinicopathological characteristics and clinical course of the patients, moreover the simultaneous measurement of both DNA lebels of BamHI W and LMP1 is very useful for diagnosis, disease monitoring and prediction of prognosis of patients. The serum EBV DNA levels maybe associated with EBV reactivation.

**13.008**

## Characterization of an EBV-Positive Intestinal Smooth Muscle Tumor Occurring Synchronously with Posttransplant Lymphoproliferative Disease in a Renal Transplant Patient

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**Background:** We report the case of a 14-year-old girl who developed PTLD with a concomitant smooth muscle tumor of the ileum following kidney transplantation. The purpose of this study was to characterize the smooth muscle tumor with respect to EBV and cellular gene expression. A correlative analysis of tumor development with the patient's EB viremia was also performed.

**Methods:** The patient's EBV serology was negative prior to transplant, but her living-related donor tested positive. Due to this mismatch the patient's EBV load in blood was followed post-transplant by semi-quantitative PCR. Primary EBV infection was detected 2 weeks after surgery. Two and one half years after transplant, PTLD was diagnosed and, 9 months thereafter, multiple abdominal lesions were surgically excised. These were analyzed by Southern blotting using a labelled EBV BamNJ fragment. Tumor sections were stained for EBNA2 and LMP1 using PE2 and CS1-4 monoclonals, respectively. The sections were also stained for Bcl-2 using an anti-human Bcl-2 monoclonal and for EBERs using a labelled nucleic acid probe.

**Results:** Histological evaluation of a cervical lymph node and of a lesion of the palate showed evidence of polymorphic PTLD. An abdominal mass excised 9 months later was shown to consist of proliferating smooth muscle cells which had initially developed within the muscularis propria of the small bowel wall, with surrounding nodular metastases. The tumor was shown to be oligoclonal and to contain both circular and linear EBV genomes. Retrospective analysis of the radiographic file confirmed that the abdominal lesions occurred synchronously with the histologically proven PTLD. EBV infection, demonstrated by EBER staining, was evident in both the primary tumor and the metastases. The smooth muscle lesions expressed EBNA2 in virtually all cells, but LMP1 was not detected. CD21 could not be demonstrated on the smooth muscle cell tumor. Bcl-2, however, was strongly expressed, particularly in the metastatic lesions. The patient's EB viral load in blood was elevated 9 months prior to diagnosis of the PTLD.

**Conclusion:** This case demonstrates the synchronous occurrence of 2 EBV-related tumors, occurring in a setting of mild immunosuppression. Further, this case supports the existence of a new EBV latency state whereby EBNA-2 expression exists without LMP1, suggesting a different mechanism of oncogenesis in smooth muscle cells.

**13.009**

## Tumorigenesis of Epstein-Barr Virus (EBV) Positive Gastric Carcinoma Cell Line, SNU-719 in Nude Mouse

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Epstein-Barr virus (EBV) is associated with about 10% of gastric carcinomas. In a process of seeking out a good model cell line for EBV associated gastric cancer, we found that one of the previously established gastric cancer lines from Korean patients is infected with type I EBV. This SNU-719 cell line was a naturally derived gastric cancer cell line and expresses similar set of latent EBV genes with EBV associated gastric cancer. In this study, we investigated tumorigenecity of the SNU-719 cell line in vitro and in vivo. SNU-719 cells grew anchorage independently in soft agar and showed low serum dependency. When inoculated to nude mice, SNU-719 cells developed tumor about 45-50 days after the inoculation but no metastasis was observed. These tumors showed the characteristics of undifferentiated carcinoma. EBERs were detected in all the tumor cells, and the tumor showed the same genotype with SNU-719 by STR genotyping. Mono- or biclonality of EBV genome was observed in tumor tissues. Not only the type of EBV but also the expression pattern of EBV genes in the developed tumor was identical to those of SNU-719 cell line. SNU-719 would serve as a useful model to clarify the tumorigenesis of EBV positive gastric cancer.

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**13.010**

**Viral microRNA Expression in EBV-associated Gastric Carcinoma**

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Recently, viral microRNAs (miRNAs) have been found to be expressed in B cells latently infected with Epstein-Barr virus (EBV). However, the expression pattern of EBV miRNAs has been studied neither in EBV-infected epithelial cells nor in EBV-associated tumors. In addition, whether EBV miRNAs undergo similar biogenesis process with eukaryotic miRNAs is not clear, yet. In this study, we tried to clarify these two points. The expression pattern of EBV miRNA in EBV positive gastric carcinoma cell lines were different from that of EBV infected B cells. Northern blot results using EBV positive B cell lines of diverse latency types suggest that the expression pattern of EBV miRNAs is affected by the latency type of EBV infection. Identical EBV miRNAs were expressed in EBV positive gastric carcinoma tissues as in SNU-719. To check whether the biogenesis of EBV miRNAs is similar with that of cellular miRNAs, expression pattern of an EBV miRNA was analyzed with or without RNA interference for Drosha and Dicer. The level of the EBV miRNA decreased in a similar way with miR-16 following siRNAs treatment. The high level expression of EBV miRNAs in EBV positive gastric carcinoma tissues suggests that EBV miRNAs may play important roles in tumorigenesis.

**Acknowledgement:** This work was supported by grant No. R01-2004-000-10847-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

13.011

## EBV-DNA Survey in Saliva for Early Diagnosis in Solid Tumour Patients After Allogeneic Stem Cell Transplantation

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**Background:** Epstein-Barr Virus (EBV) causes hepatitis, intestinal bleeding and lymphomas in immunosuppressed individuals. We have studied whether EBV-DNA in saliva is enough for survey in solid cancer patients (8 colon, 7 renal, 2 liver, 2 prostate, 1 breast cancer) after allogeneic stem cell transplantation (SCT).

**Patients and Methods:** Twenty patients (14 males, 6 females; median age 55.5 years) underwent reduced intensity conditioning (RIC) and SCT as adjuvant therapy against metastatic disease. All patients and 19 donors were EBV seropositive. Blood (EDTA) and saliva was collected for real time PCR initially twice a month, then monthly the 4th to 12th month post SCT.

**Results:** EBV-DNA was detected in the saliva >= 1 times in all the 20 patient samples. EBV-DNA was found in 14/20 patients' blood samples: 9/9 patients with an unrelated donor (URD), 5/11 with an HLA identical sibling donor (Sib). EBV-DNA load coincided in saliva and blood in 66% (108/163) of the parallel obtained samples. EBV-DNA in saliva preceded EBV-DNA finding in blood in 7/14 patients with median 30 (14-97) days or vice versa in 2 patients. In the remaining 5/14 cases, the occurrence of EBV-DNA was contemporary in blood and saliva. EBV-DNA occurred earlier in saliva in URD patients as compared to Sib patients: median 33 and 63 post SCT, respectively. EBV-DNA was detected in 9/13 patients with acute graft-versus-host-disease >= grade II. None of the patients developed EBV associated lymphomas. One representative patient is shown in figure.

**Conclusion:** EBV-DNA survey in saliva is worthy but should be confirmed with blood analysis. The risk for reactivating EBV remains in patients with solid cancer despite RIC prior to allogeneic SCT. The higher incidence of EBV in URD patients is suggested to the given antibodies against CD3+ cells in their RIC.

**13.012**

**The XLP Gene SAP is Involved in the Control of Cell Cycle/Apoptosis**

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**Background:** Deleted or mutated SAP genes that encode functionally defective protein are associated with the X-linked lymphoproliferative disease (XLP). This primary immunodeficiency is characterized by extreme sensitivity to EBV infection (fatal infectious mononucleosis) and by a high rate of lymphoma development (200 times higher than in the general population). While the lymphoma development is also attributed to the immunodeficiency, we considered additional mechanisms. Our previous results showed that SAP is a direct target of p53 and it is induced in DNA damaged lymphocytes. We studied now whether lack of the SAP function causes a defect in the control of the cell cycle/ DNA repair/ apoptosis machinery.

**Methods:** We studied the cell cycle distribution, proliferation and survival of DNA damaged/activated cells that express SAP protein.

**Results:** We compared the cell cycle distribution of healthy donor and XLP patient derived lymphoblastoid cell lines (wt p53) following DNA damage induced by gamma-irradiation. While the healthy donor derived LCLs (expressing SAP following DNA damage) arrest in the G2 phase of the cell cycle, the XLP patient derived LCLs (that lack functional SAP) do not show arrest in G2 following irradiation.

Constitutive expression of SAP in XLP patient derived LCLs (introduced by retroviral transduction) led to a significantly higher number of dead cells (40%) following DNA damage when compared to the same LCLs with no SAP expression (10%). This result is in line with the possibility that SAP is involved in the elimination of DNA damaged cells.

In Saos-2 cells (osteosarcoma line) transfected with SAP, DNA damage induced by irradiation delayed cell cycle progression. Similar results were obtained in experiments with longer observation periods. Seven days after irradiation, in the Saos-2-vector cultures there were several growing colonies, while in the SAP expressing Saos-2 cultures colonies were absent.

Upregulation of SAP in the late phases of T cell activation suggests a role in T cell homeostasis. We have established clones with variable levels of SAP from the T-ALL line CCRF-HSB2. Clones with higher SAP levels were more prompt to apoptosis when activated with PHA or PMA + ionomycin.

**Conclusions:** Our data indicates that in addition to controlling T and NK function, SAP is involved in the cell cycle control/apoptosis/DNA repair, thus contributes to the maintenance of lymphocyte homeostasis and of genomic stability.

13.013

## Induction of Distinct Gene Expression Patterns by the BARF1 Gene of Epstein-Barr Virus in Epithelial and Lymphoid Cells

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**Background:** The expression of the BARF1 gene of Epstein-Barr virus (EBV) in latent EBV infection is restricted to epithelial malignancies, e.g. gastric carcinomas (GC) or nasopharyngeal carcinomas (NPC). In addition, BARF1 is considered to be a lytic gene, because it is expressed upon induction of the lytic cycle in Burkitt's lymphoma cell lines. In the present study we analyzed the gene expression patterns of a BARF1-transfected epithelial and lymphoid cell line in order to identify cellular genes regulated by BARF1.

**Methods:** Gene expression of a BARF1-transfected lymphoblastoid (Louckes+) and a BARF1- transfected epithelial (HaCaT+) cell line were compared by cDNA microarray analysis using Affymetrix U133A chips. Of each group 6 genes were confirmed by Realtime PCR. In addition, immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) was performed on a tissue microarray (TMA) of 181 gastric carcinomas for selected genes, including 11 EBV-associated gastric carcinomas (6.1%) as tested by EBER-RNA in situ hybridization.

**Results:** The Louckes+ cells revealed 730 genes downregulated and 545 genes were upregulated, whereas the number of upregulated genes in epithelial HaCaT+ was 10 fold lower and the number of downregulated genes comparable to Louckes+. Among others, cyclin D1 expression was significantly upregulated in HaCaT+ by BARF1, but not in Louckes+. Analyzing cyclin D1 expression on a TMA of 181 gastric carcinomas revealed a significant overexpression of cyclin D1 in EBV-associated gastric carcinomas ($p<0.012$) but not in EBV-negative gastric carcinomas as tested by IHC. Cyclin D1 FISH showed that cyclin D1 overexpression was not due to gene amplification.

**Conclusion:** Expression of BARF1 induces the expression of distinct sets of cellular genes in epithelial and lymphoblastoid cells underlining the restricted BARF1 expression patterns in latent epithelial EBV infection and lytic EBV replication in lymphoblastoid cell lines. In addition, the overexpression of cyclin D1 in EBV-associated gastric carcinomas is induced by BARF1 and not due to cyclin D1 gene amplification. Next to cyclin D1 other genes have been identified in this study by cDNA microarray analysis which might help to understand the role of EBV in gastric carcinogenesis.

**13.014**

## CGH DNA Microarray Analysis as a Tool to Identify Distinct Aberrations Between EBV-Associated and EBV-Negative Gastric Carcinomas

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**Background:** Worldwide EBV is associated with approx. 10% of gastric adenocarcinomas. It has been shown recently by comparative genomic hybridization (CGH) that EBV-associated gastric carcinomas are characterized by distinct chromosomal aberrations other than EBV-negative gastric carcinomas.

**Methods:** In this retrospective study we established a new patient cohort of gastric cancer patients of 110 gastric carcinomas (GC) collected at the Institute of Pathology of Freiburg, Germany, between 2000-2005 in order to obtain EBV-associated gastric carcinoma tissue to perform CGH DNA microarray analysis. The presence of EBV was tested in the paraffin embedded tissues of the GCs by EBV-DNA PCR and by EBER-RNA in situ hybridization (RISH). The DNAs of EBV-associated (n=6) and negative (n=3) GCs were submitted to CGH DNA microarray analysis using the VYSIS Genosensor system. Selected identified genes were analyzed by immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH). The EBV status was correlated to clinico-pathological parameters.

**Results:** Six EBV-positive gastric carcinomas were identified (5.45%) by EBER-RISH. The DNA of these paraffin embedded GC tissue was submitted to CGH-DNA microarray analysis. Among others c-erbb2 amplification was detected in 2 of 3 EBV-negative gastric carcinomas but in none of the EBV-associated gastric carcinomas. Further analyses by IHC revealed that c-erbb2 overexpression was mainly found in EBV-negative gastric carcinomas (9.1%) but not in EBV-associated gastric carcinomas (0/6). C-erbb2 amplification was restricted to EBV-negative gastric carcinomas in 10 cases as tested by FISH.

**Conclusion:** Our data show that CGH-DNA microarray analysis is a valuable tool to identify distinct aberrations in archival paraffin embedded GCs. Further characterization of other identified genes is currently ongoing.

## Notes

# Abstracts

**Monday, July 10, 2006**
**Room: Poster Area/Level 2**

## Session 14: Poster Session II
## Diagnostics, Vaccines, and Therapy
## 17:30-19:00

**14.001**

## The *E. coli* Heat Labile Enterotoxin B-Subunit Enhances CD8+ T-Cell Killing of Epithelial Tumour Cells Expressing EBV LMP2

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**Background:** EBV LMP2 is a potential tumour immunotherapeutic target but EBV lymphoblastoid cell lines (LCL) are not normally susceptible to killing by uncloned LMP2-specfic CD8+ cytotoxic T-cells (CTL) *in vitro* unless pulsed with epitope peptide or if LMP2 is expressed by vaccinia virus. However, the *E. coli* enterotoxin subunit, EtxB, alters the MHC class I-dependent processing of LMP2 and enhances susceptibility of LCL to CTL (Ong et al., 2003, J. Virol. 77:4298-4305). Ultimately, it will be important to know if EtxB can enhance the killing of nasopharyngeal carcinoma (NPC) cells by LMP2-specific CTL but cell lines suitable for *in vitro* CTL studies are not yet available. Consequently, we wished to determine if EtxB could enhance the LMP2-specific CTL susceptibility of an alternative oropharyngeal tumour cell line artificially expressing LMP2.

**Methods:** The HLA A*02 oropharyngeal carcinoma cell line, H103, was stably transfected with an expression vector containing the LMP2A coding sequence. These cells (H103 LMP2) were pre-incubated with EtxB or the HLA A*02-restricted LMP2 epitope, CLGGLLTMV (CLG), or were untreated. LMP2-specific CTLs were recovered and expanded from an HLA A*02 donor and added to the H103 LMP2 target cells at an E:T ratio of 12:1. Target cell cytotoxicity was measured by lactate dehydrogenase release. The expression and distribution of LMP2 and EtxB in the H103 LMP2 target cells was assessed by Western blotting and confocal immunofluourescence microscopy.

**Results:** LMP2 protein expression was exclusively intracellular in H103 LMP2 epithelial cells and partially co-localised with internalised EtxB and a Golgi marker after 4 hours incubation. Control H103 cells were only killed by LMP2-specific CTL when pre-treated with CLG peptide. H103 LMP2 cells were also not killed unless pre-treated with CLG peptide. However, EtxB treatment of H103 LMP2 cells rendered them susceptible to killing by LMP2-specific CTL without pre-exposure to CLG.

**Conclusions:** As with EBV LCL, the susceptibility to LMP2-specific CTLs of epithelial tumour cells expressing LMP2 protein was greatly enhanced by treatment with EtxB and that cell surface LMP2 expression is not a requirement for this effect to take place. Whether this holds true for other epithelial tumour cell lines and other HLA types is currently being investigated. These results further strengthen the view that EtxB has potential as a therapeutic agent for EBV-associated tumours expressing LMP1 and LMP2.

**14.002**

## Expansion of Antigen-specific T Cells Following Stimulation with Adenoviral Polyepitope and in Vivo Modelling of Immunotherapy for EBV-associated Malignancies

**C. Smith**[1], M. Burgess[1], L. Cooper[1], M. Rist[1], N. Webb[1], E. Lambley[1], U. Dua[1], J. Seymour[2], M. Gandhi[1], R. Khanna[1]. [1]Queensland Institute of Medical Research, Brisbane, Australia; [2]Peter MacCallum Cancer Centre, Melbourne, Australia

Immunotherapeutic treatment with cytotoxic T lymphocytes (CTL) is currently being investigated as an alternative therapy for the treatment of the EBV-associated malignancies, Hodgkin's lymphoma (HL) and nasopharyngeal carcinoma (NPC). Recent evidence has indicated that during the acute stages of HL, T cells specific for the EBV antigens expressed by HL cells are unresponsive to antigenic stimulation. Therefore strategies employed to generate CTL for adoptive therapy need to overcome the unresponsive phenotype of these T cells. We have developed a novel antigen presentation system based on a replication-deficient adenovirus which encodes multiple HLA class I-restricted epitopes from EBV antigens expressed in HL (LMP1 and LMP2) which is covalently linked to EBNA1 protein (referred to as AdE1-LMPpoly). A single stimulation with AdE1-LMPpoly can overcome the unresponsiveness of these T cells from HL patients, resulting in the rapid expansion of T cells specific for all three antigens. The T cells generated from the HL patients are phenotypically similar to those produced in healthy donors, and importantly are capable of lysing targets cells, including a HL cell line, endogenously expressing the HL and NPC-associated antigens, providing evidence that the polyepitope expressing adenovirus is an effective tool for the expansion of CTL with broad HLA-specificity from HL patients. Furthermore, we have developed an in vivo model using NOD/SCID mice to test the effectiveness of the AdE1-LMPpoly expanded T cells.

**14.003**

**Evaluation of a New Commercial Assay: the 'EBV R-Gene Quantification Kit'**

**S. Fafi-Kremer**[1], G. Bargues[1], S. Magro[2], C. Barranger[2], J. Bes[2], P. Bourgeois[2], M. Joannes[2], P. Morand[1], J.M. Seigneurin[1]. [1]Laboratoire de Virologie, Strasbourg, France; [2]Argene SA, Varilhes, France

**Background:** measurement of EBV DNA load in the blood using real-time quantitative PCR has appeared as a useful marker in the clinical management of EBV-associated diseases. The aim of this study was to evaluate, in a number of unfractionnated whole blood samples, a new commercial kit for quantitative detection of EBV DNA: the 'EBV R-gene Quantification kit'. This assay was compared to our well validated 'in-house' real-time quantitative PCR.

**Methods:** reproducibility and repeatability of the R-gene assay were first tested with EBV DNA positive whole blood samples purified by three different extraction methods: the Qiamp® DNA isolation mini kit, the Qiagen's BioRobot EZ1 System, and the MagNAPure® compact instrument. In a second time, EBV DNA load was measured, after an automated extraction with the MagNAPure LC robot, on 150 whole blood specimens from transplanted patients, HIV infected individuals, patients with infectious mononucleosis, and healthy EBV-carriers. Moreover, 14 follow-up whole blood samples from transplanted patients and infectious mononucleosis patients were included in this study, using the R-gene assay (ARGENE) with the SmartCycler® 2.0 PCR platform (CEPHEID) in comparison with our 'in house' PCR using the LightCycler platform (ROCHE).

**Results:** the R-gene assay was highly sensitive (10 copies/reaction). No significant difference was observed between the three extraction methods: coefficients of variation of the intra- and inter-assays ranged from 0.56 to 18.81% and from 0.66 to 13.82 % respectively, with the highest variability for the lowest template concentration. Nevertheless, automated DNA extraction was easier to perform. Although some discrepant results were observed in occasional samples where higher viral load were mainly observed with 'in-house' PCR, a good correlation was observed between absolute values of EBV DNA loads determined by R-gene assay and 'in house' PCR ($r^2 = 0.87$). The viral load dynamics measured by the two methods in follow-up samples showed an excellent correlation, and corresponded very well with the clinical course.

**Conclusions:** the present study demonstrated that the EBV R-gene quantification kit is an EBV-specific, robust and reproducible method, compatible with manual or automated extraction. This kit is well suited in routine use for the monitoring of the EBV-associated diseases with whole blood sample as input for PCR. Our results confirm that the same extraction method, the same PCR method and the same PCR platform should be used for a more adequate patient management.

**14.004**

## Extreme Elevations of Epstein-Barr Viral Load in Pediatric Transplant Recipients: Consequences for Monitoring of the Development of Post-Transplant Lymphoproliferative Disease

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**Background:** Posttransplant lymphoproliferative disease (PTLD) associated with Epstein-Barr virus (EBV) remains a major complication with a significant cause of morbidity and mortality in transplant recipients. Its early detection is required for an appropriate intervention and successful treatment. Recently, we showed that elevations of EBV load in plasma (> 10,000 EBV copies/ml plasma) or peripheral blood mononuclear cells (PBMC) (> 5000 EBV copies/$\mu$g PBMC) were both sensitive and specific for the diagnosis of PTLD in a study of pediatric renal transplantated patients. Here, we tested these threshold values in both pediatric and adult patients after a different type of transplantation.

**Patients and Methods:** Our longitudinal study included 26 pediatric patients and 46 adult patients after heart transplantation. Five pediatric patients developed PTLD. Blood samples of patients were collected over a period of 4 to 56 months (median 12 months). EBV load in plasma and PBMC was detected by using real-time quantitative polymerase chain reaction.

**Results:** The levels of EBV load significantly differed between pediatric and adult patients ($p<0.001$, Chi-square 59.7; table 1). In adult patients, the threshold value for the development of PTLD was exceeded by only one patient in PBMC and no patient in plasma. In contrast, EBV load was critically increased in all but two pediatric patients. Although all five pediatric patients with PTLD exceeded at least once 5000 copies/$\mu$g PBMC, their level of EBV load did not distinguished them from another 19 pediatric patients without PTLD. No correlation of EBV load detected in plasma and the development of PTLD was seen in pediatric patients with elevated EBV load in PBMC.

**Conclusion:** Concerning the development of PTLD, absolute threshold values of EBV load have to be interpreted with regard to the type of transplantation and the age of the patients. As levels of EBV load are frequently elevated in pediatric patients after heart transplantation, it appears impossible to detect individuals at high risk for the development of PTLD in this patient group by monitoring of their EBV load only.

**14.005**

## Molecular and Serological Monitoring of Pediatric Patients with Nasopharyngeal Carcinoma Treated by the Protocol NPC-2003-GPOH of the German Society for Pediatric Oncology

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**Background:** Nasopharyngeal carcinoma (NPC) is a rare malignancy in children and adolescents living in central Europe. Recent studies on adult NPC patients in Southeast Asia demonstrated a correlation between the tumor burden and the level of EBV DNA in peripheral blood. We investigated the clinical significance of both EBV load and serological parameters in a population of pediatric patients in Germany.

**Patients and Methods:** 22 patients (3 female, 19 male, age range 9-26 year) with biopsy-proven stage III or IV nasopharyngeal carcinoma were treated with chemotherapy, radiotherapy and interferon beta therapy according to the protocol NPC-2003-GPOH. Blood samples from patients were longitudinally collected at diagnosis, during treatment as well as after completion of therapy. EBV DNA was detected both in plasma and in peripheral blood mononuclear cells (PBMC) by means of real-time quantitative polymerase chain reaction. For comparison, serologic titers of anti-EA-IgG, -IgM, -IgA and of anti-EBNA1-IgG were analysed in parallel plasma samples by enzyme-linked immunoassays.

**Results:** Plasma EBV DNA was detected in 93% of patients at diagnosis, but in no patient with complete remission after therapy. Cured patients became negative for EBV DNA in plasma latestly after completion of the radiotherapy. One patient with progressive disease was positive for EBV DNA in plasma at any time during treatment and showed increasing EBV load in plasma after therapy corresponding to his increasing tumor burden. Neither EBV load in PBMC nor any serological marker correlated with the course of disease in patients.

**Conclusion:** Quantification of EBV DNA in plasma is powerful for monitoring pediatric patients with nasopharyngeal carcinoma and is more informative than serology.

**14.006**

**A Mouse Monoclonal Antibody Against Epstein-barr Virus (EBV) Envelope Glycoprotein 350 Prevents EBV Infection Both in Vitro and in Vivo**

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**Background:** Active or passive immunisation of EBV-seronegative organ graft recipients prior to transplant resulting in neutralising anti-gp350 antibodies may prevent EBV transmission from a seropositive donor via the graft and thereby reduce the risk of post-transplant lymphoproliferative disease (PTLD). The aim of this study was to characterise a mouse anti-gp350 monoclonal antibody (mAb; 72A1) against EBV in scid mice, and to assess its in vivo safety and effects on acquisition of EBV during the immediate post-transplant period.

**Methods:** 72A1 hybridoma cell line was obtained from Johns Hopkins University Medical School (Baltimore, USA) and mAb was manufactured according to EU GMP guidelines. The neutralising titre was determined in vitro based on outgrowth of lymphoblastoid cell lines. Enzyme immunoassays were used to measure 72A1 mAb in plasma and detect anti-72A1 human anti-mouse antibody. Scid mice were inoculated with peripheral blood mononuclear cells (PBMC) from seronegative donors followed by administration of concentrated virus.

**Results:** In vitro, 72A1 inhibited EBV infection and immortalisation of B lymphocytes. PBMC from 4 EBV-seronegative donors were injected ip into scid mice. For each donor, 2-3x$10^7$ PBMC were inoculated into each animal in a group of 6 scid mice. For each such group of 6 mice, 3 test mice were injected with 72A1 ip the day before injection of PBMC, on the day of PBMC injection, the day after (day 1), and then three times weekly for 3 weeks whereas 3 control mice were injected at the same time with saline only. On day 1, all mice received 50μL ip of a concentrated EBV preparation (immortalising titre $10^{-4}$). 72A1 mAb prevented development of PTLD-like tumours in all of the test mice (0/12). In contrast, 67% (8/12) control mice developed PTLD-like lesions (p=0.001). In a pilot study, purified 72A1 was infused into 1 healthy adult and 4 EBV-seronegative children post-liver transplant. No adverse reactions were seen in the adult and 3 of the transplant patients. The remaining patient developed a hypersensitivity reaction.

**Conclusion:** 72A1 mAb neutralisation of gp350 prevents EBV infection and/or EBV-driven lymphoproliferation in vivo that translates into prevention of disease. In light of a hypersensitivity reaction in an infused patient, however, further clinical studies require either humanising the mAb or producing a human mAb.
(JID in press.)

**14.007**

**The Anti-cancer Drug Flavopiridol Inhibits EBNA 2-activated Transcription through Specific Inhibition of CDK9**

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**Background:** We have recently shown that the activation of transcription by EBNA 2 requires the activity of the RNA polymerase II C-terminal domain (CTD) kinase, CDK9, and that EBNA 2 stimulates CTD phosphorylation *in vivo*. Since the anti-cancer drug and CDK inhibitor, Flavopiridol, inhibits CDK9 with a much higher degree of specificity than other CDKs, we investigated whether this agent could be used to block EBNA 2-activated transcription and EBNA 2-driven proliferation.

**Methods:** EBNA 2-activated transcription was monitored using C and LMP1 promoter luciferase reporter assays in the EBV negative B-cell-line, DG75. The effects of Flavopiridol on cell viability, cell-cycle CDK activity, and apoptosis were measured using cell proliferation assays, H1 kinase assays, propidium iodide staining, and PARP cleavage assays.

**Results:** Our data showed that Flavopiridol inhibited EBNA 2-activated transcription with an IC50 of 83nM. At these relatively low concentrations of drug, we detected little toxicity and no induction of cell-cycle arrest or apoptosis indicating that the inhibitory effects of the drug were through inhibition of CDK9 and not cell-cycle CDKs. Moreover, control experiments showed that Flavopiridol inhibited CDK9-dependent HIV-1 Tat-activated transcription with an IC50 of 80nM in DG75 cells confirming specific inhibition of CDK9. We are currently testing whether Flavopiridol can be used to inhibit EBNA 2-activated transcription and thus EBNA 2-driven proliferation in immortalised B-cells providing a new way to block latent infection.

**Conclusion:** Given that Flavopiridol is showing promise as an anti-HIV agent through its ability to inhibit CDK9-dependent HIV-1 Tat-driven transcription, these results suggest that Flavopiridol may be of use in the treatment of EBNA 2-positive malignancies.

**14.008**

**'LCL-specific' CD4+ T Cells from EBV-immune and EBV-naïve Donors May Have Therapeutic Potential Against a Range of EBV-associated B Cell Lymphomas**

**J. Zuo**, H.M. Long, N.H. Gudgeon, H. Jia, A.B. Rickinson. Cancer Studies, Birmingham, United Kingdom

**Background:** Epstein-Barr virus (EBV) is associated with three B cell malignancies, post-transplant lymphoproliferative disease (PTLD) in T cell compromised patients, Burkitt's lymphoma (BL) and Hodgkin's disease (HD). The adoptive transfer of cytotoxic T cells specific for EBV antigens has been proved an effective treatment for PTLD lesions. Such T cell preparations, generated in vitro by stimulating peripheral blood mononuclear cells with the autologous EBV transformed lymphoblastoid cell line (LCL), tend to be dominated by HLA class I-restricted CD8+ T cells specific for the nuclear antigens EBNAs 3A,3B and 3C that are absent in BL and HD. However, most preparations also contain CD4+ T cells of unknown specificity that, where examined, recognise and kill LCLs in an HLA class II-restricted fashion. Our work has focused on the establishment and functional analysis of such "LCL-specific" CD4 effectors.

**Results:** Using IFN-gamma release assays as a readout, we identified CD4+ T cell clones showing HLA class II-restricted recognition of EBV-transformed LCLs but no recognition of autologous EBV-negative B lymphoblasts either alone or expressing individual MHC class II-directed EBV latent proteins from vaccinia vectors. Many such clones are cytotoxic and efficiently block autologous LCL outgrowth in co-cultivation assays. Importantly, such clones could be generated from both EBV-immune and EBV-naïve donors, strengthening the argument that they are directed against target epitopes of cellular rather than viral origin. Work to date suggests that such cells are also capable of recognizing EBV-positive and EBV-negative BL cell lines providing they express the correct HLA II restricting allele.

**Conclusions:** We infer that such novel CD4+ T cell reactivities cells are present in the LCL-stimulated effector populations and that they may have therapeutic potential in the context not just of PTLD but also of other EBV-associated B cell tumours. It will be important to identify their precise antigenic specificities.

**14.009**

**Valproic Acid Enhances the Efficacy of Chemotherapy in EBV-positive Tumors by Increasing Lytic Viral Gene Expression**

**W.H. Feng**, S. Kenney. Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

**Background:** EBV infection in tumor cells is generally restricted to the latent forms of viral infection. Switching the latent form of viral infection into the lytic form may induce tumor cell death. We have previously reported that certain chemotherapy agents increase the amount of lytic viral gene expression in EBV-positive tumor cells. In this study, we have examined valproic acid, an anti-seizure drug that also has strong histone deactylase activity, in regard to its ability to induce lytic viral gene expression by itself, or enhance the lytic-induction effect of chemotherapy.

**Methods:** Valproic acid activation of lytic EBV gene expression (with or without chemotherapy) was assessed in latently-infected, EBV-positive cell lines. Valproic acid and chemotherapy killing of EBV-positive versus EBV-negative cells was investigated in LCL and epithelial cell lines. The ability of valproic acid to inhibit growth of EBV-positive lymphoblastoid tumors in the presence and absence of chemotherapy was examined in SCID mice.

**Results:** Although valproic acid treatment alone induced only a modest increase in the level of lytic viral gene expression, it strongly enhanced the ability of chemotherapeutic agents to induce lytic EBV gene expression in EBV-positive epithelial and lymphoid cells. Furthermore, valproic acid enhanced cell killing in vitro by chemotherapeutic agents in lymphoblastoid cells and gastric cells (AGS) containing wild-type EBV. In contrast, valproic acid did not enhance the cytotoxicity of chemotherapy in lymphoblastoid cells containing a lytic-defective (BZLF1- knockout) form of EBV or in EBV-negative AGS cells. Finally, we found that the combination of valproic acid and chemotherapy was significantly more effective in inhibiting EBV-driven lymphoproliferative disease in SCID mice than chemotherapy alone.

**Conclusion:** These results suggest that short course valproic acid therapy might enhance the efficacy of conventional chemotherapy for the treatment of EBV-positive tumors, and that this effect requires the lytic form of viral gene expression.

**14.010**

**Re-Targeting Cytotoxic T Lymphocytes to LMP2 for Immunotherapy of EBV-associated Tumours**

**A.M. Swanson**, I. Johannessen, D.H. Crawford. The University of Edinburgh, Edinburgh, United Kingdom

**Background:** Re-targeting of cytotoxic T lymphocytes (CTLs) to EBV-associated malignancies by transduction of chimeric T cell receptors specific for a widely expressed virus antigen is likely to improve the efficacy of immunotherapy. In addition, utilising an antibody variable domain (Fv) as the antigen recognition site of the chimeric receptor circumvents the need for HLA-matching. The aim of this study is to re-target CTLs to latent membrane protein 2 (LMP2), a viral protein expressed across most EBV-associated tumours. We have also established SCID mouse models of EBV-associated Hodgkins lymphoma (HL), nasopharyngeal carcinoma (NPC) and gastric carcinoma (GC), for in vivo testing of the engineered CTLs.

**Methods:** Peptides mimicking the extracellular domains of LMP2 were constructed and used to screen human single chain Fv (scFv) phage libraries for reactive clones. Clone specificity was tested by ELISA. For in vivo tumour modelling, SCID mice were injected via the subcutaneous or intraperitoneal route with cell lines derived from HL (HDLM-2, L1236, L540, L591), NPC (C666.1) or GC (AGS, NUGC3). Tumours were harvested and examined by immunohistochemistry and in situ hybridisation to confirm malignancies were of the correct histology.

**Results:** Initial experiments showed selection and enrichment of phage clones specific for whole protein, but not peptide, controls. Biotinylation and circularisation of peptides improved selection methods and a number of scFv clones reactive against the LMP2 peptides were identified, with varying levels of specificity and affinity. In the in vivo subcutaneous models, cell lines derived from HL, NPC and GC gave rise to tumours. For HL, mice injected with L1236, L540 and L591 developed tumours within 4 weeks, for NPC, mice injected with C666.1 within 2 weeks, while the GC cell line AGS gave rise to tumours after 10 weeks. The histology of all tumours was unchanged from that of the original cell line

**Conclusion:** The identification of scFv clones reactive against peptides mimicking the extracellular domains of LMP2, and the establishment of relevant pre-clinical animal models, are important steps in the development of an immunotherapy efficacious for a range of EBV-associated malignancies

**14.011**

## Successful Stem Cell or Bone Marrow Transplantation for Severe Chronic Active Epstein-Barr Virus Disease in the United States

**J.I. Cohen**[1], J. Dale[1], E. Jaffe[1], S. Pittaluga[1], A. Marques[1], K. Rao[1], H. Heslop[2], C. Rooney[2], S. Gottschalk[2], M. Bishop[1], W. Wilson[1], S. Straus[1]. [1]National Institutes of Health, Bethesda, MD, USA; [2]Baylor College of Medicine, Houston, TX, USA

**Background:** Severe chronic active Epstein-Barr virus disease (SCAEBV) is characterized by markedly elevated levels of EBV DNA in the blood, EBV nucleic acid or proteins with lymphocytic infiltration in tissues, and the absence of known prior immunodeficiency. Patients usually manifest fever, lymphadenopathy, and splenomegaly and progressive cytopenias, hepatitis, hemophagocytic syndrome, or neurological disorders, as well as progressive lymphoproliferation and immunodeficiency such as to incur severe opportunistic infections or frank lymphomas. In contrast to Asian patients with SCAEBV, who typically present with EBV in their NK or T cells, patients in the United States usually have virus in their B cells.

**Methods:** We report our 20 year experience with SCAEBV patients, including the first 4 in the United States to undergo transplantation.

**Results:** Prolonged immunosuppressive treatments (e.g. prednisone, cyclophosphamide; azathioprine, cyclosporine) provided some patients temporary reductions in disease associated morbidity, but did not appear to improve the long term outcome and may have increased the risk of infection or malignant transformation. Immunomodulatory (e.g. interferons, autologous cytotoxic T cells), cytotoxic, or antiviral therapy had little effect on SCAEBV. Four patients, aged 9 to 57, underwent allogeneic bone marrow or stem cell transplantation for SCAEBV. All patients had progressive disease for 2 to 8 years before transplantation. Two patients received matched unrelated donor and two had sibling donor transplants. Three of the transplants were myeloablative and one was non-myeloablative. At 1.5 to 6 years after transplantation, all 4 patients are alive and have returned to normal activity levels. Two are EBV PCR positive in the peripheral blood and 2 remain EBV-negative. Three patients are in continuous remission, while one has slowly progressive disease, but continues to attend school and remains active.

**Conclusion:** Allogeneic transplantation is an effective treatment for some patients with SCAEBV.

**14.012**

**A Latency-Null Gammaherpesvirus Generates Protective Immunity Independent of Latent Antigens**

**Q. Jia**, I. McHardy, D. Martinez-guzman, S. Hwang, T. Rickabaugh, L. Tong, R. Sun.
UCLA, Los Angeles, CA, USA

Murine gammaherpesvirus 68 (MHV-68) has been utilized to study the fundamental principles underlying gammaherpesvirus infection and immune responses. We and others have previously shown that a recombinant MHV-68 virus that over-expresses an immediate early protein RTA is deficient, but not completely, in latency establishment.

To completely eliminate the capacity of the virus to establish latency, we have constructed a new recombinant MHV-68 in which the latency-associated locus encoding ORF72, M11 and ORF73 was replaced by a RTA expression cassette driven by an actin-CMV hybrid promoter/enhancer. This new recombinant virus, named AC-RTA, was capable of lytic replication with kinetics similar to those of the wild-type MHV-68 in vitro. The viral replication in vivo was characterized by live whole body imaging. The replication of AC-RTA virus in the nasal oral cavity was enhanced and the infection the lung was controlled by the immune system significantly faster than the wild type virus. No AC-RTA virus or viral DNA was detected in the isolated splenic cells, implying a lack of latency in spleen B cells. AC-RTA virus did not generate significant neutralizing antibody responses over a long period of time, strongly suggesting lack of any persistent infection in the body. Mononucleosis-like disease, a characteristic of latency-competent MHV-68 infection in mice, was not detected. Importantly, AC-RTA was able to protect mice against subsequent challenge by wild-type MHV-68.

Our results suggested that latent antigens are not essential for preventing latent gammaherepesvirus infection. This study suggests a new strategy for vaccine development against EBV and KSHV, using a latency-deficient and replication-competent virus.

**14.013**

## Efficient Gene Transduction of Unstimulated Normal B-lymphocytes Using an Epstein-Barr Virus-based Vector

**H. Yoshiyama**, T. Kanda, D. Iwakiri, K. Takada. Institute for Genetic Medicine, Sapporo, Japan

**Background:** Although several reports have described efficient gene transduction of human B-cell chronic lymphocytic leukemia (CLL) cells using vectors based on an adenovirus, herpes simplex virus type 1, lentivirus and EBV, none of these vectors can transduce unstimulated B-lymphocytes from healthy donors. In fact, unstimulated normal B-lymphocytes cannot be transduced efficiently by any means.

**Methods and Results:** A packaging cell derived from a Burkitt's lymphoma Akata cell line carrying the entire EBV plasmid except for the packaging signal was generated. In the packaging cells, a full lytic program could be induced efficiently without producing progeny viruses and plasmids carrying the packaging signal of EBV, the origin of EBV DNA replication for lytic infection and a gene encoding the green fluorescence protein (GFP) could be packaged as virus-like particles. Transduction of unstimulated normal B-lymphocytes with these virus-like particles was very efficient, as GFP expression was observed in > 90% of the transduced cells. Most of the transduced cells remained in the resting state.

**Conclusion:** Here, we demonstrated that the EBV-based vector efficiently transduced foreign genes in human unstimulated normal B-lymphocytes. Since most of these cells remained in the resting state, this system should prove useful for studying the effects of various genes in unstimulated B-lymphocytes and has the potential as a vector for human gene therapy. We used Akata cells for generating virus-like particles because they produce the highest levels of EBV among the numerous cell lines thus far established, and because virus production can be induced efficiently and simply, by addition of anti-Ig antibodies to the culture medium, allowing easy preparation of large amounts of viral stocks for clinical application.


# Abstracts

**Monday, July 10, 2006**
**Room: Poster Area/Level 3**

## Session 15: Poster Session II
## Gene Regulation
## 17:30-19:00

**15.001**

## Dual Regulation of LMP1-Augmented Kappa Light Chain Expression in Nasopharyngeal Carcinoma Cells by NFκB and AP-1

**H.D. Liu**, H. Zheng, M. Li, Y.G. Tao, Z.X. Lu, D.S. Hu, Y. Cao. Cancer Research Institute,Central South University, Changsha, China

**Background:** We and others have reported several human cancer cell lines of epithelial origin, including nasopharyngeal carcinoma (NPC) cell lines, expressed immunoglobulins (Igs). Moreover, our previous studies indicated expression of κ light chain in NPC cells could be up-regulated by EBV-encoded latent membrane protein (LMP1). LMP1 is known to induce gene expression via activation of several signal transduction pathways, including NFκB, AP-1 pathways. A κB site within the intron enhancer (iEκ) of Igκ gene has been implicated as a key regulator of its expression, an AP-1 binding site upstream of the iEκ has also been found to play a role in the regulation of κ gene. All these combined with our observations prompt us to examine whether the two pathways involve in LMP1-augmented κ light chain expression in NPC cells.

**Methods:** The specific NFκB inhibitor Bay11-7082 and constantly expressing dominant-negative mutant of IκBα (DNMIκBα) LMP1-positive NPC cells, as well as the selective JNK inhibitor SP600125 and stably expressing dominant-negative mutant c-Jun (TAM67) LMP1-positive NPC cells were used to determine the involvement of NFκB and AP-1 pathways. Flow cytometric analysis for intracellular Igκ staining, Western blot for Igκ in whole cell lysates and quantitation ELISA assay for Igκ in concentration of cell culture supernatants were performed. Human myeloma cell lines XG6 (expression [a] chain) and XG7 (expression κ chain) act as negative and positive control, respectively.

**Results:** Compared with vehicle control, the κ-specific mean fluorescence intensity (MFI) in NPC cells treated with Bay11-7082 and SP600125 was markedly decreased($P<0.05$). Cells overexpressing DNMIκBα or TAM67 exhibited significantly decreasing κ-specific MFI compared with their respective parental cells($P<0.05$). Western blot assay also indicated that up-regulation of Igκ expression by LMP1 was correlated with the phosphorylation of JNK1/2 and of IκBα and that SP600125 and Bay11-7082 inhibited the increase of Igκ expression induced by LMP1. However, in serum-free supernatants, the amounts of Igκ secreted by NPC cells were extremely low.

**Conclusion:** Experimental results suggest that LMP1-increased κ chain expression through activation of the AP-1, NFκB signal pathways and this ectopic expression of κ chain may mainly exist in cytoplasmic forms. The present study provided us some hints of possibility mechanism by which human cancer cells of epithelial origin produced immunoglobulins.

**15.002**

**NF-κB Binds and Activates the LMP1 Promoter in B Cells**

**P. Johansson**, A. Jansson, A. Sjöblom-Hallen, L. Rymo. Institution of Biomedicine, Gothenburg, Sweden

**Background:** It is well established that the Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) signaling pathway activates NF-κB. A TRANSFAC database search of the LMP1 promoter region suggested the presence of a binding site for NF-κB at position -89/-79 relative to the transcription start site. This led to the hypothesis that NF-κB may have a role in regulation of LMP1 expression. Here, we investigated the binding of the NF-κB to the LMP1 promoter, and its effect on promoter activity.

**Methods:** Identification of factors binding to the LMP1 promoter NF- κB site was performed using a DNA fragment corresponding to the -56/-106 promoter region that encompasses the NF-κB site as an affinity bait for protein purification. Electromobility shift assays (EMSAs) were also performed with oligonucleotides encompassing this site. To study the regulation of the promoter, different NF κB isoform expression vectors were transiently cotransfected into DG75 cells (EBV negative BL) with CAT reporter plasmids carrying a segment of the LMP1 regulatory sequence (LRS) with an intact or mutated NF-κB site in front of the reporter gene, and the effect on reporter gene expression was analyzed

**Results:** NF-κB was identified using FT-ICR tandem mass spectrometry on proteins isolated from a WW1-LCL nuclear extract by DNA affinity purification. EMSA with oligonucleotides corresponding to the NF κB binding site and cbc-Rael nuclear extract showed the presence of a specific DNA-protein complex. This complex was competed by an NF-κB consensus sequence. Both DNA affinity purification and EMSA results indicate that NF-κB binds to the suggested site in LRS. Ectopic expression of NF-κB (p50) activated the LMP1 promoter reporter plasmid via NF-κB site (-89/-79) when the site was intact. When the site was mutated, NF-κB failed to activate the promoter, indicating that promoter activation was achieved through this site.

**Conclusions:** Taken together, the results show that NF-κB can bind to the NF-κB site in the LMP1 promoter, and that exogenous expression of NF-κB can activate the promoter through this site. This suggests the presence of an autoregulatory pathway, in which the expression of the NF-κB transcription factor is up-regulated by LMP1 which in turn increases LMP1 expression.

**15.003**

## Characterization of a Cellular Protein-associated with the Q Promoter of Epstein-Barr Virus

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**Background:** EBNA 1 is one of the Epstein-Barr virus (EBV) latent genes, and is only expressed in latency I cells, such as Burkitt's lymphoma (BL) cells, where it may play an important role. The EBNA 1 transcript in latency I cells is specifically initiated by the Q promoter (Qp), which may hence serve a key function in the maintenance of EBV latent infection. Therefore, the investigation of regulation of Qp by cellular factors is the excellent way to explore how an EBV maintains latent infection in latency I cells.

**Methods and Results:** Previous data have shown that a number of hitherto unknown proteins in the BL Rael cells are bound to the promoter region of Qp. To identify these cellular proteins, we first used the DNA fragment corresponding to the -50 to -90 region of the Qp as a probe to screen an expression cDNA library of Rael cells via the yeast one-hybrid system. Three clones were positive from the yeast screening, and one of these three was highly associated with the -50 to -90 region of Qp in the yeast system, and was identified as transcription factor human immunodeficiency virus-enhancer binding protein 2 (HIV-EP2) by comparing it to GenBank sequences. We performed *in vitro* electrophoretic mobility shift assay (EMSA) to demonstrate that HIV-EP2 was indeed associated with this region of Qp in mammalian cells. To further explore the importance of HIV-EP2 in such cells, we attempted to determine the distribution of HIV-EP2 expression in human tissue by using Northern blotting for RNA localization. The results showed that the expression of HIV-EP2 occurs mainly in lymphatic and brain organs, indicating that the HIV-EP2 can be a key factor involved in the growth control of lymphatic and neuronal cells. Additionally, the results of luciferase reporter assay and RT-PCR analysis indicated that the expression of HIV-EP2 inhibited the transcription of EBNA 1.

**Conclusion:** Taken together, we have found that a cellular transcription factor, HIV-EP2, is active in repressing the promoter activity of the Qp. These results suggest that the expression of HIV-EP2 in cells may play an important role in controlling the life cycle of EBV.

**15.004**

**EBNA3A Induces the Expression of, and Interacts with, a Subset of Chaperones and Co-chaperones**

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**Background:** EBNAs 3A and 3C but not 3B are essential for the efficient immortalisation of B cells by EBV *in vitro*. It is assumed that all three proteins play an essential role in viral persistence in the human host. EBNAs 3A and 3C can repress transcription when targeted to DNA and both bind the cellular co-repressor CtBP. CtBP-containing complexes coordinate enzymatic events that convert transcriptionally active chromatin directly to a repressive or silent state. No cellular gene promoters have yet been identified as specific targets of the EBNA3 proteins.

**Methods and Results:** In order to reveal cellular genes regulated by EBNA3A, cDNAs encoding EBNA3A and an EBNA3A CtBP-binding mutant have been incorporated into recombinant adenoviral vectors. Microarray experiments comparing infection of human diploid fibroblasts with adenoviruses (Ad-) EBNA3A, Ad-EBNA3A CtBP-binding mutant and an empty Ad control have consistently shown an induction of mRNA corresponding to chaperones Hsp70 and Hsp70B and co-chaperones Bag3 and DNAJ/Hsp40 by EBNA3A. This appears to be partly dependent on binding CtBP.

Analysis of Ad-infected fibroblasts by real time PCR and Western blotting confirmed that EBNA3A and to a lesser extent the CtBP mutant, (but not EBNA3B or EBNA3C) induce expression of HSP70, HSP70B, BAG3 and DNAJ/HSP40. Both EBNA3A and the CtBP mutant activate transcription from the HSP70B promoter in transient transfection assays. Co-immunoprecipitation experiments suggest EBNA3A can complex with all these proteins in both the Ad-infected cells and LCLs.

**Conclusions:** HSP70 complexes are important in protein folding, proteasomal degradation (and MHC presentation) and have associated anti-apoptotic activity. Through specific coordinated up-regulation of chaperones and co-chaperones, EBNA3A induces all the factors necessary for an active HSP70 chaperone complex. The role of CtBP in this gene activation is at present unknown.

**15.005**

**Regulation of Transcription by EBNA3B**

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**Background:** EBNA3A and 3C can act as potent repressors of transcription when targeted to DNA by fusion with the Gal4 DNA binding domain (DBD) or by binding the cellular adaptor protein RBP-JK (CBF1). EBNAs 3A and 3C both bind the cellular co-repressor CtBP and associate with other proteins involved in transcriptional repression e.g. HDAC1 and 2. Unlike EBNA3A and EBNA3C, EBNA3B is not essential for the immortalisation of B cells and has been lost during the continuous passage of some EBV-immortalised lymphoblastoid cell lines (LCLs).

**Methods and Results:** We show that EBNA3B, when targeted to DNA by fusion to the Gal4DBD, can very effectively repress transcription. Like EBNA3A and EBNA3C, EBNA3B can also overcome EBNA2 mediated activation of EBV promoters Cp and TP1. EBNA3B does not bind CtBP or associated proteins, but binds efficiently through protein:protein interactions to all isoforms of heterochromatin-associated protein HP1 in GST-pulldown assays. EBNA3B also co-immunoprecipitates with HP1 from cells. HP1 is a key component of constitutive heterochromatin and is involved in cellular gene silencing in association with histone modifications. Protein:protein interactions with HP1 often involve a pentapeptide-binding motif PxVxL and EBNA3B contains two of these sequences (PtVvL and PvViL starting at amino acids 450 and 891 respectively). However mapping of the EBNA3B repression domain and the interaction site of EBNA3B with HP1 indicate that EBNA3B binds to HP1 in a PxVxL-independent manner. Similar analyses of the EBNA3B encoded by the Rhesus lymphocryptovirus (LCV) of Old World primates showed this also represses transcription when targeted to DNA and binds HP1.

EBNA3B, like 3A and 3C, can repress transcription, however, to our surprise we discovered that EBNA3B also acts as an extremely potent activator of the Hsp70B promoter in a luciferase reporter construct. The mechanisms of this striking activation of a reporter plasmid and the repression seen when EBNA3B is targeted to DNA are presently unknown.

**15.006**

**HLARK/RBM4 Strongly Co-stimulates Gene Activation by the Epstein-Barr Virus Nuclear Antigen 2 (EBNA2) and its Cellular Counterpart Notch-IC**

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**Background:** The Epstein-Barr virus (EBV) virus-encoded nuclear antigen 2 (EBNA2) is a key mediator of B-lymphocyte transformation by EBV and regulates viral and cellular gene expression. EBNA2 is the viral functional homologue of Notch, which also interferes in gene expression through RBPJκ. By a Yeast Two Hybrid screen we identified hlark (RBM4) as a novel interaction partner of EBNA2.

**Methods:** Luciferase reporter gene assays, Western Blots, Yeast two hybrid analysis.

**Results:** The hlark gene encodes a protein of the RNA recognition motif (RRM) family which was detectable by monoclonal antibodies in EBV-transformed B-cells. Coexpression of EBNA2 and hlark in EBNA2 deficient P3HR1 cells led to an increase in LMP1 protein expression as compared to EBNA2 alone. In luciferase reporter assays hlark increased the EBNA2 mediated activation of the viral LMP1 promoter four- to ten-fold but did not affect stimulation of the viral C-promoter. It also rescued the activity of several EBNA2-mutants while a RNA binding deficient mutant of hlark was inactive. Furthermore we found a strong enhancement of the Notch-IC-mediated activation of the cellular HES-1 promoter by hlark. We were not able to confirm the yeast two hybrid results by co-immunoprecipitation of hlark and EBNA2. Coexpression of hlark and EBNA2 or Notch shows that the proteins do not mutually influence their expression levels. Finally, hlark did not increase the mRNA level of the luciferase reporter gene.

**Conclusion:** Our data identify hlark as a novel promoter-specific coactivator of EBNA2 and Notch. The EBNA2/hlark interaction is either very labile or mediated through a third factor as co-immunoprecipitation from cell extracts expressing both proteins was not possible. The results of our studies imply that hlark apparently acts post-transcriptionally as it did not increase the mRNA- but the protein-level of the luciferase reporter.

**15.007**

**DNA Mthylation Status of the EBV BamHI W Promoter and Promoter Activity in Novel EBV-positive Burkitt and Lymphoblastoid Cell Lines**

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**Objectives:** The EBV latent cycle promoter Wp, located in each tandemly arranged copy of the BamHI W repeat in the EBV genome, drives expression of the six viral nuclear antigens (EBNAs) during the early stages of virus-induced B cell transformation. Thereafter EBNA2 and EBNA1 activate an alternative EBNA promoter, Cp, and Wp-initiated transcription declines dramatically. While transcription interference and DNA methylation have both been suggested as possible mechanisms which contribute to Wp downregulation, Wp activity is never completely silenced in LCLs. By contrast, both Wp and Cp are extensively methylated in most EBV-positive Burkitt lymphoma (BL) cell lines in which EBNA1 is selectively expressed from a third promoter Qp. In this work we have analysed the Wp methylation status in rare LCLs, recently established using wild-type EBV isolates, and novel Burkitt Lymphoma (BL) cell clones, derived from tumours carrying EBNA2-deleted EBV genomes, which express EBNA transcripts exclusively from Wp.

**Results:** Using a combination of bisulphite sequencing and methylation-sensitive PCR, we show that in standard LCLs in which Cp is the dominant EBNA promoter, some Wp copies always remain devoid of methylation. By contrast, Wp sequences in two rare Wp-using LCLs showed a markedly different pattern in which regulatory regions of the promoter known to bind critical cellular transcription factors were largely hypomethylated. Moreover, analysis of LCLs transformed with a recombinant EBV carrying just two BamHI W copies, showed that Wp was almost entirely unmethylated, a pattern never seen before in established LCLs. Extending the analysis to Wp-using BL clones also revealed that all resident copies of Wp appeared to be predominantly hypomethylated, particularly at functionally important regulatory elements.

**Conclusions:** Our studies on EBV-infected B cells with atypical patterns of Wp usage emphasise the strong correlation between Wp methylation and promoter activity.

**15.008**

**EBV Exploits the BSAP/Pax5 Cellular Transcription Factor to Ensure B Cell-specific Activation of Its Growth-transforming Programme**

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**Objectives:** The EBV BamHI W promoter Wp drives the initial transcription of all six EBV nuclear antigens (EBNAs) during the early stages of B cell transformation in vitro. In earlier work we identified two functionally important regulatory regions upstream of Wp, UAS2 which binds the ubiquitously expressed transcription factor YY1, and UAS1 which binds a number of factors including CREB/ATF, RFX and notably two binding sites for the B cell specific activator protein BSAP/Pax5. In this current work we have generated recombinant EBV genomes in which specific deletions or mutations have been introduced into the tandemly arranged copies of Wp, and then assayed for B cell transformation in vitro.

**Results:** While deletion of the cell lineage independent UAS2 region had no effect on B cell transformation efficiency, deletion of the B cell specific UAS1 element inactivated Wp and completely abrogated B cell growth transformation. To further investigate the role of BSAP in Wp activation, we also created a recombinant EBV in which base substitutions were engineered into both BSAP sites; these mutations had previously been shown to inhibit Wp activity in reporter assays and disrupt binding of BSAP to Wp sequences in bandshift assays. This new virus carrying Wp sequences with crippled BSAP sites also showed no detectable Wp transcription upon infection of resting B cells and was completely deficient in transforming activity.

**Conclusions:** Our findings dramatically underline the importance of the B cell specific regulatory element UAS1 and in particular the essential role of BSAP in initiating the B cell growth transforming programme.

**15.009**

## EBNA5 Enhances the Formation of Insoluble Protein Aggregates from Transiently Expressed Reporter Genes

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**Background:** EBNA5 together with EBNA2 are the first viral proteins expressed in freshly infected B lymphocytes. The two proteins together have been shown to induce G0 to G1 transition in resting cells and both belong to the group of viral proteins that are required for efficient transformation of B cells. EBNA5 enhances EBNA2-induced transactivation of the LMP1 promoter and it is involved in pre-mRNA processing. Moreover, EBNA5 has been reported to interact with a number of cellular proteins.

**Methods:** The standard EBNA5 expression vector employed contained seven copies of the W1W2 exons and one copy each of the Y1 and Y2 exons under the control of the CMV promoter. To investigate the function of the EBNA5 protein, CMV promoter containing Luciferase and CAT reporter plasmids were co-transfected with the standard or mutated EBNA5 expression vectors into DG75 cells, and the effect on the transient expression of the reporter genes was analyzed. A protocol for the semi-quantitative determination of the relative amounts of reporter proteins by immunoblotting was developed.

**Results:** Previously, EBNA5 was shown to repress the expression of reporter plasmids. Here we investigated whether EBNA5 modulated the expression in the cytoplasm at the mRNA or protein level. Transfection experiments demonstrated that the decrease in the levels of reporter activity in the cytoplasm obtained at high levels of EBNA5 was closely paralleled by a corresponding decrease in the levels of immunoreactive reporter polypeptide. In contrast, the level of reporter gene mRNA showed a significant increase with increasing amounts of pCI-EBNA5. We noted that large amounts of reporter polypeptides had accumulated in the nuclear fraction and that EBNA5 seemed to contribute to this process. The distribution of Luc reporter polypeptide between the soluble and insoluble cell protein fractions was determined in time-course and dose-response transfection experiments. In the presence of EBNA5, there was a very significant accumulation of insoluble luciferase aggregates over time as compared with cells without EBNA5. Increasing amounts of EBNA5 dramatically stimulated the accumulation of insoluble luciferase polypeptide in the cells, whereas the level in the soluble fraction showed a decline.

**Conclusions:** Taken together, the data suggest that EBNA5-induced repression of gene expression is, at least in part, performed at the post-transcriptional level. Furthermore, EBNA5 enhances the formation of insoluble protein aggregates from transiently expressed reporter genes in the cells.

**15.010**

**BZLF1 Regulates the TNF-alpha Receptor Promoter through its Effects on C/EBPα and C/EBPβ**

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**Background:** The immediate-early protein BZLF1 (Z) prevents TNF-alpha signaling in the host cell by inhibiting expression of the TNF-alpha receptor, TNFR1. The mechanism for this effect, however, is unknown. As the TNFR1 promoter (TNFR1p) contains a potential C/EBP binding motif, and Z directly interacts with C/EBPα (Wu et al. 2003), we hypothesized that Z inhibits the ability of one or more C/EBP family members to activate this promoter.

**Methods:** Regions of the TNFR1p were linked to the CAT reporter gene. The effect of C/EBPα and C/EBPβ on TNFR1p activity in the presence or absence of Z was determined. EMSAs were performed to ascertain if C/EBPα or C/EBPβ bind to the TNFR1p. Affinity chromatography was performed with a GST-Z fusion protein and *in vitro* translated C/EBPα and C/EBPβ proteins to study protein interactions. To determine the effect of endogenous C/EBPs on TNFR1 expression, we employed siRNA to knockdown C/EBPα and C/EBPβ.

**Results:** Both C/EBPα and C/EBPβ activated the TNFR1p, and sequences in the TNFR1p between $^{-}$154/$^{+}$35 were sufficient for this turn on. Interestingly, Z prevented this activation. Preliminary results indicate that siRNA knockdown of endogenous C/EBPα inhibits TNFR1 protein expression. EMSAs showed that Z does not bind to the TNFR1p; however, C/EBPα and C/EBPβ both bind to the 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/EBPα and C/EBPβ activation, and reversed the ability of Z to inhibit the promoter. Affinity chromatography experiments demonstrated that C/EBPα and C/EBPβ both interact with GST-Z. Mutation of Z residue 204 decreased the interaction between Z and C/EBPs, and reduced the ability of Z to inhibit the TNFR1p.

**Conclusions:** A C/EBP binding site is required for C/EBPα and C/EBPβ to activate the TNFR1p. Although Z enhances C/EBPα-mediated activation of certain EBV promoters (Wu et al. 2003), our results indicate that Z inhibits C/EBPα and C/EBPβ activation of the TNFR1p. These results suggest the Z effect is mediated by direct interaction between Z and C/EBPα and C/EBPβ, not by Z binding directly to the promoter. Z inhibition of C/EBPβ may serve to prevent activation of inflammatory cytokines in EBV-infected cells, helping EBV evade the host immune response.

**15.011**

**Decoy Receptor 3 Expression is Upregulated Upon EBV Reactivation**

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Epstein-Barr virus (EBV) belongs to gamma-herpesvirus subfamily that establishes latent B lymphocyte infection in about 90% of the world population. Latent EBV infection is associated with several human malignancies including nasopharyngeal carcinoma (NPC). People carrying latent EBV in their B cells not only maintain cytotoxic T cells directed against proteins made by the virus but also shed small quantities of infectious virus during the latent infection. How EBV latency avoids an active immune response is an important question, and whether the disruptions of the virus-host balance contribute to the EBV-associated tumorigenesis needs to be addressed. Decoy receptor 3 (DcR3) is a soluble decoy receptor belonging to the tumor necrosis factor receptor (TNFR) superfamily. It is expressed in several malignant tumor types and has been postulated to help tumor cells to gain survival advantage. We hypothesized that EBV might up-regulate DcR3 expression and DcR3 overexpression might implicate in EBV-associated cancer formation, such as nasopharyngeal carcinoma (NPC).

In this study we focused on (1) identification of EBV genes regulating DcR3 expression, and (2) the possible mechanism by which the EBV gene regulating DcR3 expression. We first established that DcR3 was upregulated upon EBV infection and reactivation. We demonstrated that DcR3 protein level and promoter activity were up-regulated by an EBV lytic trans-activator Rta in both over-expression system and in EBV-reactivated NPC cell line. Further enhanced DcR3 level was detected when over-expression of a transcriptional co-transactivator CREB-binding protein (CBP) in the presence of Rta, suggesting that Rta cooperates with CBP to up-regulate DcR3. Furthermore, Rta might transduce cellular phosphatidylinositol-3 kinase (PI3K) signaling to stimulate DcR3 expression.

**15.012**

## Epstein-Barr Virus Nuclear Antigen 3C May Modulate Nm23-H1's Metastasis Suppression Potential

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**Background:** Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus with oncogenic potential. EBV is associated with a number of human cancers. Nm23-H1 is metastatic suppressor protein, known to be down-regulated in human invasive breast carcinomas.

**Methods:** 293 embryonic kidney cell line and human osteosarcoma epithelial cell line were maintained in DMEM supplemented with fetal bovine serum. EBV-negative Burkitt's lymphoma BJAB cell line was maintained in RPMI 1640. The constructs pA3M Nm23-H1, pA3M EBNA3C, pSG5 EBNA3C and pCMV Nm23-H1 were transfected into the cells by electroporation or lipofectamine.

**Results:** Nm23-H1 is normally expressed in the cytoplasm. However, when co-expressed with EBV nuclear antigen 1 (EBNA1) or EBV nuclear antigen 3C (EBNA3C), Nm23-H1 translocates from the cytoplasm to the nucleus. EBNA3C and EBNA1 interact with Nm23-H1 in vitro and in vivo. Nm23-H1 activates the E-cadherin promoter and up-regulates E-cadherin, which is also known to be a suppressor of metastasis. Thus, Nm23-H1 may suppress metastasis through induction of the cell adhesion molecule E-cadherin. Interestingly, EBNA3C also activated the E-cadherin promoter and induced expression of E-cadherin. In cells transfected with Nm23-H1 or EBNA3C, levels of b-catenin the major downstream effecter of E-cadherin were also higher than control cells. Normally, b-catenin is predominantly localized to the cell membrane. However, some b-catenin signal was observed in the nucleus when EBNA3C or both Nm23-H1 and EBNA3C were expressed in cells.

**Conclusions:** These results suggest that EBV infection may affect the ability of Nm23-H1 to regulate the metastatic potential and possible signaling pathways in viral infected cells.

**15.013**

## EBNA-LP Transcriptional Coactivation through Interaction with Histone Deacetylase 4

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**Background:** EBNA-LP is an essential gene for immortalization of B-cells by EBV. It functions as a strong transcriptional coactivator with EBNA-2 on both viral and cellular promoters. The mechanism by which it coactivates transcription remains unknown. Here, we show that EBNA-LP effect is due at least in part to its interaction with Histone Deacetylase 4 (HDAC4) and altering its subcellular localization.

**Methods:** We used reporter gene analysis both in BJAB (EBV-) or Akata (EBV+) cells to study the effects of HDACs as well as other proteins on EBNA-2 and/or EBNA-LP activities. Subcellular fractionation followed by co-immunoprecipitations was used in order to study how EBNA-LP affected HDAC4 distribution.

**Results:** We show that EBNA-LP interacts with HDAC4 in both the nucleus and cytoplasm of B cells, and that interaction is required for coactivation of transcription Also, EBNA-LP presence significantly decreases nuclear HDAC4, indicating that EBNA-LP targets HDAC4 subcellular distribution in order to coactivate transcription. Accordingly, overexpression of HDAC4 represses EBNA-LP and anchoring HDAC4 in the cytosoplasm, by overexpression of 14-3-3 proteins, enhances EBNA-LP and reverses HDAC4 repression.

**Conclusion:** Our data indicates that EBNA-LP coactivation activity relies on its capacity to interact with transcriptional repressor Histone Deacetylase 4 and preventing it from shuttling around the cell.

**15.014**

## Downregulation of the Pro-apoptotic Nbk/Bik Gene During the EBV Growth Programme

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Inhibition of host cell apoptosis is central to the EBV strategy and may play an important role in the development of related malignant disease. The cellular Bcl-2 family BH3-only protein Nbk/Bik is a potent inducer of apoptosis which it triggers through a p53 independent pathway. Loss of Bik expression has been implicated in the development of cancers and the sensitization of tumour cells to various therapeutic agents. Here, we show that Bik is repressed in B cell lines in response to the EBV growth programme (Type III cells). Inactivation of EBNA2 function in the conditional LCL EREB2.5 led to the de-repression of bik. Rescue of these cells following retrovirus transduction with wild-type EBNA2 restored bik repression. Use of the EREB2.5 derivative P493-6 to uncouple the EBV and c-myc growth programmes indicated a co-operative role for both in bik repression. Expression of EBNA2 in EBV-negative BL-derived cells and an RBP-J/CBF1 knockout cell line led to the transcriptional repression of endogenous bik and a decrease in the level of BIK protein. Transient transfection assays using bik promoter-reporter constructs showed repression of the bik promoter both by EBNA2 and a non-CBF1-interacting EBNA2 mutant. Treatment of LCLs and BL Type III cell lines with the Histone Deacetylase (HDAC) inhibitors or a demethylating agent led to de-repression of bik indicating a role for epigenetic mechanisms in maintaining the repressed state of this gene in this cell context. Expression of exogenous BIK in LCLs or BL Type III cells led to apoptotic cell death, with a crucial role for the BH3 death domain of this protein. We conclude that EBNA2 plays a key role in repressing the pro-apoptotic bik gene and that this host-virus interaction contributes to preventing the apoptotic death of infected B cells that are proliferating on the viral growth programme.

**15.015**

**Biogenesis of EBV MicroRNAs and Regulation of BHRF1 Expression**

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**Background:** EBV BHRF1 is expressed in early stage of lytic infection and plays an important role in protecting infected cells from apoptosis. About 20 years ago, a set of mRNAs containing BHRF1 orf were identified polyadenylated at the BHRF1-specific polyadenylation site in latently infected B cells. However, the expression of BHRF1 at the protein level is not confirmed in latently infected B cells.

Most recently, it was reported that three EBV-encoded microRNAs (miRNAs) were located adjacent to the BHRF1 orf. This genomic organization raises the possibility that BHRF1 transcripts could be processed by ribonuclease III enzyme Drosha for expression of miRNAs, which may result in blocking expression of BHRF1 protein.

**Methods:** We inserted a BHRF1 gene with longer 5'-untranslated region (5'-UTR) and 3'-polyadenylation site into a vector under control of CMV promoter. The resultant construct was mutated by a serial of substitutions and deletions, and then transfected into 293 cells. The effects of mutations on expression of BHRF1 protein and miRNAs as well as mRNA level were analyzed by Western and Northern blot analysis.

**Results:**

1. Mutation of miRNA sites blocked the expression of miRNAs, but did not result in increase in BHRF1 protein level.
2. 5'-UTR of BHRF1 contains sequences which can affect mRNA level.
3. Presence of intron sequences of about 400 nt inhibited the translation of BHRF1. Removal of these sequences by RNA splicing significantly increased the BHRF1 protein level.
4. The sequences upstream of intron sequences inhibited the RNA splicing in cis.
5. Drosha can process the BHRF1 mRNA for producing miRNAs. Cutting by Drosha at microRNA site removed off the sequences that inhibit RNA splicing. However, splicing did not occur even after Drosha processing because Drosha-truncated RNA still contains intron.

**In Summary:** Drosha can block the translation of BHRF1 through inhibition of RNA splicing, but not by destroying primary BHRF1 mRNA.

**15.016**

**Epstein-Barr Virus EBNA-3A Inhibits Differentiation of Muscle Cells**

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EBV EBNA-3A is one of a limited number of latency-associated proteins essential for immortalization of primary B lymphocytes by EBV. Its function is not fully understood, but it binds to the transcription factors RBP-Jkappa and CtBP. EBNA-3A can function as an immortalizing oncogene in rodent fibroblasts, which requires intact CtBP binding motifs. The association with RBP-Jkappa has been demonstrated by our lab and others to repress transcription mediated by EBNA-2 through RBP-Jkappa in reporter gene assays. However, this function is seen when EBNA-3A is highly overexpressed. To determine whether stable EBNA-3A expression could counter the transactivating potential of EBNA-2 from endogenous cellular promoters, we decided to use a muscle cell line that can be induced to differentiate in vitro. This cell line has been used to demonstrate that Notch inhibits differentiation of muscle cells, and that EBNA-2 also inhibits differentiation. Our initial objective, therefore, was to determine whether EBNA-3A could repress this function of EBNA-2 and restore the ability of the cells to differentiate. Surprisingly, we found that expression of EBNA-3A alone inhibited differentiation. Levels of myogenin, regulated by the muscle cell-specific bHLH transcriptional regulator MyoD, failed to be upregulated in the presence of EBNA-3A. Mutation of the RBP-Jkappa binding site of EBNA-3A had no effect, whereas deletion of the CtBP binding site abolished activity. To determine whether this activity was a result of sequestering CtBP, we examined whether EBNA-3C, which also binds to CtBP, functioned in a similar manner. Unlike EBNA-3A, EBNA-3C did not affect differentiation, suggesting a unique role for the interaction of EBNA-3A with CtBP. These data suggest that EBNA-3A blocks differentiation by inhibiting the function of MyoD, directly or indirectly, in a manner that requires association with CtBP. Furthermore, it raises the intriguing possibility that EBNA-3A may regulate the activity of bHLH proteins expressed in B lymphocytes.

**15.017**

**LMP2A Auto-regulates Its Expression Independent of EBNA2**

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Recent microarray analysis of LMP2A transgenic mice (TgE) indicated that expression of EBV LMP2A during B cell development induces global changes in transcription in a manner similar to that seen in Hodgkin and Reed-Sternberg cells of Hodgkin's Lymphoma, suggesting LMP2A may play a role in disease pathogenesis (Portis et al, J Virol 77:105). Interestingly, in EBV associated malignancies such as Hodgkin's Lymphoma and Nasopharyngeal Carcinoma, LMP2A is expressed independent of its viral transcriptional activator, EBNA2. Further analysis of splenic B cells from TgE mice revealed that several factors important in lymphopoiesis are altered in a way that is consistent with activation of the Notch pathway. As EBNA2 and activated Notch1 (Notch1-IC) are functional homologues, we sought to determine whether expression of LMP2A results in constitutive activation of Notch1, which would provide insight into its EBNA2-independent transcription. RT-PCR and Western blot analysis confirmed that Notch1 is constitutively activated in B cells expressing LMP2A. Previously, it has been shown that Notch1-IC can substitute for EBNA2 to activate the LMP2A promoter. To ascertain whether LMP2A is able to auto-regulate its expression through Notch1 activation, the LMP2A promoter was cloned into a promoter-less luciferase reporter vector and transiently transfected into B cell and epithelial cell lines. Our data suggest that the LMP2A promoter is activated only when co-transfected with LMP2A or EBNA2, but not when transfected alone. Analysis of a panel of LMP2A mutants demonstrated that signaling via the N-terminus of the protein is important for mediating auto-regulation. Constitutive activation of Notch1 and activation of its own promoter suggest that LMP2A exploits the Notch pathway in order to control its own expression independent of other viral genes.

**15.018**

**Regulation of Sp100 Intracellular Localization by EBNA-LP and Cellular Factors**
**C. Echendu**, R.S. Peng, J. Tan, P.D. Ling. Baylor College of Medicine, Houston, TX, USA

Epstein-Barr Virus (EBV) efficiently immortalizes human B cells in vitro and this requires expression of EBNA-LP, a gene-specific transcriptional co-activator of EBNA2. We have recently shown that EBNA-LP interacts with and re-localizes Sp100 and its associated protein, HP-1 (heterochromatin protein 1), from Promyelocytic Leukemia Nuclear Bodies (PML NBs). Sp100 localization outside of PML NBs was correlated with gene-specific coactivation of EBNA2 and the HP-1 interaction domain was important for this function. However, it remains unclear how Sp100 is normally regulated in and out of PML NBs. One possibility is that Sp100 is recruited to nuclear bodies through interactions with cellular adaptors, and is competed off by EBNA-LP during initial stages of EBV infection in B cells. We have identified a putative cellular protein involved in this process. Additionally, we used PCR mutagenesis to identify sequences within the amino-terminal 150 amino acid residues of Sp100 that mediate Sp100 homo oligomerization as well as interactions with EBNA-LP. In addition to shedding light on the mechanistic contributions of EBNA-LP in EBV-mediated B cell immortalization, these findings may help identify putative cellular proteins/pathways mimicked by EBNA-LP.

## Notes

# Abstracts

**Monday, July 10, 2006**
**Room: Poster Area/Level 3**

## Session 16: Poster Session II
## Immune Mechanisms
## 17:30-19:00

16.001

## Tonsillar Natural Killer Cells Restrict Epstein-Barr Virus-induced B Cell Transformation after Activation by DCs

**T. Strowig**[1], G. Bougras[1], F. Brilot[1], D. Thomas[2], W.A. Muller[2], C. Munz[1]. [1]Rockefeller University, New York, NY, USA; [2]Weill Medical College, New York, NY, USA

**Background:** Natural Killer cells (NK cells) are innate lymphocytes that play an important role in the control of infections and the immune surveillance of tumors. In particular during primary infections with herpes viruses, they are thought to limit the viral burden until virus-specific T cells are able to control viral infection. Dendritic cells are potent activators of both the innate and adaptive immune system (DCs) and act as sentinels for the immune system. Hence, we asked whether DCs are able to activate NK cells enabling them to limit EBV-mediated B cell transformation.

**Methods:** A regression assay was used to evaluate the ability of NK cells to restrict EBV-mediated B cell transformation upon activation by DCs. Briefly, B cells were infected with EBV before adding DCs and NK cells. Numbers of transformed B cells were compared between cultures with and without NK cells or specific NK cell subsets purified from human peripheral blood or secondary lymphoid organs such as tonsils and spleens, in the presence or absence of differently matured monocyte-derived or splenic DCs.

**Results:** Characterization of NK cell activation by DCs showed that DCs matured with double-stranded RNA produced high levels of NK cell-stimulating IL-12 and thereby strongly activated NK cell proliferation and cytokine secretion. In line with a direct antiviral function of NK cells from secondary lymphoid organs, DCs elicited 50-fold stronger interferon-g (IFN-g) secretion by tonsillar NK cells than by peripheral blood NK cells, reaching levels that inhibited EBV-induced B cell lymphomagenesis. In fact, 100-fold less tonsillar than peripheral blood NK cells were required to achieve the same control over EBV-transformed B cells in vitro. The protective effect of IFN-g was confirmed using transwell experiments, antibody-mediated blocking and by addition of recombinant cytokine. Further characterization showed that only a small subset of NK cells, CD56brightCD16- NK cells, was responsible for limiting B cell transformation. Interestingly, these NK cells are enriched in secondary lymphoid organs like tonsils and lymph nodes, and, hence, are strategically positioned to rapidly respond to pathogens at these sites.

**Conclusions:** These results suggest that NK cell activation by DCs limits primary EBV infection in tonsils until adaptive immunity establishes immune control of this persistent and oncogenic human pathogen.

**16.002**

**Identification of Novel CD4+ T Cell Epitopes within Epstein-Barr Virus Proteins**

**E.K. Vetsika**, M.F. Callan. Imperial College London, London, United Kingdom

**Background:** Epstein-Barr virus is a common DNA gamma-herpesvirus, which infects more than 90% of the human population and then persists for life. Primary infection in adults may cause acute infectious mononucleosis and persistent infection may be associated with the development of numerous malignancies. EBV is under continuous surveillance by the immune system. The contribution of CD8+ T cells to controlling the EBV infection is well characterized whereas the role of CD4+ T helper cells has been studied to a lesser extent.

**Objective:** To identify peptides from EBV proteins that represent novel HLA class II restricted epitopes.

**Methods:** We used ProPred, a peptide prediction computer program to identify peptides that had a high probability of binding to common HLA class II alleles. 64 peptides were selected based on predicted binding to one of the twenty-one most common HLA class II alleles with no more than one peptide being selected for each EBV protein. The 64 peptides were synthesized and arranged in pools for use as a source of antigen. Separated peripheral blood mononuclear cells (PBMCs) from thirty EBV-seropositive healthy donors were screened for reactivity to the peptide pools using an ELISpot assay to detect IFN-gamma secretion. Where T cells were reactive with the peptide pools further experiments were performed to identify individual target peptide(s). In order to reveal whether CD8+ or CD4+ T cells were responding to the identified target peptides PBMCs were either depleted of CD4+ T cells or CD8+ T cell prior to the assay.

**Results:** We identified eighteen peptides from EBV proteins that stimulated IFN-gamma expression by CD4+ T cells from healthy EBV-seropositive individuals. Two of these represent previously reported epitopes whereas the other sixteen represent novel epitopes. We selected eight of the peptides and examined their capacity to stimulate CD4+ T cells taken from six patients with primary EBV infection and acute infectious mononucleosis. Five of the peptides were recognized, with half of the donors responding to the most commonly recognized peptide.

**Conclusion:** The use of ProPred allowed the identification of peptide sequences from EBV proteins that are recognized by CD4+ T cells from EBV infected individuals and thus represent CD4+ T cell epitopes. The majority of these epitopes have not previously been reported in the literature.

**16.003**

## LMP1 and LMP2 Epitope-Specific CD4+ T Cell Clones able to Recognise and Kill EBV Immortalised Lymphoblastoid Cell Lines (LCLs)

T.A. Haigh[1], X. Lin[2], E.P. Hui[3], A.T.C. Chan[3], A.B. Rickinson[1], **G.S. Taylor**[1]. [1]CRUK Institute for Cancer Studies, University of Birmingham, Birmingham, United Kingdom; [2]Hong Kong Cancer Institute, Sir YK Pao Cancer Centre, Prince of Wales Hospital, Chinese University of Hong Kong, Hong Kong, China; [3]Department of Clinical Oncology, Prince of Wales Hospital, Chinese University of Hong Kong, Hong Kong, China

**Background:** There is considerable interest in the potential of T cells to treat cancer. CD4+ T cells specific for EBV-encoded latent membrane proteins (LMPs)-1 and 2 could potentially be used to target EBV associated malignancies such as Hodgkin's, NK and T cell lymphomas and nasopharyngeal carcinoma, which express LMPs, are HLA class II-positive and apparently possess normal class II processing function.

**Methods:** Healthy EBV-immune donors with appropriate peptide reactivities in Elispot assays were used to produce CD4+ T cell clones specific for LMP1 and LMP2 epitopes by *in vitro* reactivation and the clones assayed for their HLA class II restriction and ability to recognise EBV-transformed B lymphoblastoid cell lines. (LCLs).

**Results:** CD4+ T cell clones were raised against 7 newly identified epitopes, 4 within LMP1 and 3 within LMP2, and all clones were mapped to individual HLA-DR or -DQ restricting alleles. Almost all clones showed significant direct recognition of HLA class II-matched LCLs by interferon gamma release, at levels between 0.1% and 60% of those seen against the LCL optimally loaded with epitope peptide. All but one of the clones were cytotoxic and killed peptide-loaded targets by a perforin-dependent, rather than by a Fas ligand-dependent, pathway. Clones showing the strongest LCL recognition by interferon gamma release also showed LCL killing in chromium release assays and inhibited LCL outgrowth in long-term co-culture assays. Cyclosporin A, which blocks LCL-induced cytokine production but not cell killing, did not significantly affect outgrowth inhibition, reinforcing the likely importance of cytotoxicity for this effect.

**Conclusion:** These results emphasise the therapeutic potential of LMP specific CD4+ T cells as direct effectors targeting LMP-positive malignancies. Additionally, such clones will be important reagents to examine the antigenic processing of the highly hydrophobic LMPs.

16.004

## Distinct Memory CD4+ T Cell Subsets Mediate Immune Recognition of Epstein Barr Virus Nuclear Antigen 1 in Healthy Virus Carriers

**K.N. Heller**, C. Munz. The Rockefeller University, New York, NY, USA

**Background:** Epstein Barr virus (EBV) specific CD4+ T cells most consistently recognize the EBV nuclear antigen 1 (EBNA1) in healthy virus carriers. Since EBNA1 is the only protein expressed in all EBV associated malignancies and an efficient T cell response against this protein might assist in the prevention of EBV associated tumorigenesis, we have focused on the characterization of the EBNA1 specific CD4+ T cell composition. It is already well established that EBNA1 specific effector CD4+ T cells are primarily TH1 polarized. The objectives of this study were to determine the frequencies and phenotypic characteristics of EBNA1 specific memory CD4+ T cells in healthy virus carriers.

**Methods:** Following informed consent, peripheral blood from healthy volunteers was stimulated with overlapping peptides covering the immunogenic EBNA1 aa400-641 sequence. EBNA1 specific CD4+ T cell proliferation was determined from peripheral blood mononuclear cells (PBMC) isolated via Ficoll gradient by Carboxy-fluorescein-succinimidyl ester (CFSE) dilution. IFN-gamma secretion by EBNA1 specific CD4+ T cells was assessed by intracellular cytokine staining from whole blood. Frequency and surface marker phenotype of proliferating and cytokine producing EBNA1 specific CD4+ T cells were analyzed.

**Results:** The majority of healthy EBV carriers (18/20) harbored EBNA1 specific CD4+ T cells. While EBNA1 specific IFN-gamma secretion was detected in 0.03% of circulating CD4+ T cells, 0.26% proliferating CD4+ T cell precursors were detected for EBNA1. By their expression of the homing marker to secondary lymphoid organs, CD62L, half of the EBNA1 specific CD4+ T cells were effector memory cells ($T_{EM}$, CD62L-) and half central memory cells ($T_{CM}$, CD62L+). Only $T_{EM}$ cells were found to secrete IFN-gamma in response to EBNA1, but all subsets expressed CXCR3, and not CCR4 and CXCR5. Therefore, EBNA1 specific CD4+ T cells are TH1 committed, but only half of them are effector memory T cells, supported by a proliferating central memory compartment.

**Conclusions:** EBNA1 mediated immune control of chronic EBV infection includes a substantial reservoir of $T_{CM}$ CD4+ TH1 precursors, which continuously fuels TH1 polarized effector cells. We suggest that this balance of central memory and effector memory CD4+ T cells is required for the immune control of persistent viral infections.

**16.005**

## Epstein-Barr Virus Immediate-Early Protein RTA Negatively Regulates the Function of Interferon Regulatory Factors

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**Background:** Activation of certain interferon regulatory factors (IRFs) is essential for the induction of type I interferon (IFN) and induction of innate antiviral responses. While other herpesviruses have evolved many mechanisms to block IFN responses little is known about mechanisms utilized by Epstein-Barr virus (EBV) to overcome immune responses of host cells. We have shown that the EBV immediate-early (IE) protein Zta inhibits the function of IRF7 and that Zta physically interacts with IRF7. We hypothesized that the other major EBV IE transactivator, Rta, also is involved in IRFs regulation.

**Methods:** IFN promoter-reporter construct and luciferase assays were used to measure IRF-dependent promoter activity. To investigate Rta-dependent effects on IRF protein level we co-expressed different wild-type and mutant forms of Rta and IRFs and determined protein levels with Western blots. In vitro ubiquitination assays were carried out to determine whether Rta affects IRF7 ubiquitination. We used Northern blotting and RT-PCR to examine whether Rta regulates RNA levels of IRFs.

**Results:** We show that the EBV IE transactivator, Rta, also affects function of IRF7 as well as other IRFs. We found that expression of Rta decreased IFN promoter activity induced by Sendai virus infection as well as IRF-dependent promoter activity. Expression of Rta reduces levels of IRF1, 2, 3, and 7 but not 5 or 9. Both the N and C-termini and the nuclear localization signal (NLS) of Rta are required for this activity. Rta does not affect ubiquitination of IRF7, but decreases IRF RNA levels. Moreover endogenous RNA and protein levels of the principal IFN regulatory factor, IRF7, are down-regulated during the course of cytolytic EBV infection.

**Conclusion:** Since IRF7 and 3 are necessary for type I IFN responses, inhibition of their expression by EBV Rta may contribute to the ability of the virus to evade innate and immune host responses during lytic EBV infection.

**16.006**

**The Latent Membrane Protein 1 Primes EBV Latency Cells for Interferon Production: Implication for the Pathogenesis of Systemic Lupus erythematosus**

D. Xu, K. Brumm, **L. Zhang**. University of Nebraska, Lincoln, NE, USA

Latent membrane protein 1 (LMP-1) plays important roles in EBV transformation and latency. Previously, we have found that LMP-1 induces the expression of several interferon (IFN)-stimulated genes and has antiviral effect (Zhang et al., 2004. J Biol Chem. 279:46335). In this report, a novel mechanism related to the antiviral effect of LMP-1 is identified. We show that EBV type III latency cells, in which LMP-1 is expressed, are primed to produce robust levels of endogenous IFNs upon infection of Sendai virus. This priming action is similar to IFN-mediated priming effect. The priming action of EBV latency cells is due to the expression of LMP-1. In addition, LMP-1-mediated activation of NF-κB and IFN regulatory factor 7 (IRF-7) are apparently involved in the priming action in human B cells. These data strongly suggest that LMP-1 may prime EBV latency cells for IFN production and the antiviral property of LMP-1 maybe an intrinsic part of EBV latency program. Systemic lupus erythematosus (SLE; also known as lupus) is an autoimmune disease. EBV is among the environmental factors that are suspected of predisposing to SLE. IFN has been proposed to be the driven force for SLE development and is apparently a hallmark cytokine expressed in SLE patients. Our results about LMP-1 and IFN might provide a link between EBV and SLE. Because EBV-infected cells are more frequent and LMP-1 is expressed in SLE patients, therefore, EBV latency cells may serve as surrogate profession IFN-producing cells and may be partially responsible for the high IFN levels in SLE patients. This EBV-IFN connection might provide a novel target for future SLE therapies.

**16.007**

## Nk Cells Produced IFNγ and Potentiated T Cell Activation and Cytotoxic T Cell Generation in EBV-infected Cord Blood Cell Cultures

**A. Liu**, A. Holmgren, G. Klein, E. Klein. Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden

**Background:** Epstein-Barr virus (EBV)-specific immunity is not transferred from mother to child. Therefore, cellular interactions in primary infection can be studied in cord blood mononuclear cell (CBMC) population. We found that the immunomodulators potentiated NK and T cell activation in the EBV infected cultures. B cell growth induced by EBV was inhibited in these cultures. NK cells are known be involved in the development of adaptive immunity. We tested therefore the EBV infected CBMC that NK cells were depleted.

**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:** NK cells produced IFNγ in the infected and immunomodulator containing cultures. T cell activation, B cell growth inhibition and the production of IL15 and IL12 were downregulated in the cultures devoid of NK cells. These were due to lack of IFNγ. These effects could be restored by addition of IFNγ. Cytotoxic T cells precursors were present in the EBV infected cultures, but only if the initial population contained NK cells.

**Conclusion:** IFNγ production of NK cells had a pivotal role in the activation of T cells and generation of cytotoxic T cells. Our results suggest the following scenario: EBV infected and activated B cells. These were recognised by NK and T cells. NK cells produced IFNγ and T cells were primed. PSK and Trx80 activated the monocytes which then produced cytokines IL-15 and IL-12 respectively in the presence of the primed T cells and IFNγ. The posed T cells were activated by both cytokines for function. This strategy may be exploited for generation of T lymphocytes inhibiting the proliferation of EBV infected B cells.

**16.008**

**Control of Epstein-Barr Virus Infection in Vitro by T Helper Cells Specific for Virion Glycoproteins**

J. Mautner. University of Technology, Munich, Germany

Epstein-Barr virus (EBV) establishes lifelong persistent infections in humans by latently infecting B cells, with occasional cycles of reactivation, virus production, and reinfection. Protective immunity against EBV is mediated by T cells, but the role of EBV-specific T helper (Th) cells is still poorly defined. Here, we study the Th response to the EBV lytic cycle proteins BLLF1 (gp350/220), BALF4 (gp110), and BZLF1 and show that glycoprotein-specific Th cells recognize EBV-positive cells directly; surprisingly, a much higher percentage of target cells than those expressing lytic cycle proteins were recognized. Antigen is efficiently transferred to bystander B cells by receptor-mediated uptake of released virions, resulting in recognition of target cells incubated with <1 virion/cell. T cell recognition does not require productive infection and occurs early after virus entry before latency is established. Glycoprotein-specific Th cells are cytolytic and inhibit proliferation of lymphoblastoid cell lines (LCL) and the outgrowth of LCL after infection of primary B cells with EBV. These results establish a novel role for glycoprotein-specific Th cells in the control of EBV infection and identify virion proteins as important immune targets. These findings have implications for the treatment of diseases associated with EBV and potentially other coated viruses infecting MHC class II-positive cells.

## Notes


# Abstracts

**Monday, July 10, 2006**
**Room: Poster Area/Level 3**

## Session 17: Poster Session II
## Comparative Systems
## 17:30-19:00

**17.001**

## Comparative Analysis of Cercopithicine Herpes Virus 15 Encoded Zta with EBV-encoded Zta

**Q. Hope**, M. Meyer, A.J. Sinclair. Univeristy of Sussex, Brighton, United Kingdom

**Background:** Cercopithicine Herpes virus 15 infects Rhesus monkeys. It encodes a version of Zta, ZtaRh that displays high homology with ZtaEBV; only eleven amino acid differences are found within the bZIP and adjacent CT regions. We anticipate that major functions of Zta will be conserved across these two species of virus, but that if differences were found they would aid the rapid elucidation of the molecular mechanisms by which Zta functions.

**Methods:** The coding sequence of ZtaRh was assembled and tagged with poly-histidine. Domain swap mutants were generated using site directed mutagenesis. DNA-binding assays were undertaken in vitro with a variety of methylated and non-methylated probes by EMSA. Cell cycle assays were quantitated using FACs and the activation of the EBV-lytic cycle was quantitated using real-time PCR.

**Results and Conclusions:** The ability and specificity of ZtaRh interaction with DNA was questioned because there are two amino acid differences in the basic (DNA contact) region and the region immediately adjacent to the ZIP region of ZtaEBV has been shown to influence the DNA-binding specificity of Zta (see C.Schelcher abstract). Quantitative analysis of the ability of ZtaRh to interact with a selection of ZREs (AP1, Ori lyt, ZRE1, ZIIIB and both methylated and non-methylated versions of ZRE2 and ZRE3) was undertaken using EMSA. This revealed that the DNA-binding specificity of ZtaEBV was fully conserved in ZtaRh. We compared the ability of ZtaRh and ZtaEBV to promote cell cycle arrest. This phenotype appears to be mediated by several mechanisms in EBV and ZtaRh contains mutations throughout the regions implicated. Preliminary results indicate that ZtaRh is compromised in its ability to promote cell cycle arrest. Domain swap mutants between ZtaRh and ZtaEBV have been generated and are being assessed for their cell cycle phenotype. This analysis should allow us to rapidly identify residues that are key for this function.

**17.002**

**Differential Translation Efficiency of EBNA-1 Encoded by Lymphocryptoviruses influences Endogenous Presentation of CD8+ T cell Epitopes**

**J. Tellam**[1], G. Connolly[1], M. Rist[1], N. Webb[1], C. Fazou[1], F. Wang[2], R. Khanna[1].
[1]Queensland Institute of Medical Research, Brisbane, Australia; [2]Brigham and Women's Hospital, Boston, MA, USA

Lymphocryptoviruses (LCV) which infect human and Old World primates display a significant degree of biological and genetic identity. These viruses use B lymphocytes as primary host cells to establish a long-term latent infection and express highly homologous latent viral proteins. Of particular interest is the expression of a nuclear antigen referred to as EBNA-1 which plays a crucial role in maintaining the viral genome in virus-infected B cells. Using human and Old World primate homologues of EBNA-1 we show that the internal repeat sequences differentially influence their in vitro translation efficiency. Although the glycine-alanine repeat domain of human LCV Epstein-Barr virus (EBV)-EBNA-1 inhibits its self synthesis, the repeat domains within the simian LCV homologues of EBNA-1 do not inhibit self synthesis. As a consequence, simian LCV EBNA-1 expressing cells are more efficiently recognized by virus-specific CTLs when compared to human EBV-EBNA-1 even though both proteins are highly stable in B cells. Interestingly, we also show that similar to human EBNA-1, CD8+ T cell epitopes are predominantly derived from newly synthesized protein rather than the long-lived pool of stable protein. These observations provide evidence which indicate that immune recognition of EBNA-1 can occur without compromising the biological maintenance function of this protein.

**17.003**

## MHV-68 Inoculation of Neonatal Mice Causes Persistent Infection and Inflammation of the CNS Mimicking Cerebral EBV Infection

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Infection with the Epstein-Barr virus (EBV), a gamma-herpesvirus, is associated with various neurological complications ranging from facial palsy to encephalitis. The molecular pathogenesis of these complications has not been elucidated, efficient therapeutic regimens are not available. We established a model system for neurologic gamma-herpesvirus diseases by inoculating newborn BALB/c mice intranasally with MHV-68. All types of inflammation observed in children in response to EBV ranging from menigitis to severe focal encephalitis can be observed in MHV-68 infected mice.
For characterization of the pathogenesis the kinetics of the viral appearance in the CNS and the related inflammatory response were determined and linked to each other.
At different time points post infection (p.i.) solid organs and blood of infected (n=30) and control mice (n=7) were checked for viral load by PCR, for viral activity and localisation by IFA, and for cellular infiltrates by H.E. staining and immunhistochemistry. Six days p.i. the viral load in brain tissue exceeded viremia significantly indicating intracerebral viral replication. Viremia was maximal one week p.i. and decreased rapidly, maximal viral load in brain was gained on day 12 p.i. and remained positive for months. Cellular infiltrates were initially detected 9 days p.i. (T-lymphocytes), their extent increased 16-48 days p.i. and decreased again 100 days p.i. They co-localize with viral activity. A strong positive correlation was observed between CNS viral load and severity of inflammation, particularly in the temporal lobe. The inflammation reaction can be associated with severe cellular damages. Apoptotic activity was detected in the necrotic areas.
Taken together, MHV-68 leads to delayed, persistent CNS infection followed by inflammation patterns observed in patient with cerebral EBV infection. High viral loads in CNS are indicative for severe inflammation which can trigger apoptosis.

**17.004**

**EBV Nuclear Antigens EBNA3C & EBNA1 Promote Metastasis and Can Overcome Metastasis Suppressor Effect of Nm23H1 in Nude Mice Model**

**R. Kaul**, M. Murakami, T. Choudhuri, E.S. Robertson. University of Pennsylvania, Philadelphia, PA, USA

**Background:** Studies from our group have demonstrated that EBV nuclear antigens EBNA3C and EBNA1 interact with metastasis suppressor Nm23-H1 both in vitro and in vivo. We hypothesize that the association of EBV nuclear antigens with Nm23H1, a known suppressor of cell migration modulates the activity of this master regulator in regulating gene expression of specific cellular molecules involved in migration of cells in vivo.

**Methods:** This was tested in a nude mice model to analyze the metastasis potential of the human breast cancer cell line MDA-MB-231T stably expressing EBNA3C, EBNA1 with Nm23H1. Six week old Nu/Nu nude mice were injected with cancer cells in the fourth mammary fat pad and maintained for 3 months. The mice were sacrificed either when tumor mass grew to more than 10% of body weight or after 3 months whichever was earlier. The tumorigenicity and metastasis potential of cancer cells were evaluated using various physical and histopathological parameters.

**Results:** The results showed that the presence of EBNA3C or EBNA1 in cancer cells lead to a significant increase in their metastasis potential. Stable exogenous expression of Nm23H1 in these cells led to significant reduction in metastasis potential which was then dramatically rescued by presence of the EBV nuclear proteins. Interestingly, the presence of EBNA1 or EBNA3C alone did not seem to have any significant effect on the tumorigenic potential of the cancer cells; however, the presence of both EBNA1 and EBNA3C together dramatically increased the tumorigenicity potential.

**Conclusion:** Results clearly demonstrated a significant role of Nm23H1-EBV protein interactions in the enhancement of the metastasis potential of EBV positive cells. The study confirms our hypothesis and provides a clear experimental model for determination of the role of EBV latent antigens in cell migration and metastasis.

**17.005**

## Real-time Monitoring of Murine Gamma-Herpesvirus 68 Replication in vivo Using Molecular Imaging

**S. Hwang**, L.M. Tong, T.T. Wu, R. Sun. UCLA, Los Angeles, CA, USA

**Background:** Murine gamma-herpesvirus 68 (MHV-68) has been used as a model system for the study of gamma-herpesviral pathogenesis and interaction with the host immune system. Intranasal infection of MHV-68 into mice establishes acute lytic replication in the lung followed by persistent latency in the spleen. However, viral replication in the whole body is not well characterized. Therefore, we introduced a bioluminescent imaging system of small animal model to monitor the replication of MHV-68 in vivo non-invasively and repetitively.

**Method:** We constructed a recombinant MHV-68 expressing firefly luciferase driven by viral M3 promoter (MHV-68/M3FL). After multiple route of infection, we investigated the spatial and temporal progression of MHV-68 infection in vivo.

**Results:** Luminescent signal from MHV-68/M3FL showed consistent replication kinetics with the previous literatures. Furthermore, we identified novel sites of viral replication, such as nose, salivary gland, thymus and mesenteric lymph nodes. Interestingly, MHV-68 infection progresses to different organs with different kinetics and the clearance of lytic viral replication happens locally, not globally. After initial clearance of acute infection in the lung, persistent viral replication was detected in multiple organs for several months, suggesting the spontaneous reactivation of MHV-68. The reactivation of MHV-68 from latency can be detected after the treatment of immunosuppressant cyclosporine A. This demonstrates that host immune system actively controls MHV-68 reactivation.

**Conclusion:** Our results show that bioluminescence imaging can be successfully used for the real-time monitoring of MHV-68 replication in vivo, and further applied for the characterization of mutant virus and development of vaccine.

**17.006**

## Phylogenetic Relationships and Geographic Distribution of the Japanese Macaque (Macaca fuscata) Lymphocryptovirus, Inferred from Glycoprotein B Sequences

**V. Saechan**[1], T. Ishida[2]. [1]The University of Tokyo, Tokyo, Japan; [2]University of Tokyo, Tokyo, Japan

Evolutionary studies of gamma-herpesviruses have been based on many gamma-specific genes, such as BCRF1 gene, K2 gene, gene 02 set encoding DHFR, and DPOL gene. The gene encoding the virion glycoprotein B (gB) of which C-terminus contains important cellular-sorting signals and regulates virally induced membrane fusion. The gB gene was also found to be a good tool for evaluating herpesvirus phylogeny since its orthologues exist in all mammalian herpesviruses.. A lot of primate species have been shown to harbor lymphocryptoviruses (LCVs) as their natural hosts. Japanese macaques (Macaca fuscata) onre of the member of Old world monkeys also have been demonstrated to have an EBV related virus, which was associated malignant lymphoma in a Japanese macaque. However, the phylogenetic relationships of LCV of Japanese macaque (MfusLCV) with others LCVs have not been studied. The aim of this study is to determine the sequences of gB gene of MfusLCV and figure out their phylogenetic relationships.

Eight Japanese macaque lymphoblastoid cell lines that had been shown to express EBV cross reactive antigens were the source of MfusLCV. The nucleotide sequences of gB genes were determined by using PCR-direct sequencing method, and aligned by using MEGA version 3.0 program.

The complete sequence (2,595 bp) of gB gene for eight MfusLCVs were determined. The gB gene of MfusLCVs showed 84-93% and 61-62% identity to Old World and New World primate LCVs, respectively. Based on the results of pairwise nucleotide sequence comparisons and phylogenetic analyses, the gB sequence was proven to be highly informative to distinguish Old World primate LCVs from those of New World primates. MfusLCV is close to LCV of rhesus macaques but a distinctive primate LCV. Sequences of the gB gene of MfusLCV isolates were clearly divided into two distinct geno-groups that reflect geographic locations as well as the host monkeys' genealogic relations. The gB gene is thus another candidate for a tool for MfusLCV evolutionary study.

In summary, complete nucleotide sequence of the gB gene of MfusLCVs was determined. Sequence analyses of the gB gene give an insight into the genealogic relationships of both LCV and their hosts.

**17.007**

## Rhesus Lymphocryptovirus Latent Membrane 2A Activates Beta-catenin Signaling and Inhibits Differentiation in Epithelial Cells

**C.A. Siler**, N. Raab-Traub. University of North Carolina-Chapel Hill, Chapel Hill, NC, USA

**Background:** Rhesus lymphocryptovirus (LCV) is a gamma-herpesvirus closely related to Epstein-Barr virus (EBV). 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 beta-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 beta-catenin signaling pathways. Cellular fractionations were performed and analyzed by Western blot to ascertain cytoplasmic and nuclear levels of beta-catenin. Stable cell lines were also used in a methyl cellulose 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 (GSK3beta) and Forkhead transcription factor (FKHR). Cellular fractionation experiments revealed increased levels of cytoplasmic and nuclear beta-catenin in LCV LMP2A expressing cells. In LCV LMP2A expressing cells treated with the Akt inhibitor, triciribine, the translocation of beta-catenin to the nucleus was abolished whereas total endogenous levels were not affected. In methyl cellulose-induced differentiation assays, the expression of cellular differentiation markers was reduced in LCV LMP2A expressing epithelial cells.

**Conclusions:** The expression of LCV LMP2A in epithelial cells induces Akt activation, GSK3beta inactivation and accumulation of beta-catenin in both the cytoplasm and nucleus. The nuclear translocation, but not accumulation of beta-catenin is dependent on Akt activation. 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. The similarities underscore the similarity of LCV to EBV and its suitability as an animal model for studying EBV pathogenesis in epithelial cells.


# Abstracts

**Monday, July 10, 2006**
**Room: Poster Area/Level 3**

## Session 18: Poster Session II
## Human Infection
## 17:30-19:00

**18.001**

## Sequence Variations in the EBER Locus Do Not Correlate with the EBNA2 Type of Epstein-Barr Virus

K.B. Chang, Y.K. Jeon, M.K. Kim, **W.K. Lee**. Division of Bioscience and Bioinformatics, Myongji University, Yongin, Republic of Korea

Two different EBV types, EBV-1 and EBV-2, display characteristic sequence differences in EBNA2 and EBNA3 family genes. It has been reported that small, but EBV type-related, sequence differences are also present in the EBER locus, which is located 40-kb upstream of the EBNA2 gene. In particular three variations within the upstream promoter region of the EBER2 gene, which give restriction polymorphisms for AluI and NflIII enzymes, are proposed to be highly correlated with the EBNA2 type of the virus. In this study we sequenced the EBER2 region in a panel of 19 type 1 and 4 EBV-2 isolates. We found that all isolates, with the exceptions of one type 1 and one type 2, had AluI and NflIII polymorphisms, which are previously proposed to be EBNA2 type 2-related, suggesting little correlation of these variations to the EBNA2 type 1. Furthermore, phylogenic tree analysis revealed that the EBER variations did not co-segregate with the EBNA2 allelic types of the isolates. These results indicate that the sequence variations in the EBER locus do not correlate with the EBV types

**18.002**

**Heterophile Antibodies Trigger Phagocytosis of Red Cells by Macrophages in Epstein-Barr Virus-Associated Hemophagocytic Syndrome**

W.C. Hsieh, I.J. Su. Division of Clinical Research, National Health Research Institutes, Tainan, Taiwan

Hemophagocytic syndrome (HPS) is a fatal complication of viral infections. In X-linked lymphoproliferative disorders and hemophagocytic lymphohistiocytosis, Epstein-Barr virus (EBV) is the major causative agent. In EBV-associated HPS, red cells (RBC) are predominantly phagocytosed by macrophages in lymphoid tissues. To understand pathogenic mechanisms of RBC phagocytosis triggered by EBV infection, we adopted a rabbit model of EBV-associated HPS, previously established by using EBV-related Herpesvirus papio (HVP). Kinetics of virus-host interaction was followed serially. Using flow cytometry, we detected autoantibody-coated RBCs that emerged at peak virus load during weeks 3 to 4 after HVP inoculation and increased rapidly thereafter, reaching almost 100% reactivity immediately before mortality. The appearance and increase of autoantibody-coated RBCs closely correlated to serum reactivities with Paul-Bunnell (PB) heterophile determinants. Preabsorption of infected serum with RBCs removed the majority of heterophile reactivities. After observing emergence of heterophile antibodies before RBC phagocytosis developed in lymphoid tissues, we demonstrated that RBC phagocytosis by activated macrophages occurred only when RBCs were coated with heterophile antibodies. Therefore, heterophile antibodies appear to encourage development of RBC phagocytosis in conditions or diseases associated with overt cytotoxic T cell response and macrophage activation in response to EBV infection. Our observations explain the frequent association of HPS with EBV and pathogenesis of hemophagocytosis in EBV-associated HPS.

**18.003**

## Increased Expression of EBV Genes in Systemic Lupus erythematosus

**B.D. Poole**, E.J. Brown, J.M. Guthridge, J.B. Harley, J.A. James. Oklahoma Medical Research Foundation, Oklahoma City, OK, USA

**Background:** Epstein-Barr virus (EBV) has been implicated in systemic lupus erythematosus (lupus) through humoral cross-reactivity with initial autoantibody targets, immunization with viral peptides leading to lupus autoimmunity, EBV serology and DNA associations, and observations of lupus-specific alterations in EBV humoral and cellular immunity. Altered EBV transcription in lupus-prone individuals may affect the development of lupus by increasing the amount of available antigen, altering cell death, or viral modulation of the immune system.

**Methods:** PBMCs were isolated from 10 lupus patients and race, age, and sex-matched controls. Epstein-Barr virus was incubated with $1X10^6$ PBMCs/ml in 1:1 B95-8 supernatant: RPMI 1640 with 10% FBS and 1 ug/ml cyclosporine A. Cells were incubated for 12 days at 37°C, 5% $CO_2$. EBV gene expression was also assessed in uninfected cells. RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). Gene expression levels were measured by real-time PCR using Syber Green reagents (ABI, Foster, CA). Significance was calculated using the Wilcoxon signed-rank test.

**Results:** Expression levels of 15 out of 16 EBV genes were increased following viral exposure in the lupus patient-derived samples compared to the controls. The increase was significant for 5 EBV genes: BLLF1, 3.2-fold ($p<0.004$); LMP-2, 1.7-fold ($p<0.008$); EBNA-1, 1.7-fold ($p<0.01$); BcRF1, 1.7-fold ($p<0.02$), and BXLF2, 1.97-fold ($p<0.02$). EBV gene expression was tested by RT-PCR without in vitro infection in 6 SLE and 9 control samples. Transcripts for EBER-1, BLLF1, BcRF1, LMP-1, EBNA-1, and EBNA-LP were found in higher proportions of patients than controls, with the difference in LMP-1 proportion reaching statistical significance (50% of patients, 11% of controls $p<0.05$). LMP-2 expression was not seen in patients but was found in 11% of controls. BZLF1 expression was not detected in this small patient group.

**Conclusions:** EBV gene expression is increased in SLE. Both new infection and persistent infection are affected, and both latent and lytic genes are over-expressed. Previously published work found BZLF1 expression in freshly isolated cells from patients but not controls. Although our system did not detect BZLF1 in these cells, we observed expression of lytic genes in both patients and controls. Increased amounts of EBNA-1 may lead to more cross-reactive antibodies. Increased lytic gene expression may lead to more cell death, and increased immune activation.

**18.004**

**HERV-K18 Superantigen as a Potential Risk Factor in Multiple Sclerosis**

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**Background:** Results of numerous epidemiological studies strongly support an association between infection with the gamma-herpesvirus, Epstein-Barr Virus (EBV), and risk of multiple sclerosis (MS). Recently, an EBV-inducible superantigen, the envelope protein (Env) of the human endogenous retrovirus-K18 (HERV-K18), was identified and cloned.

**Methods:** To test whether there is an association between HERV-K18 Env and MS, we compared the allelic and genotypic distribution of the three alleles of HERV-K18 Env between 207 MS patients and 416 matched healthy controls in a case-control study nested within two large ongoing cohorts.

**Results:** Overall, we found a significant association between HERV-K18 Env genotype (Chi-squared p-value: 0.03) or HERV-K18 Env allele frequency (Chi-squared p-value: 0.002) and risk of MS. In an additive model using allele K18.2 as the reference, the relative risk (RR) of MS was 1.23 for allele K18.1 (p=0.30) and 1.76 for allele K18.3 (p=0.006). The association between allele K18.3 and MS was slightly attenuated when adjusted for history of infectious mononucleosis and number of HLA-DRB1*1501 alleles, but remained significant (RR=1.70, p=0.046).

**Conclusion:** These results suggest that genetic variations in HERV-K18 may influence MS risk and deserve further investigation in larger studies.

**18.005**

**Tracking EBV Infection of B Cell Subsets in Vivo in Primary and Persistent Infection**

**S. Chaganti**, A.I. Bell, A.B. Rickinson. CRUK Institute for Cancer Studies, Birmingham, United Kingdom

**Background:** How EBV selectively colonises the memory B cell compartment during primary infection, in particular how important germinal centre (GC) transit might be, remains controversial. Furthermore, though some EBV-positive lymphomas are derived from GC cells or from atypical GC emigrants, the extent to which EBV-infected cells transit the GC compartment during asymptomatic virus carriage remains uncertain. Here we investigate EBV infection of different B cells subsets in vivo both during primary and persistent infection.

**Methods:** Peripheral blood B cells, selected by anti-CD19 coated beads were co-stained with anti-IgD and CD27 monoclonal antibodies (MAbs). Highly purified naïve (IgD+ CD27-), conventional memory (IgD- CD27+) and atypical memory (IgD+ CD27+) B cells were isolated by FACS sorting. Tonsillar B cells, isolated after CD3 depletion, were co-stained with anti-CD38, IgD, and CD27 monoclonal antibodies. Highly purified naïve (CD38lo IgD+ CD27-), memory (CD38- IgD- CD27+), GC (CD38+), and plasma cells (CD38hi) were isolated by FACS sorting. EBV genome load in each subset was estimated by quantitative PCR.

**Results:** In agreement with previous literature, we find EBV predominantly confined to the memory B cell compartment in peripheral blood in both primary and persistent infection. However, in many cases there is also a significant genome load in IgD+ CD27+ atypical memory B cells, a subset held to be GC-independent in origin. In chronic carrier tonsils, as reported, EBV is found in the memory but not in the naive B cell compartment; however there is very little if any infection of the GC fraction in such tonsils. By contrast, in acute IM tonsils, overall loads are >1000-fold higher, with the highest loads consistently seen in the CD38+ GC cell and CD38hi plasma cell fractions. We are currently analysing further the phenotype of these CD38+ cells from IM tonsils to see if they express other markers typical of GC cells.

**Conclusions:** We observe frequent EBV infection of IgD+ CD27+ (atypical) memory B cells as well as the IgD- CD27+ (GC-dependent) memory compartment in the blood of virus carriers. Studies on tonsils from acute IM patients are in progress and could help to resolve uncertainties over the route of viral entry into the memory B cell pool

**18.006**

## CD8-mediated EBV-specific IFN-γ Responses Tend to be Suppressed While EBV Viral Loads are Increased after an Episode of Acute Clinical Malaria in Kenyan Children

**A.M. Moormann**[1], P.S. Ogolla[1], K. Chelimo[2], P.O. Sumba[2], D.J. Tisch[1], R.W. Novince[1], J.W. Kazura[1], R. Rochford[3]. [1]Case Western Reserve University, Cleveland, OH, USA; [2]Kenya Medical Research Institute, Kisumu, Kenya; [3]SUNY Upstate Medical University, Syracuse, NY, USA

Holoendemic malaria (perennial and intense exposure) and early-age EBV infection are two co-factors associated with the high incidence of endemic Burkitt's lymphoma found in equatorial Africa. The mechanisms by which repeated *Plasmodium falciparum* infections could impact EBV persistence and immunity have not been well described. To address this question, we measured EBV-specific IFN-γ T cell responses and EBV viral loads from Kenyan children during an episode of acute uncomplicated clinical malaria and again 4-weeks post recovery. To assess the capacity to respond to malaria, we also examined responses to merozoite surface protein 1 (MSP-1), which is expressed during blood stage infection. PBMC were cultured with HLA Class I-restricted peptides for EBV lytic and latent antigens or the MSP-1 42-kDA recombinant protein and IFN-γ response was measured by ELISPOT assay. We found that IFN-γ responses to EBV latent and lytic peptide pools tended to decrease while those to MSP-1 42-kDa recombinant protein significantly increased. Furthermore, mean EBV viral loads as measured by RTQ-PCR significantly increased after an episode of symptomatic malaria. These data suggest that malaria-induced suppression of EBV-specific T cells allows for an increase in the number of EBV infected B cells which could increase the risk for emergence of a malignant clone. However, malaria-induced immunosuppression appears to be selective rather than global as demonstrated by the increase in malaria-specific responses.

**18.007**

**Alterations in B Cell Subsets Following Acute Malaria in Children: Implications for EBV Persistence and the Etiology of Burkitt's Lymphoma**

**R. Rochford**[1], A. Amolo[2], K. Chelimo[2], R. Ploutz-Snyder[1], A. Moormann[3]. [1]SUNY Upstate Medical University, Syracuse, NY, USA; [2]Kenya Medical Research Institute, Kisumu, Kenya; [3]Case Western Reserve University, Cleveland, OH, USA

Despite the strong epidemiologic link between Plasmoidum falciparum malaria and endemic Burkitt's lymphoma (eBL), the mechanism by which P. falciparum malaria promotes eBL is unknown. Two possible but not mutually exclusive mechanisms have been proposed: suppression of T-cell immunity or activation of B cells. In this study, we investigated whether an episode of acute malaria in young children results in increases in the memory B cell subset, the site of long-term EBV latency. We characterized the B cell phenotype in the peripheral blood of 15 children aged 2-5 years during episodes of acute clinical malaria, post recovery and of six healthy age matched controls who were parasitemic but asymptomatic. We observed a significant increase in CD19+ B lymphocytes following recovering from malaria. Characterization of the B cell subsets in the peripheral blood based on expression of IgD+CD38+ revealed a significant increase in the numbers of naïve B cells while there was a decrease in memory B cells after recovery period from acute malaria. This observation suggests that increases observed in EBV viral load during episodes of acute malaria are not due to expansion of peripheral memory B cell population. Further analysis of the peripheral B cell phenotype has identified an expansion of CD10+CD27- B cells in children following an episode of acute malaria with up to 25% of total CD19+ B cell pool residing in this subset. This cell population has recently been identified as a transitional B cell and elevated levels in peripheral circulation are thought to be indicative of B cell dysfunction. Interestingly, BL tumors express CD10 and it has been thought that these tumors derive from CD10+ germinal center B cells. Elevation of CD19+CD10+ B cells following an episode of acute malaria raises the possibility that these tumors arise from the peripheral B cell pool.

**18.008**

## Plasma Titers of Antibodies Against Epstein-Barr Virus BZLF1 and Risk of Multiple Sclerosis

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**Background:** Results of recently conducted prospective studies have demonstrated that the presence of high titers of anti-EBNA1 or anti-EBNA complex IgG antibodies in healthy individuals is a strong risk factor for multiple sclerosis (MS). Antibodies to BZLF1, the product of the homonymous early lytic gene, have been found to be related to risk of nasopharyngeal carcinoma, but have not been previously measured in MS studies. Therefore, we examined whether high levels of anti-BZLF1 IgG antibodies also predict MS risk.

**Methods:** We conducted a nested case-control study among women in the Nurses Health Study and Nurses Health Study II cohorts. There were 18 cases of MS with a blood sample collected prior to the onset of disease. Each case was matched with one control on year of birth and cohort. IgG antibodies to BZLF1 were measured by ELISA and relative risks were estimated using conditional logistic regression.

**Results:** Compared with their matched controls, the optical density (OD) values of serum BZLF1 antibody to EBV were higher among MS cases (OD, 0.80 vs. 0.44; p-value 0.05). We found BZLF1 antibodies to be a predictor of MS, with a relative risk of 3.6 (95% CI, 0.9, 14.8). However, this association was somewhat attenuated after adjusting for EBNA1 antibody titers (RR 3.3; 95% CI, 0.3, 31.7), and disappeared after further adjustment for antibody titers to EBNA2 (RR 0.76; 95% CI, 0.1, 5.9). Using a stepwise regression procedure, IgG antibody titers to EBNA2 and EBNA1 were first selected in the regression model, and no further contribution to MS risk was provided by the anti-BZLF1 IgG antibodies.

**Conclusion:** Results of this study suggest that antibody titers to EBNAs are the strongest predictor of MS risk, and little further contribution may be provided by measuring anti-BZLF1 antibodies, but this needs to be confirmed in larger investigations.

**18.009**

## Multiple Sclerosis Patients Make a Unique Antibody Response to EBNA-1 Prior to the Diagnosis of MS

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Multiple Sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) characterized by the presence of lesions or plaques of varying sizes throughout the white matter of the CNS. During MS, white blood cells are drawn to regions of the white matter of the CNS resulting in the destruction of the myelin sheath that surround nerve cell axons in the brain and spinal cord. Both antibodies and reactive immune cells to myelin antigens seem to have a role in the development of these lesions. Previous studies have shown a strong temporal association between elevated levels of antibodies directed against various EBV proteins and increased risk of developing MS in serial collections of patient sera. This study seeks to understand the potential role for EBV (and specifically EBNA-1) in triggering MS by analyzing the fine specificity of the anti-EBNA-1 response in MS patients. We have constructed the unique overlapping decapeptides of EBNA-1 to analyze the humoral response to this protein in MS patients as compared to controls. Twenty-eight patients and 28 matched controls were selected from the Nurse's Health Study with stored serum samples available. Eighteen of the samples were taken from patients before the onset of symptoms of multiple sclerosis, and 10 were from before diagnosis of MS but after symptom onset. The fine specificity of the antibody response to EBNA-1 was analyzed in all patients and controls. Interestingly, we found that MS patients respond differently to EBV at the humoral immune level than do normal individuals. One epitope was identified to which MS patients have a significantly higher antibody response- GGGAGAGGGA. Although controls do exhibit binding to this sequence, patient binding is significantly higher ($p=0.0001$). Additionally, parts of this sequence are present on several different decapeptides so we were able to determine that critical sequence for this response is AGAGGGA. The majority of peptides containing this sequence had significantly higher binding in MS patients than in controls.

These findings demonstrate a significant difference in the anti-EBNA-1 antibody response in MS patients that is present even before diagnosis of MS. Although further studies will be required to analyze the pathologic potential of these anti-EBV antibodies this data further supports the role for EBV in the etiology and/or pathogenesis of MS.

**18.010**

## Frequency and Phenotype of EBV-specific Cytotoxic T Lymphocytes during Intermittent EBV Reactivation in Healthy Individuals

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**Background:** Persistent EBV infection is tightly controlled by virus-specific memory $CD8^+$ T cells which in the blood are known to be of intermediate effector memory phenotype, predominantly expressing CD45R0 and to a smaller extent CD45RA depending on the specificity for either latent or lytic epitopes. However we noted that EBV infection can reactivate intermittently over time, even in apparently healthy EBV carriers, and changes in the frequency and phenotype of memory $CD8^+$ T cells have not yet been assessed in this context. We hypothesized that viral reactivation leads to expansion and/or activation/differentiation of the pool of the virus-specific T cell pool.

**Methods:** Blood samples from 14 healthy donors were collected at regular intervals for a period of one year. Each sample was screened for plasma viremia and increases in PBMC viral load as parameters of EBV reactivation using quantitative real-time PCR. Moreover, PBMC were subject to ELISPOT analysis using the autologous lymphoblastoid cell line and appropriate CD8 EBV epitope peptides as stimulators. Certain memory T cell populations were also visualized by HLA-peptide tetramer staining and co-stained with activation and differentiation markers.

**Results:** Ten donors exhibited signs of asymptomatic EBV reactivation at least once over the study period, with detectable plasma viremia - indicative of ongoing replication - in eight individuals. In six cases, viremia was accompanied by an increase in most epitope-specific T cell frequencies. Likewise, increases in viral load were preceded by or concurrent with a boost in T cell frequencies covering both latent and lytic antigens in eight patients. Coincident with EBV reactivation, donors exhibited general up-regulation of some activation markers, in particular CXCR1, on EBV-specific as well as on some other T cell populations in the blood.

**Conclusion:** We find that in healthy EBV carriers the pool of EBV-specific $CD8^+$ T lymphocytes is being expanded and activated during spontaneous virus reactivation events and that numbers of virus-specific T cells are not constant throughout. Intermittent EBV reactivation provides a constant challenge to the host's adaptive immune system.

18.011

## Comparison of Serological and Virological Parameters Between Infectious Mononucleosis and Asymptomatic Primary Epstein-Barr Virus Infection

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**Background:** Primary Epstein-Barr virus (EBV) infection can result in either asymptomatic (AS) infection or infectious mononucleosis (IM). We hypothesize that the serological and virological parameters of these infection states show discernible differences.

**Methods:** Twenty IM patients and 10 AS patients were followed prospectively at up to 6 timepoints from the time of diagnosis to one year post-infection. Estimation of VCA IgM, VCA IgG (including avidity), EA and EBNA IgG titres by immunofluorescence tests and quantitation of virus loads in blood and saliva by Taqman assays were performed.

**Results:** Serological screening of almost 1000 patient samples of Hong Kong Chinese children showed that seroconversion to EBV appeared after one year and rapidly progressed to 50% by 5 years and reaching 80% and 100% at 10 and 18 years, respectively. The highest incidence of IM occurred in young children at 1 to 5 years, corresponding to the pattern of EBV seroprevalence in our population. Both IM and AS patients had high VCA IgG titres at the time of diagnosis. In IM patients, VCA IgG was of low avidity at diagnosis, converting to high avidity at about 3 months. IgM disappeared over 3-6 months whilst IgG was maintained at high titres. EA IgG emerged slightly later than VCA IgG. EBNA IgG emerged latest at about 3 months in IM patients and even later in AS patients. The serological profile of each patient defined the timing of EBV infection, allowing comparison of virus loads between IM and AS patients. A typical kinetics profile was seen in peripheral blood (PB) of IM patients with high virus loads at diagnosis followed by a brisk fall during the first 1-2 weeks, a transient rise between 1-3 months and then a gradual decline towards one year. Interestingly, the virus loads in PB at one year post-IM were approximately one log higher than those found in healthy virus carriers. In AS patients, the virus loads in PB were generally two logs lower than those of IM patients and the level gradually fell to those of healthy virus carriers within one year post-infection. High virus loads were present in the saliva of IM patients throughout the first year post-infection whilst lower saliva virus loads comparable to those of healthy virus carriers were detected in AS patients.

**Conclusion:** Different serological profiles and high EBV loads distinguish infectious mononucleosis from asymptomatic primary EBV infection. The mechanisms of such differences are currently unclear.

**18.012**

## Mannose-binding Lectin Genotypes and Susceptibility to Epstein-Barr Virus Infection in Infancy

**J. Friborg**[1], R.F. Jarrett[2], A. Koch[1], P. Garred[3], J. Freeland[2], A. Andersen[1], M. Melbye[1].
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**Introduction:** Differences in age at EBV acquisition have been associated with differences in socioeconomic status, crowding and hygienic standards, while individual factors influencing time of infection are sparsely described. Mannose-binding lectin (MBL) is a serum lectin participating in the innate immune defense by opsonizing various microorganisms for phagocytosis. The MBL gene (MBL2) is located on chromosome 10 and three variant alleles have been described. About 4-8% of individuals are homozygous or are compound heterozygous for the variant alleles in whom only trace amount of a dysfunctional MBL are found. MBL insufficient individuals are more susceptible to a range of infections, especially in early infancy before the maturation of the adaptive immune system, but the influence of MBL2 polymorphisms on EBV infectivity is unknown.

**Methods:** In a community-based cross-sectional study, MBL2 genotypes and presence of EBV antibodies were determined in plasma samples from 247 children aged 2 months to 4 years. Levels of IgG and IgM antibodies to the EBV-viral capsid antigen (VCA) were determined using ELISA assays. By combining the MBL2 genotypes, a MBL-sufficient group (YA/YA, YA/XA, YA/O, XA/XA) (N=234) and a MBL-insufficient group (XA/O + O/O ) (N=13) were defined, with virtually undetectable amounts of functional MBL in the blood of the latter group.

**Results:** Anti-VCA IgG/IgM seropositivity was significantly lower in the MBL-insufficient (38.5%) compared to the MBL-sufficient group (88.6%) ($p=0.01$), just as time to EBV seroconversion was increased for MBL-insufficient children ($p<0.0001$).

**Conclusion:** The present study indicate that polymorphisms in the MBL2-gene, determining MBL serum levels, are associated with susceptibility to EBV infection in infancy, as MBL-insufficient children acquired the infection significantly later than MBL-sufficient children.

## Notes


# Abstracts

**Monday, July 10, 2006**
**Room: Poster Area/Level 3**

## Session 19: Poster Session II
## Burkitt & Hodgkin Lymphoma
## 17:30-19:00

**19.001**

**Specific Killing of EBV-positive Hodgkin`s Lymphoma Cells by a CD30 Promoter-dnEBNA1 Gene Therapy Vector**

**G. Kapatai**, H. Parry, R.J. Jones, A.T.H. Burns, L.S. Young, P.G. Murray. University of Birmingham, Birmingham, United Kingdom

**Background:** Overexpression of CD30 is restricted to several malignancies which include Hodgkin`s Lymphoma (HL). In contrast, only a minority of normal cells express this surface receptor. CD30 expression is regulated at the transcriptional level by a minimal promoter that has a cell type restricted activity, thus making it a promising tool for specific treatment of gene therapy to Hodgkin and Reed-Sternberg (HRS) cells. The Epstein-Barr virus (EBV) genome is present in HRS cells of EBV-associated HL, where it expresses several latent genes including EBNA1. Since EBNA1 is essential for the maintenance of the viral episome, it is an ideal target for developing new approaches to the treatment of EBV-associated tumours.

**Results:** We have previously demonstrated the ability of a VP22-dnEBNA1 fusion protein to induce EBNA1 degradation and cell death in EBV-positive, but not EBV-negative, epithelial cell lines. We now show that, as well as killing EBV-infected epithelial targets, the dnEBNA1 construct is also capable of inducing cell death in EBV-positive HL cells. Furthermore, we have generated a vector in which expression of the VP22-dnEBNA1 fusion protein is regulated by a CD30 promoter. Expression of VP22-dnEBNA1 from this vector results in the killing of EBV-positive, CD30 expressing tumour cells, but has no effect on CD30-negative tumour cells.

**Conclusion:** Our results demonstrate the potential of using dnEBNA1 construct in the treatment of a broader range of EBV-positive malignancies. Importantly, a vector in which dnEBNA1 construct is driven by the CD30 promoter selectively kills CD30 expressing, EBV-positive HL cells. This suggests a potentially safe means to the specific targeting of gene therapy to EBV-positive HL cells.

**19.002**

## Expression Profiling of Hodgkin's Lymphoma Identifies CCL20, an EBV Target that Modifies T Cell Recruitment to the Tumor Site

**K.R.N. Baumforth**[1], A. Birgersdotter[2], G.M. Reynolds[1], W.B. Wei[1], M. Vockerodt[3], C.B. Woodman[1], L.S. Young[1], I. Ernberg[2], P.G. Murray[1]. [1]University of Birmingham, Birmingham, United Kingdom; [2]MTC, Karolinska Institute, Stockholm, Sweden; [3]Georg August University of Goettingen, Goettingen, Germany

**Background:** A proportion of Hodgkin's lymphomas (HL) carry the Epstein-Barr virus (EBV), an oncogenic herpesvirus, 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. The more frequent association of EBV with the mixed cellularity subtype suggests that the virus may influence the nature of the tumor cell microenvironment.

**Methods:** Microarray analysis was used to compare gene expression profiles of EBV-positive and EBV-negative primary HL tumors with that of normal germinal centre B cells. Differentially expressed genes were compared to a list of EBV-regulated genes derived from a microarray analysis of paired EBV-positive and EBV-negative HL cell lines. RT-PCR, ELISA and immunohistochemistry were used to validate gene expression changes. Transwell assays were used to assess the chemotaxis of PBMCs towards conditioned media from HL cell lines.

**Results:** Microarray analysis revealed differences in gene expression between EBV-positive and EBV-negative tumors, which included the upregulation of CCL20 in EBV-positive tumors. CCL20 was also up-regulated in both EBV-positive HL cell lines. Furthermore, by immunohistochemistry, CCL20 expression was also observed more frequently in the HRS cells of EBV-positive primary tumours. Higher levels of CCL20 in conditioned media from EBV-positive HL cells led to increased chemotaxis of CCR6 positive PBMCs.

**Conclusion:** EBV infection of HRS cells increases the migration of CCR6 positive PBMCs through the up-regulation of CCL20. These CCR6 positive cells include regulatory T cells which may induce a localised immunosuppression and suppress CTL responses to EBV-infected tumor cells.

**19.003**

**Three Restricted Forms of EBV Latency Counteracting Apoptosis and Affecting the Cellular Differentiation Status of c-myc Expressing Burkitt Lymphoma Cells**

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**Background:** EBV is aetiologically linked with Burkitt Lymphoma (BL) but its contribution to lymphomagenesis, versus that of the chromosomal translocation activating c-myc expression, remains unclear. This is in part because the full virus growth transforming programme is not expressed in BL. Instead EBV is detected in a restricted Latency I form of infection characterised by expression of only one latent antigen EBNA1 from the BamHI Q promoter. Here we describe an endemic BL, Awia, which uniquely is heterogeneous at the single cell level for EBV gene expression.

**Methods:** Limiting dilution analysis was employed to generate single cell clones of Awia-BL. These clones were firstly screened by DNA PCR to determine EBV genome status and then subsequently selected clones were analysed by western blotting techniques and quantitative RT-PCR assays to determine EBV latency. The effect of the different forms of EBV latency on the apoptosis sensitivity of the BL clones was tested using ionomycin and surface IgM ligation as apoptotic triggers. Finally microarray analysis was carried out on the isogenic Awia-BL clones to determine the influence of EBV gene expression on cellular transcription.

**Results:** Analysis of single cell clones of Awia-BL revealed cells displaying three forms of restricted EBV latency: Latency I expressing the nuclear antigen EBNA1 from a wild-type virus genome, Wp-restricted latency expressing EBNAs 1, 3A, 3B, 3C and -LP from an EBNA2-deleted genome, and a novel EBNA2+/LMP1- latency in which all EBNAs including EBNA2 are expressed without the latent membrane proteins LMPs 1 and 2. Comparison with rare EBV-negative clones from the same tumour showed that each form of infection provides the c-myc-expressing BL cells a specific degree of protection from apoptosis. In addition microarray analysis on these isogenic Awia-BL clones showed that the EBNA profile influences the differentiation status of the BL cell on the germinal centre to plasmacytoid differentiation pathway. These findings may reflect viral functions that are important not just for BL pathogenesis but also for EBVs normal strategy of persistence in the B cell system.

**Conclusion:** Our work suggests firstly that EBV acts as an anti-apoptotic rather than a growth-promoting agent in BL and secondly that individual EBNA proteins have direct effects on the B cell phenotype that are independent of the LMPs.

**19.004**

## EBV Genome Loss from Endemic Burkitt Lymphoma Cell Lines and Its Effects on Cell Phenotype

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**Background:** EBV's growth transforming function (Latency III infection) appears central to the pathogenesis of post-transplant lymphoproliferative disease but the virus' contribution to Burkitt Lymphoma (BL) remains uncertain since all but one of the transforming genes (EBNA1) are usually down-regulated in BL cells (Latency I infection). Work with the sporadic BL line Akata, which spontaneously generates rare loss clones in late passage, suggests that EBV can influence the BL cell phenotype. We attempted to build on this work by focusing on early passage endemic BL lines and screening for EBV-loss clones.

**Methods:** Cell clones from limiting dilution cultures were initially screened for EBV status and viral load using quantitative real time DNA PCR. Any EBV-loss clones were compared with EBV-positive counterparts for cell growth, apoptosis sensitivity, expression of Bcl-2 family member proteins and, by microarray analysis, for global cellular gene transcription.

**Results:** From 11 endemic EBV-positive cell lines analysed in early passage, only three (Eli-BL, Mutu-BL and Awia-BL) generated EBV-loss clones; these three lines did not have unusually low genome copy numbers or low EBNA1 levels, but they did show a tendency for asymmetric genome segregation in mitotic figures. On all three BL backgrounds (as in Akata-BL), EBV-loss clones were consistently more sensitive than EBV-positive clones to apoptosis induced by surface IgM ligation or ionomycin; this could not be explained by any observed difference in expression of the Bcl-2 family member proteins. Contrary to the Akata-BL literature, for these endemic BLs clonal growth capacity in low serum or in soft agar was less strongly linked to EBV status. By microarray analysis, while EBV-positive and EBV-loss clones show very similar cell transcription profiles, some genes are differentially expressed; their relevance to the above differences are being investigated.

**Conclusions:** EBV-loss clones can be generated from a subset of early passage endemic BL cell lines. EBV Latency I infection appears to provide a small but significant protection from apoptosis in the three different endemic BL cell backgrounds upon which it could be examined.

**19.005**

## Plasma Epstein-Barr Virus (EBV) DNA Is a Biomarker for EBV-Positive Hodgkin's Lymphoma

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**Background:** Latent Epstein-Barr virus (EBV) genomes are found in the malignant cells of approximately one-third of Hodgkin Lymphoma (HL) cases. Detection and quantitation of EBV viral DNA could potentially be used as a biomarker of disease activity.

**Methods:** Initially EBV-DNA viral load was prospectively monitored from peripheral blood mononuclear cells (PBMC) in HL patients. Subsequently, we analysed viral load in plasma from a second cohort of patients. A total of 58 HL patients (31 newly diagnosed, 6 relapsed and 21 in long-term remission) were tested. Using real-time PCR, 43 PBMC and 52 plasma samples were analysed.

**Results:** EBV-DNA was detectable in the plasma of all EBV-positive HL patients prior to therapy. However, viral DNA was undetectable following therapy in responding patients (p=0.0156), EBV-positive HL patients in long-term remission (p=0.0011), and in all patients with EBV-negative HL (p=0.0238). Conversely, there was no association seen for the EBV-DNA load measured from PBMC in patients with active EBV-positive HL patients as compared to EBV-negative HL, or patients in long-term remission. EBV-DNA load in matched plasma/PBMC samples were not correlated.

**Conclusions:** We show that free plasma Epstein-Barr virus (EBV) DNA has excellent sensitivity and specificity, and can be used as a non-invasive biomarker for EBV-positive HL and that serial monitoring can predict response to therapy. Further prospective studies are required to further evaluate the use of free plasma EBV-DNA as a biomarker for monitoring response to treatment in patients with EBV-positive HL.

**19.006**

**The EBV-encoded Latent Membrane Protein -1 Imposes on Germinal Centre B Cells, a Hodgkin Reed-Sternberg-like Gene Expression Signature**

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Classical Hodgkin's lymphoma (cHL) is a distinct malignancy of the immune system, associated with the Epstein Barr virus (EBV), where the tumour cells are mainly derived from germinal-centre (GC) B cells that have survived the GC reaction in absence of a high affinity BCR. Furthermore, the malignant Hodgkin Reed Sternberg cells diplay an unsual phenotype, characterized by the lack of B cell specific genes, deregulated activation of signaling pathways, the enhanced expression of immunomodulators and anti apoptotic proteins. Signaling through the latent membrane protein 1 (LMP1) of EBV is likely to be important for transformation of GC B cells leading to the development of tumours such as cHL. However, the contribution of LMP1 to the pathogenesis of these tumours is still unknown. We have developed a non-viral vector based approach to express LMP1 in primary tonsillar human GC B cells. Gene expression profiling revealed that many LMP1 induced transcriptional changes in GC B cells are similar to those observed in cHL, including the characteristic loss of B cell specific genes and the activation of apoptotic regulators, intracellular signaling molecules and transcription factors. These findings suggest that LMP1 expression in GC B cells may be an early and critical step in the virally induced transformation of GC B cells leading to the development of tumours such as cHL.

## Notes

# Abstracts

**Monday, July 10, 2006**
**Room: Poster Area/Level 3**

## Session 20: Poster Session II
## Nasopharyngeal Carcinoma
## 17:30-19:00

**20.001**

## DNAzymes Targeted to EBV-encoded Latent Membrane Protein-1 Induce Apoptosis and Enhance Radiosensitivity in Nasopharyngeal Carcinoma

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Epstein-Barr Virus (EBV) is involved in the carcinogenesis of several types of cancers such as nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma. The latent membrane protein (LMP1) encoded by EBV is expressed in the majority of EBV-associated human malignancies and has been suggested to be one of the major oncogenic factors in EBV-mediated carcinogenesis. Therefore, genetic manipulation of LMP1 expression may provide a novel strategy for the treatment of the EBV-associated human cancers. Deoxyribozymes (DNAzymes) are catalytic nucleic acids that bind and cleave a target RNA in a highly sequence-specific manner. We have designed several LMP1-specific DNAzymes and tested their effect on cell proliferation and apoptosis in LMP1-positive cells. Here, we show that active DNAzymes down-regulated the EBV oncoprotein LMP1 and inhibited cellular signal transduction pathways abnormally activated by LMP1. This down-regulation of the LMP1 expression was shown to be associated with a decrease in the level of antiapoptotic Bcl-2 and a increase of Caspase-3 and -9 activity in the nasopharyngeal carcinoma cell line CNE1-LMP1, which constitutively expresses the LMP1. When combined with radiation treatment, the DNAzymes significantly induced apoptosis in CNE1-LMP1 cells, leading to an increased radiosensitivity both in cells and in a xenograft NPC model in mice. The results suggest that LMP1 may represent a molecular target for DNAzymes and provide a basis for the use of the LMP1 DNAzymes as potential radiosensitizers for treatment of the EBV associated carcinomas.

**20.002**

**Immortalization of Nasopharyngeal Epithelial Cells and Their Applicatons in EBV Study**

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Nasopharyngeal carcinoma (NPC) is a common disease in Hong Kong and Southern provinces of China, and is closely associated with Epstein-Barr virus (EBV) infection. Immortalized nasopharyngeal epithelial cell system with well-characterized properties will be useful in elucidation the role of EBV infection in NPC. Our laboratories have been involved in the establishment and characterization of immortalized nasopharyngeal epithelial cells using telomerase, SV40T and HPV16E6E7. These immortalized cell lines resemble and share many properties of premalignant nasopharyngeal epithelial cells. The NP69 cells(immortalized by SV40T) could be infected by EBV and highly responsive to the transformation property of EBV oncogenes including LMP1. Recently, we have established a near diploid nasopharyngeal epithelial cell line (NP460) using telomerase alone. The NP460 is unique in haboring a discrete deletion of p16 and RASSF1A inactivation. The properties of these immortalized nasopharyngeal epithelial cell lines and their applications in EBV studies will be presented.

**20.003**

**Regulation of EBV- Encoded Latent Membrane Protein1 Expression in Nasopharyngeal Carcinoma**

D. Sun, D.N. Van, X.N. Zhang, I. Ernberg, **L.F. Hu**. MTC, Karolinska Institute, Sweden

Nasopharyngeal carcinoma (NPC) distinguishes itself from other epithelial malignancies by its significant association with Epstein-Barr virus (EBV). The EBV-encoded latent membrane protein 1 (LMP1) is expressed in about 65% of NPCs. It is an oncogenic protein and could transform both B cell and epithelial cell in vitro. LMP1-expressing NPCs show different growth pattern and outcome from those lacking LMP1. However, regulation of LMP1 transcription in NPC is not fully understood. There are two active promoters for initiating LMP1 expression in NPCs : a short 2.8 kb transcript controlled by a proximal promoter (EDL1) and a long 3.5 kb transcript controlled by a distal promoter (L1TR). In this study, the activation of LMP1 promoters in NPC biopsies was first detected by northern blot and then divided the tumor samples into four different categories according to their transcripts: a) EDL1+/L1TR+; b) EDL1+/ L1TR-; c) EDL1-/L1TR+; and d) EDL1-/L1TR-. The methylation status of EDL1 promoter between EDL1+ (a and b) and EDL1- (c and d) groups was compared. The bisulfite sequencing results show that the EDL1 promoter was heavily methylated in the EDL1- NPC subgroup, while methylation was rarely detectable in EDL1+ NPCs. The distal promoter L1TR was free of methylation in all NPC samples with no correlation with the L1TR transcript. When an EBV carrying cell lines of gastric carcinoma with a silenced LMP1 long transcript was treated with histone deacetylation inhibitor TSA, the expression of LMP1 long transcript could be induced, while demethylating agent 5-aza-dc had no such an effect. Chromatin immunoprecipitation experiments show that TSA treatment resulted in acetylation of the H3, H4 histones binding to the L1TR region. We found that two promoters of LMP1 could contribute either separately or simultaneously to the functional LMP1 expression in NPCs, and activation of these two promoters is regulated by different epigenetic mechanisms. While EDL1 is regulated by DNA methylation, L1TR is controlled by histone modification.

**20.004**

**Nasopharyngeal Carcinoma in Children and Adolescents – A Multicenter Study**

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**Background:** To determine the efficacy of preradiation cisplatin and fluorouracil (5FU) chemotherapy and of adjuvant IFN-β-therapy in advanced stage nasopharyngeal carcinoma in children and adolescents and the impact on event free survival, especially in 'high risk' patients.

**Methods:** 59 patients (58 'high risk' patients and one 'low risk' patient, median age 13 years, range 8 - 25) were treated at 27 hospitals in the co-operative GPOH-study NPC-91. The stage II patient received irradiation as initial therapy. 57 patients received three courses of preradiation chemotherapy (methotrexat 120 mg/m2, day 1, cisplatin 100mg/m2, day 1 and 5FU 1000 mg/ m2 /d, days 1 through 5). After chemotherapy patients were treated with uniform radiation therapy stratified by stage. The cumulative radiation dose to the primary sites was 59.4 Gy with single doses of 1, 8 Gy. A total dose of 45 Gy was delivered to the neck area. After irradiation all high and low risk patients were treated with 105 U/kg body weight recombinant IFN-β three times a week for 6 months.

**Results:** 59 patients were eligible for the analysis of the NPC-91-GPOH-study. Except for one stage II patient, all were American Joint Committee on Cancer (AJCC) stage III-IV at presentation. VCA-IgA was found in 90% our tested patients. After combined modality therapy, a 91, 37% complete clinical response rate was accomplished. 54 patients are still in first continuous remission with a median follow up of 48 months (range 10–108 months). In three patients metastases (Bone, bone and lung and lung) were observed 14, 15 and 18 months after diagnosis and two patients had local relapse of tumor, 9 and 11 months after diagnosis. Mucositis grade III or IV developed in all patients, in 2 (3.5 %) of the patients acute cardiotoxicity occurred.

**Conclusions:** Because of the rarity of this disease in children, only a multiinstitutional trial result in an improved treatment outcome. It is the greatest study of NPC in children and adolescents.The good results of preradiation chemotherapy, radiation therapy and IFN-ß encourage continuing the trial. The dose of radiotherapy will be reduced in patients with effective neoadjuvant chemotherapy. The new study NPC-2003-GOPH and the preliminary results will be presented.

**20.005**

## Epstein-Barr Virus BART Gene Products

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**Background:** The BART RNAs were proposed to express various proteins including RPMS1 and A73 but recently the BART microRNAs have been reported to be derived from the introns spliced out of the BART RNAs (Cai et al, PloS Pathog, 2006 2: e23).

**Methods:** We prepared monoclonal antibodies that react specifically with artificially expressed RPMS1 and A73. An A73 expresion vector was transfected into 293 cells to study effects on calcium flux. Southern blotting and methylation specific PCR were used to fine map the unmethylated region around the BART promoter.

**Results:** We have not yet reliably detected endogenous expression of RPMS1 or A73 protein in EBV infected cell lines but studies are continuing. However, we identified an effect of artificially expressed A73, which modulated calcium flux in some stably transfected cell lines.

In two NPC cell lines, the demethylated region near the BART promoter includes downstream sequences that we previously found to be required for promoter activity in transfection assays. Specific C residues between 138733 and 138894 in the EBV-wt map are unmethylated, consistent with a role in BART promoter function. Analysis of RNA from the C666.1 cell line shows that BART RNA is detected both in the nucleus and the cytoplasm. The RNAs detected on Northern blots have been related to cDNA structures of the BART RNAs using a series of Northern blot probes. In C666.1 cells the main EBV RNAs detected in Northern blots of cytoplasmic polyA+ RNA correspond to several genes normally expressed in the early lytic cycle.

**Conclusions:** DNA methylation analysis supports our previous data suggesting the BART promoter requires sequences both upstream and downstream of exon I. The effects of A73 on calcium flux in transfected cell lines and our previous observations on RPMS1 suggest that the proteins are genuine gene products but the proteins have still to be demonstrated reliably in EBV infected cells. Some C666.1 NPC cells appear to express a subset of early lytic cycle genes, which might contribute to the EBV lytic cycle antibody response that is characteristic of nasopharyngeal carcinoma.

**20.006**

**EBV Serology, not EBV-DNA Load, Predicting Distant Metastases in a Juvenile Caucasian NPC Patient: Response on EBV Lytic Cycle Induction Therapy**

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Case Report: A juvenile Caucasian female with EBV EBER-RISH positive NPC (T4N2M0). She showed characteristic IgA and IgG responses to EBNA1, VCA-p18 and Early Antigens (EA) at diagnosis, however without detectable EBV DNA in blood (LightCycler QT-PCR). Complete remission was achieved by combined chemo- and radiotherapy and additional Interferon-beta, accompanied by strongly declining IgG and IgA anti-EBV reactivities in subsequent months. Eighteen months later, without clinically apparent signs or symptoms of recurrent disease, IgA and IgG responses to VCA-p18 and EA increased again, but not to EBNA1. Five months after the first increases in EBV-serology, lung metastases were found during a routine check-up. This correlated with peak levels in anti-EBV IgA/IgG reactivity. No EBV-DNA was detected during follow-up. After surgical removal of lung metastases and gemcitabine/epirubicine treatment, intra-abdominal lymph node metastases and new lesions in the mediastinum appeared, again without detectable EBV-DNA in blood. Two courses of novel treatment were given, consisting of 5-fluorouracil (5-FU) plus the histone deacetylase inhibitir valproic acid, followed by valganciclovir. The rationale for this was to induce the viral lytic cycle and subsequent eradication of EBV-positive tumour cells by antiviral drugs. This treatment resulted in the first detectable EBV DNA levels in blood (8069 copies/ml), but was discontinued as disease progressed. We conclude that in this patient EBV serology was useful in predicting distant NPC metastasis after initial complete remission, in contrast to EBV DNA load. To our knowledge this is the first NPC patient treated with 5-FU/valproic acid/valganciclovir, which apparently induced EBV DNA replication.

**20.007**

**Sequence Variation and Functional Significance of the EBV-encoded Genes**
D.J. Li, S.J. Mai, **Y.X. Zeng**. Sun Yat-sen University Cancer Center, Guangzhou, China

We have previously cloned and sequenced the whole genome of an EBV strain GD1 isolated from a Cantonese nasopharyngeal carcinoma (NPC) patient from Guangdong Province, China, and found numerous sequence variations as compared with the prototype B95.8. To further study the functional significance of such sequence variations, we focused on several EBV-encoded genes with well-known important functions, including EBNA1 and BARF0. Our preliminary results suggest that the mutations within those genes are of important biological meaning and may indicate the existence of subtypes of EBV with stronger oncogenic potential toward NPC development. Based on our and other findings from many other labs, we would like to propose the establishment of world-wide network for collection and sequence determination of EBV isolates from blood cells and throat washings, with an aim to fully understand the distribution of the EBV subtypes geographically and ethnically, and the disease association as well.

**20.008**

**Antibodies to Gp350/220 Can Enhance Infection of a CR2-negative Epithelial Cell**
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Glycoprotein gp350/220 is the most abundant protein in the EBV envelope and has the largest apparent mass. It initially tethers virus to CR2 at a distance of several nanometers from the B cell surface. Cell-free virus can infect CR2-negative epithelial cells in culture by binding to a gHgL receptor, although infection is inefficient. Glycoprotein gp350/220 is not required. We recently found that monoclonal antibodies to gp350/220, which inhibit EBV infection of B cells, also enhance infection of epithelial cells as judged by expression of green fluorescent protein from a recombinant virus. The effect could not be duplicated by non-neutralizing antibodies to gB or gp78 or by antibody to gp42. $F(ab')_2$ fragments retained the ability to enhance infection, but Fab fragments were unable to enhance unless anti-mouse antibody was added to cross link them. Enhancement by intact antibody was increased by addition of soluble protein AG, which also increases cross-linking. The antibodies had no significant effect on virus binding and infection could not be blocked by pretreating cells with antibodies to alpha five beta one integrins. Saliva from EBV-seropositive individuals that could reduce infection of B cells also enhanced infection of epithelial cells and the effects could be partially reversed by depletion of antibody. We propose that cross-linking of gp350/220 with antibody patches the protein in the virus envelope and facilitates access of more important players to the epithelial cell surface. These players are not likely to include gHgL, which already have good access to the gHgL receptor, but may include gB, which increases the efficiency of epithelial cell infection when its expression is increased. The results may have implications for development of nasopharyngeal carcinoma in high risk populations in which elevated antibody titers to EBV lytic cycle proteins are prognostic.

**20.009**

**Induction of Twist by Latent Membrane Protein-1 Causes Epithelial-Mesenchymal Transition and is Associated with Metastasis of Nasopharyngeal Carcinoma**

**T. Horikawa**, J.S. Pagano. Lineberger Comprehensive Cancer Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

**Background:** Nasopharyngeal carcinoma (NPC) is highly invasive and metastatic. EBV-encoded LMP1 is a primary oncogene and is also associated with upregulation of invasion and metastasis factors both in cell culture models and in NPC. Recently, transcriptional factor Twist-a basic helix loop protein essential for mesoderm formation and neural crest migration-was shown to mediate EMT and induce cellular motility during tumor progression and play an essential role in metastasis in breast cancer. Epithelial-Mesenchymal Transition (EMT) is a vital process for morphogenesis during embryogenesis whereby epithelial cells lose cell-cell contacts, and is also implicated in oncogenic progression. Based on these facts, we focused on the association of Twist with LMP1 and its contribution to the highly metastatic character of NPC.

**Methods:** Expression of Twist and LMP1 in NPC tissues and in different EBV-positive and negative cell lines was analyzed by western and northern blot analyses. Wound migration and Matrigel invasion assays were performed to assess cell motility and invasiveness.

**Results:** In LMP1-positive NPC, Twist is frequently expressed, whereas LMP1-negative NPC rarely express Twist. Twist expression levels are increased in diverse EBV-infected cells that express LMP1, but not in parental EBV-negative cells. Transfection of LMP1-expressing plasmid into AdAH cells, an EBV-negative nasopharyngeal epithelial cell line, induced Twist protein and mRNA expression. Coexpression of IκB with LMP1 showed that NF-κB is essential for induction of Twist. Constructs expressing either LMP1 CTAR1 or CTAR2 induce Twist, but to a lesser extent than wild-type LMP1, consistent with the activation of NF-κB by both signaling domains. Interestingly, transfection of LMP1 in Madin-Darby canine kidney (MDCK) epithelial cells results in loss of E-cadherin-mediated cell-cell adhesion, activation of mesenchymal markers, induction of cell motility and increase in invasiveness, which are markers of EMT. There is corresponding up-regulation of Twist protein in LMP1-transformed MDCK cells. Further, expression of Twist siRNA reversed LMP1-induced EMT and suppressed cell motility.

**Conclusion:** There is a close association between expression of Twist and EBV LMP1. Induction of Twist by LMP1 appears to cause up-regulation of cell motility and EMT, which may in part contribute to the metastatic nature of NPC. Possible correlation of Twist levels and stage of NPC are under study.

**20.010**

## Expression and Regulation of Discoidin Domain Receptor Gene Family in Nasopharyngeal Carcinoma

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Nasopharyngeal carcinoma (NPC), an Epstein-Barr virus (EBV)-associated epithelial carcinoma, are poorly understood its molecular mechanisms in highly metastatic potential. We attempted, therefore, to explore the alterations of gene expression that responsible for promoting metastasis of NPC. By exploiting real-time quantitative PCR analysis, we targeted the detection of DDRs (discoidin domain receptor) tyrosine kinase family gene expression profiling in NPC and its related control tissues. Of note, the up-regulation of DDR2 was found unique to NPC, but almost undetectable in controlled nasal epithelium. Additionally, the expressions of DDR2 in NPC and NPC meta were significantly higher than that of other head and neck cancer tissues. By immunohistochemical staining, DDR2 was predominantly distributed in NPC tumor cells but less in infiltrating lymphocytes. On the other hand, the expression of DDR1 transcripts showed no statistical difference among all categories of tissues tested. The expressions of EB viral transcripts were determined in our study. The correlation between viral gene products and DDR2 expression would be discussed.

**20.011 (cancelled)**

## Functional Advantage of NPC-related V-val Subtype of Epstein-Barr Virus Nuclear Antigen 1 Compared with Prototype in Epithelial Cell Line

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**Background:** Epstein-Barr virus (EBV) is closed associated with nasopharyngeal carcinoma (NPC) and the viral nuclear antigen 1 (EBNA1) plays a crucial role in viral latency. Three EBNA1 subtypes, P-ala (prototype), V-thr and V-val can been detected from healthy carriers in Guangzhou area in Southern China. A close relation of V-val EBNA1 with NPC was suggested by its preference to infect NPC cells. We therefore investigate the functional difference among these 3 EBNA1 subtypes in human epithelial cell line.

**Methods:** The whole coding sequence of P-ala, V-thr and V-val EBNA1 subtypes were amplified and cloned into pGFP-C2 vector, and transfected into 293 cells respectively. Effects of EBNA1 expression on cell doubling time, cell cycle distribution, and cloning efficiency were examined using stably transfected cells. In order to evaluate the maintenance activity and expression level of EBNA1-plasmid in transient transfection assay and after 14-days selection of G418, the average percent of GFP-expressing cells and the average intensity of green fluorescence in GFP-expressing cells were examined by FCM and divided by the value at 18h post-transfection to normalize transfection efficiency.

**Results:** No significant difference on cell doubling time or cell cycle distribution was found among P-ala, V-thr and V-val EBNA1 transfected cells. The colony-formation efficiency of P-ala-EBNA1/293 (32.67%) was significant lower than V-thr (39%), V-val (41.67%) and vector (46%) transfected cells ($P<0.05$, t test) (fig), which indicate V-thr and V-val subtypes were less toxic than P-ala EBNA1 for 293 cells. In EBNA1-plasmids maintenance and expression assay, no difference was found among EBNA1 subtypes in transient transfection, while the ratio of average intensity of fluorescence after 14-days selection over that at 18h post-transfection was gradually higher from P-ala (0.48), V-thr (0.57) to V-val (0.62) EBNA1 transfected cells, and there is a significant difference between V-val and P-ala-EBNA1 ($P<0.05$, t test) (table).

**Conclusion:** The results show that V-val subtype of EBNA-1 has functional advantages over the prototype in cloning efficiency and plasmid expression. We suggest that V-val EBNA1 might contribute to the tumorigenesis of NPC by increasing the expression of itself or other viral or cellular genes.

**20.012**

## Increased Intraepithelial Regulatory T-cells in Undifferentiated Nasopharyngeal Carcinoma

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Undifferentiated nasopharyngeal carcinoma (NPC) is an epithelial tumor that is consistently associated with Epstein-Barr virus (EBV) latent infection. The tumor is characterized histopathologically by an abundant infiltration of T-lymphocytes. The presence of these tumor infiltrating lymphocytes is considered to be important in the development of NPC. Of these tumor-infitrating lymphocytes, the CD4+CD25+ regulatory T-cells (Treg) that functionally suppresses the immune response mediated by T-cells may induce antigen-specific and local immune tolerance at tumor site. Recent studies showed that Treg population was increased both in the circulation and tumor infiltrating lymphocytes of cancer patients, suggesting the accumulation of Tregs in tumor environment and suppression of tumor-specific T-cell responses. In this study, we evaluate the presence of intraepithelial Treg population on fifty primary NPCs and fifteen normal nasopharyngeal epithelial samples. By immunohistochemical analysis, T-cell markers including CD4, CD25, CD3, CD8, FOXP3, TIA-1 and Granzyme B were investigated. We detected abundant total T-cell (CD3+) in both tumor infiltrates and stromal cells adjacent to normal epithelia. The prevalence of resting cytotxic T-cells and NK cells (TIA-1+ cells) and activated cytotoxic T-cells (Granzyme B+ cells) showed no significant differences in normal and tumor tissues. However, CD25+ and FOXP3+ T-cells were significantly increased in NPC samples compared to normal tissues ($p<0.001$), indicating the expansion of Treg population in tumor microenvironment. The presence of Treg population in primary NPC tumors was also confirmed by multiparameter flow cytometric analysis. We found 30.96±7.18% of tumor infiltrating lymphocytes exhibited Treg cell immunophenotypes with CD4+CD25+FOXP3+ expression. The findings showed that CD4+CD25+ Tregs expressing FOXP3+ selectively accumulate in tumor tissues and may contribute to antitumor immune dysfunction during the progression of NPC. The prognostic value of intraepithelial Tregs in NPC patients is being investigated.

**20.013**

## Efficient EBV Infection of Epithelial Cells by Transfer from Resting B Cells: Monitoring Early Events Post-infection

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**Background:** Epstein-Barr virus (EBV) infects B cells efficiently through binding of the viral glycoprotein gp350 and the complement receptor CD21. EBV can also infect epithelial cells in vivo, but this has been very difficult to reproduce in vitro using cell-free virus preparations. We have shown that delivering the virus from the surface of resting B cells increases epithelial cell infection rates by $10^3$ to$10^4$-fold (Shannon-Lowe, PNAS, 2006); we now go on to look at events immediately post- infection.

**Methods:** Recombinant EBV strains (with a GFP cassette) bound to the surface of freshly-isolated resting B cells were briefly co-cultured with epithelial cells, the B cells were then washed off and infection of epithelial cells studied by (i) fluorescence in situ hybridisation (FISH) for EBV genome, (ii) FISH or Q-RT-PCR assays for viral transcripts, (iii) EBV antigen and GFP expression.

**Results:** We find by FISH that the EBV genomes rapidly transfer from the point of contact between B cell and epithelial surfaces into the epithelial cell nucleus within 1 hr. Many cells then go on initially to express EBER RNAs, showing that levels of infection are greater than apparent from the GFP marker. Some infected cells enter lytic cycle, with BZLF1 expression and occasional full replication, while other infected cells show transient EBNA1 expression form the BamHI Q promoter; both routes of infection are associated with LMP1 expression. In most cells the infection is not sustained and the genome is gradually lost with serial passage over time.

**Conclusions:** Transfer from the B cell surface represents an efficient means of infecting primary epithelial cultures and a range of epithelial cell lines in vitro, thereby providing an experimental system through which to study the viral and cellular factors governing the type of infection established.

**20.014**

## Consistent Overexpression of c-IAP2 Protects NPC Cells against an Unusual Form of Apoptosis

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**Background:** NPC oncogenesis involves both latent EBV-infection and specific alterations of cellular gene expression. In order to better understand the cellular part of NPC oncogenesis, gene expression profiling was performed on NPC xenografts. Our subsequent investigations were focused on genes consistently over-expressed in NPC specimens, especially c-IAP2 (chromosome 11q22.2).

**Methods:** Over-expression of the c-IAP2 gene was confirmed by quantitative PCR with comparison of malignant and non-malignant NPC cells. The c-IAP2 protein was detected by Western blotting and immunohistochemistry. Functional inactivation of c-IAP2 in NPC cells was performed using a small, cell permeable, IAP-inhibitory molecule mimicking the effect of the Smac/Diablo protein (smac mimic).

**Results:** The c-IAP2 gene was shown to be consistently over-expressed at both RNA and protein levels. Its overexpression was retained even when it was reduced to a single copy in a context of 11q22-23 loss of heterozygosity. This suggested that NPC cells were somehow addicted to its high expression. This dependence was confirmed using a Smac peptido-mimetic which, in our system, induced a selective decrease of c-IAP2 with no significant effect on X-IAP or survivin. At a concentration of 50 nm, this agent was sufficient to induce apoptosis of NPC cells in the absence of any other stimulus. This apoptosis was documented by typical morphological aspects on electron micrographies as well as an increase in the sub-G1 fraction on cell cycle analysis. Because caspases are the usual targets of IAP proteins, we expected to find biochemical evidence of caspase activation. This was not the case. A possible involvement of lysosomal proteins is suspected.

**Conclusion:** C-IAP2 is one more anti-apoptotic protein intensely expressed in NPC cells in addition to Bcl2, Bcl-XL and A20. Its inhibition induces an unusual process of apoptosis which is currently under investigation.

**20.015 (cancelled)**

## Detection of Aberrant Methylation in Patients of Nasopharyngeal Cancer by Multiplex Methylation-Specific PCR

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**Background:** A growing evidence demonstrating that aberrant methylation in gene promoter is an alternative way to activate oncogene and inactivate tumor suppressor gene in nasopharyngeal cancer (NPC). It led to a development of multiplex methylation-specific PCR (MMSP) which can be used as an early diagnosis tool. The aim of this study was to test the methylation status in brushing samples of Indonesian NPC patients.

**Methods:** Thirty-three matched NPC specimens deriving from paraffin-embedded tumor tissue and brushing samples of the same patients were taken from Dr. Sardjito Hospital, Yogyakarta, Indonesia. The sample DNA was modified by bisulfite treatment and amplified by methylation-specific PCR. Five target genes were involved in a simultaneous PCR reaction containing two EBV genes (EBNA-1 and LMP-1) and three tumor suppressor genes (DAP-kinase, RassF1A, and CHFR). The expression level and pattern from different markers was evaluated semi-quantitavely by quality control.

**Results:** EBNA-1 expression was detected in 96.9% of paraffin and 93.9% of brushing samples while LMP-1 expression in 57.6 and 48.5% respectively. The detected frequency of hypermethylation in DAP-kinase was 54.5% of both paraffin and brushing samples while in RassF1A was 78.8% of paraffin and 69.7% of brushing samples. We found that 90% (30) of paraffin and 84% (28) of brushing samples showed aberrant methylation in at least one of tumor suppressor genes.

**Conclusion:** A high frequency (93.3%) of aberrant methylation was detected in brushing samples of NPC compared to paraffin samples which was regarded as 100%. It indicated that MMSP has a value in NPC for early diagnosis.


# Abstracts

**Tuesday, July 11, 2006**
**Room: Grand Ballroom (Level 2)**

## Session 21: Signal Transduction
## 08:30-10:00

**21.001**

**LMP1-CTAR2 Determines a Unique Type of TRADD Signaling which is Required for IKKbeta Activation by LMP1**

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The TNF-receptor 1-associated death domain protein (TRADD) is the key player in apoptotic signal transduction of TNF-receptor 1. TRADD binding to TNF-receptor 1 (TNFR1) is mediated by a direct interaction of the death domains (DD) of both molecules. TRADD is also recruited by the CTAR2 domain of the Epstein-Barr virus oncogene latent membrane protein 1 (LMP1), albeit with unclear functions in LMP1 signaling. To characterize the role of TRADD in LMP1 signal transduction, we generated the first genetic TRADD knockout by sequentially deleting both TRADD alleles from human DG75 B-cells. In TRADD-/- B-cells, we found that LMP1-CTAR2 activation of IKKbeta is in fact TRADD-dependent, whereas JNK activation by LMP1 is TRADD-independent. Furthermore, TRADD is required for the recruitment of IKKbeta to LMP1. Although transmitting signals through TRADD, LMP1 rather induces cell transformation than apoptosis. Therefore, we asked whether the unique TRADD binding site of LMP1 encodes the non-apoptotic phenotype of LMP1-induced TRADD signaling. To address this question, we replaced the death domain of LMP1-TNFR1, a constitutively active fusion protein of the LMP1 transmembrane domain and the TNFR1 signaling domain, with the 17 C-terminal amino acids 370-386 of LMP1. The resulting chimera showed the unique LMP1-type interaction with TRADD, which was independent of an intact TRADD death domain. The TRADD binding site of LMP1 was fully functional in the TNFR1 context inducing LMP1-type TRAF6-dependent NF-kappaB signaling, but did not activate apoptosis. Our results show that TRADD is a critical mediator of LMP1-CTAR2 signaling to NF-kappaB. Moreover, amino acids 370-386 encompass the complete TRADD binding site of LMP1, which determines the unique and non-apoptotic type of TRADD signaling.

**21.002**

**LMP1 Signaling in IKKg Deficient Cells**

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The latent infection membrane protein (LMP-1) is expressed in B lymphocytes latently infected with EBV and is one of five latent genes shown to be essential for EBV-induced transformation of B lymphocytes, for LCL establishment and continued proliferation. LMP-1 functions as a constitutively activated TNF-Receptor. The C-terminal-activating regions (CTAR1/TES1 and CTAR2/TES2) of LMP-1 act as docking sites for complexes of signaling proteins (TRAFs, TRADD, RIP) that trigger activation of the transcription factor NF-kB.

LMP1 activates NF-kB by two pathways: canonical and noncanonical. CTAR2/TES2 activate an IKKb/IKKg dependent pathway, resulting in IkBa phosphorylation, ubiquitination, degradation, and translocation of p50/ Rel heterodimers to the nucleus.

CTAR1/TES1 activates a NIK/IKKa dependent pathway resulting in p100 phosphorylation, ubiquitination, cleavage, and translocation of p52/Rel heterodimers.

The experiments reported here were undertaken to investigate a suggested third NF-kB activating pathway. I-TAC gene expression is IKKb dependent but IKKg and IKKa independent. Because cells lacking IKKg/NEMO are unable to assemble the IKK complex, canonical signaling with IkBa proteolysis and p50/p65 translocation into the nucleus is presumed to be impossible. However, our lab showed p65 nuclear translocation in IKKg deficient LMP1 expressing MEFs. The data presented here verify this experiment and show p65 translocation into the nucleus of LMP1 expressing IKKg- Jurkat cells. Furthermore, EMSAs showed that LMP1 induced nuclear translocation of p50, p52, and p65 in IKKg- Jurkat cells. Further, we found that expression of F-dN-IkBa inhibits the NF-kB-activating effect of LMP1 in both IKKg- and WT Jurkat cells, which implies that for LMP1 mediated NF-kB activation in IKKg- Jurkat cells phosphorylation and degradation of IkBa is required. Moreover we show that WT LMP1 and LMP1, expressing CTAR2 only, activates NF-kB stronger in IKKg- than in WT Jurkat cells, consistent with the hypothesis that LMP1 can activate IKKb in the absence of IKKg.

**21.003**

**LMP Interacts with UBE1L (Ubiquitin Activating Enzyme E1-like Protein) to Mediate NF-kappaB Activating Signals**

**K.M. Izumi**. University of Texas Health Science Center, San Antonio, Texas, USA

Latent infection membrane protein 1 (LMP1) is hypothesized to usurp signaling adapters of tumor necrosis factor receptors (TNFR) to activate NF-kappaB and thus alter gene expression that enables EBV to transform B-lymphocytes into long-term proliferating lymphoblastoid cell lines (LCLs). A yeast two hybrid screen reveals residues 355-386, which comprise C-terminal activating region 2 and transformation effector site 2 (CTAR2/TES2), are able to retrieve UBE1L from an LCL cDNA library. LMP1 interaction with UBE1L is hypothesized to be important in transformation because UBE1L may capture and transfer ISG15, a ubiquitin-like peptide, onto an E2 (conjugating enzyme). TNF receptor associated factors (TRAFs) interact with CTAR2/TES2 through TRADD (TNFR associated death domain protein), and TRAFs are likely E3s (ligating enzyme) that transfer ubiquitin from an E2 such as Ubc13 onto I-kappaB that is phosphorylated by the I-kappaB kinase complex. Ubiquitination of I-kappaB and 26S proteasome mediated degradation are critical for activation of NF-kappaB. Yeast two-hybrid analysis that UBE1L interacts specifically with CTAR2/TES2, and co-immune precipitation analyses confirm interaction in mammalian 293T human embryonic kidney cells. Co-immune precipitation analyses further reveal endogenous UBE1L interacts with LMP1 from an LCL transformed by a recombinant EBV expressing Flag-tagged LMP1 gene product. In transiently transfected 293T cells, reporter assays reveal UBE1L has no endogenous activating abilities but when co-expressed with LMP1, UBE1L is able to synergize CTAR2/TES2 in activating an NF-kappaB responsive reporter. Reporter assay analyses reveal mutation of a critical UBE1L cysteine to alanine abrogates synergism with LMP1 in NF-kappaB activation. These results support the model that LMP1 CTAR2/TES2 recruits UBE1L to start an enzyme series that targets I-kappaBs for ubiquitin or ubiquitin-like modification. I-kappaB modification and destruction activates NF-kappaB, altering the expression of genes that are likely critical in B-cell transformation.

**21.004**

**LMP1 Transmembrane Intermolecular Interactions that Mediate Nf-kB Activation**

**V. Soni**[1], T. Yasui[2], E. Kieff[1]. [1]Brigham, Boston, MA, USA; [2]Osaka University, Osaka, Japan

These experiments investigate the role of the EBV LMP1 six Transmembrane domains (TM) in constitutive homotypic aggregation and c-terminal cytosolic domain (CT) mediated NF-kB activation. The role of intermolecular interactions in constitutive NF-kB activation was studied by cotransfecting two mutually exclusive and signaling incompetent LMP1 expression plasmids in HEK293. The data confirm that TM1-2 and TM1 F38WLY41 are critical for LMP1 intermolecular interactions and signaling. Further: (i) TM1-2 expressed from one vector induced TM3-6CT, expressed from a second vector, to activate NF-kB at 60% of WT LMP1 levels. (ii) TM1-2 induction of TM3-6CT mediated NF-kB activation was critically dependent on TM1 F38WLY41. (iii) TM1-2 also induced TM3-4 CT to mediate 41% of WT LMP1 NF-kB activation; this activation was independent of TM1 F38WLY41. (iv) TM1-2 and TM1-4 could also weakly induce TM5-6CT mediated NF-kB activation; TM1 F38WLY41 were required. (v) While TM5-6 Y159, W164W165, or W173 were individually unimportant for TM1-2 induction of TM3-6CT mediated NF-kB activation, TM3-6 W98 was important for TM1-2 induction of TM3-6CT mediated NF-kB activation and for TM1 F38WLY41 effect. (vi) TM3-6 W98 was also important for intermolecular interaction with TM1 F38WLY41. These data support a model in which TM1-2 has a key role in functional intermolecular interactions with TM1-2, TM3-4, or with TM5-6, that TM1 F38WLY41 is particularly important in intermolecular interactions involving TM3-6, and that TM3-6 W98 is important for TM1-2 effects through TM3-6.

**21.005**

**The EBV Encoded LMP-1 Protein Blocks Interferon-alpha Signaling in Human B Cells by Interacting with and Inhibiting Tyk2 Phosphorylation**

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**Background:** Epstein-Barr virus (EBV) is a tumor virus associated with a variety of cancers. Latent Membrane Protein-1 (LMP-1) is the EBV oncoprotein essential for B cell immortalization by EBV. Regulation of B cell signaling via the C-terminus of LMP-1 is required for immortalization of B cells by EBV. LMP-1 has a polytopic transmembrane domain which regulates cell signaling via an as yet undefined mechanism that does not require the LMP-1 C-terminus. We have developed an affinity purification and mass spectrometric approach to identify novel interactors associated with native LMP-1 signaling complexes from EBV-immortalized lymphoblastoid cells. The non-receptor tyrosine kinase Tyk2 surfaced as a potential LMP-1 interactor in our initial screen. Tyk2 is a member of the janus kinase family and functions as a signaling intermediate in the Type 1 Interferon (IFN) pathway. IFN alpha activates cell surface IFN receptors resulting in the activation of associated janus kinases and downstream STAT signaling. The end result of IFN alpha signaling is the establishment of an antiviral and antiproliferative state in the cell.

**Methods and Results:** LMP-1 was purified from EBV+ lymphoblastoid cells by immunoaffinity chromatography and purified complexes were analyzed by LC/MS/MS. Tyk2 was among the proteins identified by mass spec as a potential LMP-1 interactor. The physical and functional interaction between LMP-1 and Tyk2 was studied in EBV+ lymphoblastoid cells and human B lymphoma cell lines. The interaction between LMP-1 and Tyk2 was independent of the CTAR domains in the C-terminus of LMP-1. LMP-1 prevented tyrosine phosphorylation of Tyk2 and inhibited IFN alpha-stimulated STAT2 nuclear translocation and ISRE transcriptional activity. The effect of LMP-1 on IFN signaling was specific for Type 1 IFNs since LMP-1 had no effect on IFN gamma signaling. Long-term culture of EBV+ lymphoblastoid cells in IFN alpha was associated with outgrowth of a population expressing elevated LMP-1 protein levels, suggesting that cells expressing higher levels of LMP-1 survived the anti-proliferative selective pressure imposed by IFN alpha.

**Conclusions:** Together, these results show that LMP-1 can protect EBV+ cells from the IFN alpha-stimulated antiviral/antiproliferative response, and suggest that prolonged IFN-alpha treatment of EBV+ individuals has the potential to encourage the outgrowth of cells expressing elevated, and therefore potentially growth-transforming, levels of LMP-1.


# Abstracts

**Tuesday, July 11, 2006**
**Room: Grand Ballroom (Level 2)**

## Session 22: Immunology
## 14:00-15:30

**22.001 (cancelled)**

## The Intertwinement of Epstein-Barr Virus Infection and T Cell Receptor Predictability

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T cell lymphocytes remain the backbone of defence against intracellular pathogens. Recognition of infected cells relies almost exclusively on the T cell receptors (TcR) ability to sterically feel for a complimentary, 3-dimensional "match" in pathogen-derived peptides (p) presented by MHC molecules. Moreover, since our immune system cannot know in advance the exact sequence and shape of the pMHC 'puzzle' we will encounter, it covers all bases by producing millions of naïve cells (endowing each with a unique receptor 'solution'). Indeed, based on direct estimates, the T cell repertoire in any one human is simply epic (upwards if 2.5 x 10[7] in vivo from a theoretical 10[15-20] combinations) and, in light of this diversity, it was originally thought that it would be extremely improbable for two separate individuals to engage a common pathogen with T cells bearing the same receptor. However, recent evidence suggests this may not be the case and that shared T cells (or so called "public" T cells) may be much more prevalent then previously thought. In this study, we investigated whether public T cells were involved in Epstein-Barr virus (EBV) infection and, if so, to what degree. Using a large cohort of healthy, unrelated, EBV+ individuals, we sequenced >1500 TcRs specific for various highly immunogenic EBNA1 and BZLF1 peptides. Across all donors, we found multiple public T cell expansions with classic TcR fixed gene bias and bias at areas of known hyper-variability. Furthermore, we found public T cells were mobilised within hours of primary EBV infection (suggesting antigen-driven selection) and sustained, in large numbers, for at least 12 months post infection. Moreover, crystal structures of several public TcR/pMHC complexes revealed an extremely good atomic "fit" which provided a sound rationalization for their immunodominance in the T cell pool. Interestingly, many of the public receptors we encountered contained no random modifications but were comprised entirely of genomic material. This implies, remarkably, that we are all imprinted with the same tracks of DNA that encode a set of almost-perfect receptors for EBV defence. Indeed, it is certainly conceivable that such receptors are the genomic relics of a long-ago fought battle between EBV and humans. Darwinian selection forces may have driven their emergence in human populations since they would have conferred the bearers with an excellent pre-emptive defence net against an almost certain EBV encounter.

**22.002**

## Epstein-Barr Virus-associated Infectious Mononucleosis Leads to Long-term Global Deficit of IL-15ralpha Expression and IL-15 Responsiveness in the T Cell Pool

**D. Sauce**[1], M. Larsen[1], S.J. Curnow[2], A.M. Leese[1], P. Moss[1], A.D. Hislop[1], M. Salmon[2], A. Rickinson[1]. [1]Institute for Cancer Studies, University of Birmingham, Birmingham, United Kingdom; [2]MRC, Centre for immune regulation, Birmingham, United Kingdom

**Background:** In murine systems two cytokines, IL-7 and IL-15, are important in determining the overall size of T cell pools and in the selection and maintenance of memory populations. To address the situation in man, we have followed infectious mononucleosis (IM) patients from acute Epstein-Barr virus (EBV) infection into asymptomatic virus carriage by monitoring EBV-specific CD8+ T cell populations for IL-7 and IL-15 receptor alpha (IL-Ra) expression.

**Methods:** EBV-specific T cells were identified by staining with HLA/peptide teramers; cytokine receptor levels were analysed by antibody staining and cytokine responsiveness by cytokine-induced STAT5 phosphorylation and cell proliferation assays.

**Results:** All cells within the highly expanded, activated CD8+ T cell pool in acute IM blood, including EBV epitope-specific populations, were IL-7Ra-negative. After disease resolution, the bulk CD8+ T cell population soon recovered IL-7Ra expression. However EBV-specific memory cells recovered expression much more slowly and then only in a subset of cells, paralleling the situation seen in mouse models of persistent virus infection. Though mouse T cells do not lose IL-15Ra upon activation, the situation proved different in man. All CD8+T cells in acute IM blood were IL-15Ra-negative and, remarkably, remained so for as long as the post-IM patients were followed. More surprisingly, this IL-15Ra deficit affected not just EBV-specific CD8+ populations but the whole peripheral T and NK cell pool. Moreover, this global lack of receptor expression correlated with a marked defect in IL-15 responsiveness in vitro, assessed by STAT5 phosphorylation and proliferation assays. This absence of IL-15Ra was seen in all 22 IM patients studied up to 14 years post-IM, but was not seen in patients after cytomegalovirus-associated mononucleosis, nor in 25 healthy EBV carriers with no history of IM, nor in 30 EBV-naïve individuals.

**Conclusions:** We conclude that symptomatic primary EBV infection leaves a permanent scar on the immune system. Given the widespread and persistent nature of the IL-15Ra down-regulation on T and NK cells (and possibly on other cell types), we suggest that impairment of IL-15 signalling may have longer-term consequences for the health of post-IM patients.

**22.003**

## Increased Frequency and Broadened Specificity of Latent EBV Nuclear Antigen 1-Specific T Cells in Multiple Sclerosis

**J.D. Lunemann**[1], N. Edwards[2], P.A. Muraro[2], S. Hayashi[3], J.I. Cohen[3], R. Martin[2], C. Munz[1]. [1]Rockefeller University, New York, NY, USA; [2]NIH NINDS, Bethesda, MD, USA; [3]NIH NIAID, Bethesda, MD, USA

Epidemiological studies consistently demonstrate that patients with multiple sclerosis (MS) are almost universally infected with Epstein-Barr virus (EBV) and that the risk of developing MS increases with the level of EBV-specific antibody titers. The EBV-encoded nuclear antigen-1 (EBNA1) maintains the viral episome in replicating infected human B cells, and EBNA1-specific CD4+ T cells have been identified as crucial part of the EBV-specific immune control in healthy individuals. We studied 20 untreated EBV seropositive patients with MS and 20 healthy EBV carriers matched demographically and for the expression of MS-associated HLA-DR alleles for their immunological control of EBV latency at the level of EBNA1-specific T cells. Using 51 overlapping peptides covering the C-terminal of EBNA1 (aa 400-641), peptide-specific CD4+ memory T cells in patients with MS were found to be strikingly elevated in frequency, showed increased proliferative capacity, and an enhanced interferon-gamma production. In contrast to EBNA1, T cell responses to 3 other latent and 3 other lytic immunodominant EBV antigens and HCMV epitopes did not differ between patients and controls indicating a distinct role for EBNA1-specific T cell responses in MS. CD4+ T cells from healthy virus carriers preferentially recognized multiple epitopes within the center part of the C-terminal domain, whereas the stimulatory epitopes in MS patients covered the entire sequence of EBNA1 (aa 400-641). Quantification of EBV viral loads in PBMC by real-time PCR showed higher levels of EBV copy numbers in some patients with MS, although the overall difference in viral loads was not statistically significant compared to healthy virus carriers. We suggest that the accumulation of highly antigen-sensitive EBNA1-specific Th1 cells in MS is capable of sustaining autoimmunity by cross-recognition of autoantigens or by TCR-independent bystander mechanisms.

**22.004**

## The Switch from Latent to Productive Infection in EBV Infected B Cells Is Associated with Sensitization to NK Cell Killing

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**Background:** Following activation of EBV infected B cells from latent to productive (lytic) infection, there is a concomitant reduction in the level of cell surface MHC class I and an impaired antigen presenting function that appears to facilitate evasion from EBV-specific $CD8^+$ cytotoxic T cells. In other herpesviruses, most notably human cytomegalovirus, evasion of virus-specific $CD8^+$ effector responses via downregulation of surface MHC class I is supplemented with specific mechanisms for evading NK cells. This study addressed the question of whether EBV also has NK cell evasion strategies.

**Methods:** $EBV^+$ Akata cells showing a latency I pattern of gene expression were stably transfected with a pHEBO-based plasmid containing a reporter gene regulated by a promoter sequence derived from the 5' region of the BMRF1 EBV gene. The chimeric reporter gene encodes a transmembrane protein comprising the extracellular and transmembrane regions of rat CD2 fused to GFP in the cytosol. The derived cell line, called AKBM, expressed the CD2/GFP gene with kinetics of an early lytic cycle gene following ligation of the BCR (J.Immunol 2005, 174:6829). The AKBM model allowed the immunomagnetic isolation of viable lytic cycle populations that were used as targets in cytotoxicity assays with primary NK cells and two well-characterised NK cell lines.

**Results:** Whereas latent AKBM cells were resistant to killing by all NK populations studied, these effectors efficiently killed cells activated into lytic cycle. Susceptibility to NK lysis coincided with downregulation of HLA-A,-B and -C molecules that bind to the KIR family of inhibitory receptors on NK cells, and also with downregulation of HLA-E molecules binding the CD94/NKG2A inhibitory receptors. Conversely, ULBP-1, a ligand for the NK cell activating receptor, NKG2D, was elevated and susceptibility to NK cell lysis could be partially reversed by blocking ULBP-1 with specific antibodies.

**Conclusions:** These results highlight a fundamental difference between EBV and HCMV with regards to evasion of innate immunity.

**22.005**

## EBV and Old World Gamma-1 Herpesviruses Encode a Cd8 T Cell Immune Evasion Gene Expressed During Lytic Cycle Replication

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The CD8 T cell response to epitopes derived from EBV lytic cycle proteins is highly focussed on proteins expressed early in the viral replication cycle. These CD8 T cells differentially recognise cells replicating EBV depending on the temporal expression of the target protein. Thus, T cells specific for proteins expressed early in the replication cycle efficiently recognise their cognate epitopes while those specific for late expressed proteins recognise their targets poorly. Taken with the observation that cells replicating EBV have reduced TAP mediated transport of peptides into the endoplasmic reticulum, an explanation for this phenomena is that EBV encodes a CD8 immune evasion mechanism. To identify such a mechanism, genes unique to the Old World gamma-1 herpesviruses were cloned from EBV and expressed in antigen expressing cells to determine whether these EBV gene products could interfere with CD8 T cell recognition of target cells. Eight candidate genes were screened for immune evasion function with the protein encoded by BNLF2a, expressed early in viral replication, found to disrupt CD8 T cell mediated killing of target cells. Expression of BNLF2a prevented translocation of peptides into the endoplasmic reticulum and greatly reduced levels of surface class I MHC. Similarly, expression of the BNLF2a homologues found in Old World gamma-1 herpesviruses were also capable of causing down regulation of surface class I MHC. We propose that expression of BNLF2a by EBV during lytic cycle replication is one factor driving the focussing of CD8 response to epitopes derived from proteins expressed at the initiation of viral replication, that is, prior to BNLF2a function.

**22.006**

## Impaired T Cell Recognition during Productive EBV Infection: Involvement of ORFs BGLF5 and BNLF2a

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**Background:** The fact that replication of EBV takes place in the face of a fully functional immune system indicates that the virus has acquired effective strategies to elude the host immune response. After all, ongoing virus production takes place in immune-competent healthy individuals, as well as in mononucleosis patients demonstrating massive proliferations of functional EBV-specific T cells. Recent studies indicate that MHC class I molecules are downregulated at the surface of cells supporting EBV replication. Moreover, the efficiency of lytic cycle epitope presentation falls dramatically as the lytic cycle progresses. These observations are indicative of early events in the lytic cycle which compromise T cell recognition. This study aimed to identify the EBV gene products responsible for immune evasion during productive EBV infection.

**Methods:** Biochemical and functional studies on antigen presentation require pure populations of cells supporting the lytic cycle of EBV. We have developed a novel strategy to isolate such populations, based on the expression of a reporter gene under the control of an EBV early lytic-cycle promoter. This system allowed us, for the first time, to evaluate the integrity of MHC class I-restricted antigen processing and presentation in the course of lytic EBV infection.

**Results:** Within hours after induction of the lytic phase, a marked downregulation of MHC class I surface expression was observed. Pulse-chase experiments revealed a complete shutoff of cellular protein synthesis. This phenomenon relies on accelerated global mRNA turnover mediated by EBV ORF BGLF5, the viral DNase of which homologs are present in all herpesviruses. In addition, the import of antigenic peptides into the ER by the Transporter associated with Antigen Processing (TAP) appeared to be blocked soon after initiation of the lytic cycle. At that stage, steady-state levels of TAP proteins were unaffected. The inhibition of TAP was caused by the BNLF2a gene product, a non-essential protein to which no function had been attributed before. The BNLF2a protein blocks the interaction of peptides and of ATP with TAP, thereby preventing the translocation of peptides into the ER.

**Conclusion:** In this study we have identified two EBV early lytic cycle proteins—encoded by the ORFs BGLF5 and BNLF2a—which compromise the presentation of EBV-derived antigenic peptides to MHC class I-restricted T cells. The combined effects of these gene products may explain the decreased efficiency of presentation of EBV lytic cycle antigens in vivo, reflected by the hierarchy of immunodominance displayed in the context of primary EBV infection.

## Notes

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