FUNCTIONAL ANALYSIS AND CHARACTERIZATION OF EPSTEIN BARR VIRUS LATENT MEMBRANE PROTEIN 2B

by

Monica Jo Tomaszewski-Flick

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This dissertation was presented

by

Monica Jo Tomaszewski-Flick

It was defended on

May, 12, 2008

and approved by

Velpandi Ayyavoo, Assistant Professor, Infectious Diseases and Microbiology

Graduate School of Public Health, University of Pittsburgh

Paul Kinchington, Associate Professor, Ophthalmology & Molecular Genetics and

Biochemistry, School of Medicine, University of Pittsburgh

Simon Watkins, Professor, Cell Biology; Physiology; Immunology

School of Medicine, University of Pittsburgh

Dissertation Advisor: David T. Rowe, Associate Professor, Infectious Diseases and

Microbiology, Graduate School of Public Health, University of Pittsburgh

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Monica Tomaszewski-Flick, PhD

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Epstein Barr virus persists in the human host by establishing a latent infection following primary infection. The virus periodically reactivates; producing virus that can infect new cells or be shed in saliva to infect new hosts. EBV is also implicated in malignant B cell proliferation in the immunocompromised and a variety of haemopoetic cancers, indicating that it is of public health significance.

The LMP2 gene of EBV encodes 2 protein isoforms: a 497aa proein (LMP2a) and a 378aa protein(LMP2b). These isoforms are identical, with the exception of an N-terminal cytoplasmic signaling domain of 119aa encoded in the LMP2a exon 1. The remaining residues (including the entirety of LMP2b) encode an integral membrane protein consisting of 12 transmembrane spanning regions with short alternating intracellular and extracellular connection loops.

Most research on the LMP2 isoforms has focused on the LMP2a protein and it's role in blocking B-cell receptor mediated signaling, degradation of associated proteins, and transformation. LMP2b, lacking the obvious signaling domain, has been largely ignored. Recently studies have suggested that LMP2b is a negative regulator of LMP2a.

In the following studies, we have evaluated the contribution of LMP2b to the block in BCR signaling using LMP2b expressing BJAB cell line. Our results demonstrate that LMP2b has the ability to singularly block BCR signaling.

LMP2 proteins have been described at both the plasma membrane as well as in the intracellular membranes of cells. Our studies indicate that the intact 12-TM region of the LMP2 proteins is necessary for intracellular localization. Through progressive deletions of 2TM segments from both the N- and C-terminal ends of the protein, we find that an intact 12-TM domain is necessary for localization, and there are at least 2 domains required for multimerization.

The role of LMP2 in immortalization is also contested, with groups reporting that LMP2a is both necessary and dispensable for immortalization. We utilized an established system of recombinant EBV construction to demonstrate that LMP2a, but not LMP2b plays a role in establishment and maintenance of viral latency.

Taken together, these results indicate a function for LMP2b in signaling and immortalization separate from LMP2a.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

There are many people that need to be thanked for their help in the generation of this work. They have given me scientific, social, and moral support. They have, on occasion, talked me out of very silly things.

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Lastly, I would like to thank my advisor, Dr. David Rowe: He is patience personified. He is amazing at surprising times.

1.0 EPSTEIN-BARR VIRUS:

1.1.1 Classification:

Epstein-Barr virus (EBV) is a human herpesvirus (HHV-4), which is the prototype of the gammaherpesviruses[1]. The gammaherpesviruses are subdivided into the gamma 1, *Lymphocryptovirus*, and gamma 2, *Rhadinovirus*. EBV is the only human *Lymphocryptovirus*. Lymphocryptoviruses are very similar to each other in structure and gene organization. Herpesviruses share genes for nucleotide metabolism, genes that encode for proteins that replicate viral DNA, mediate cell-virus fusion, and structural components for packaging of viral DNA.

EBV was initially described by the team of Epstein, Achong and Barr in 1964[2]. They were able to identify the characteristic herpesvirus virion in electron micrographs of tumor cells derived from Burkitt's lymphoma growing in culture. The virus was found to be different from other herpesviruses by being unable to replicate in other cultured cells and being unable to be identified using antibodies against known herpesviruses. Epidemiologic studies established a link between EBV and Burkitt's lymphoma. Also in the 1960's, EBV was found to efficiently immortalize primary B cell cultures into cell lines that could grow indefinitely[3]. These characteristics presented EBV as the first candidate human tumor virus.

1.1.2 Virus and Genome Structure:

EBV has a characteristic herpesvirus virion structure, ranging in size from 200-300 nm. The nucleocapsid is constructed of 162 capsomeres. This is surrounded by the protein tegument and an outer envelope with external glycoprotein spikes. Some of these glycoproteins are homologous to HSV (gH, gL, gB gM and gN). The most abundant of these glycoproteins are p350/220 and gp110[4].

The EBV genome is a linear, double stranded DNA that is approximately180 kb long. (Figure 1) The genomic structure has several defining features:

- Tandem, repeated, 0.5 kbp of the same sequence at both termini
- Internal direct repeats
- Short and long largely unique sequences

Duplicate regions near the end of the unique long (U_L) region consist of multiple highly conserved GC rich tandem 125bp repeats and 2kbp of adjacent unique DNA. Since the number of nucleotide pairs in this repeat section is not divisible by 3, different reading frames are generated.

The terminal repeats are duplicated during viral DNA replication and may vary in number. The number of terminal repeats is inversely proportional to the mRNA transcribed from exons bridging these repeats (e.g. LMP2).

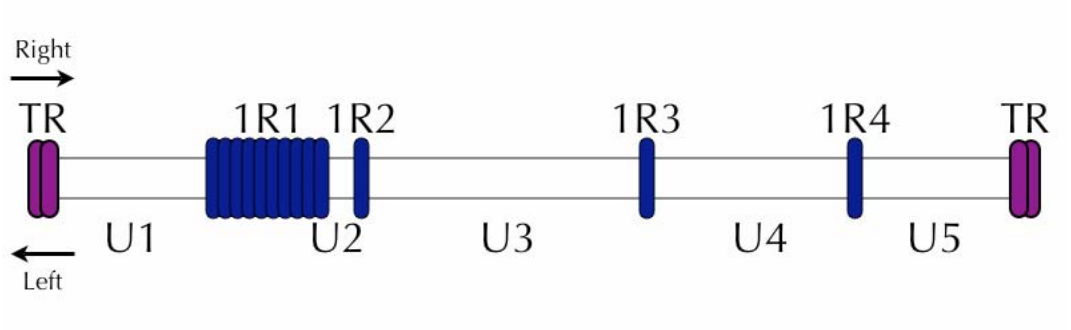

Figure 1: Linear Organization of the EBV genome.

The organization of the EBV genome is divided into 5 unique sections, (U1-5), 4 internal repeat sections, (IR 1-4), and 2 terminal repeats, (TR).

1.1.3 Virus Replication Cycle:

The cell surface protein CD21 on B-cells mediates adsorption, with gp350/220 being the requisite ligand on the viral envelope. The virus can also infect epithelial cells, although the cell surface receptor for this tropism has not been identified.

When the CD21 is engaged by the viral glycoproteins, an associated protein CD19 may have an affect mediating the activation of B cells via tyrosine kinases. Infected cells make viral mRNA, enlarge and express activation and adhesion molecules. The membrane of the cell and viral envelope fuse passing the viral capsids into the cytoplasm of the cell[5].

The viral DNA is then released and transferred to the nucleus, where circularization of the genome occurs and transcription from the Wp promoter is initiated[6]. EBNA-2 and EBNA-LP are produced to move the cell into the G1 phase of the cell cycle[7]. The infected cells can now proliferate, depending on a high density of cell and autocrine production of cytokines that mediate B-cell growth. As the cells

mature, the dependence on these autocrine factors becomes reduced. In this latent state, the viral episome is faithfully replicated once a cell cycle[8]. The replicated episomes are tethered to host chromosomes via EBNA1 and are partitioned to daughter cells.

Occasionally, these infected cells are induced into lytic replication. This begins with the expression of the immediate early genes, BRLF1 and BZLF1. These proteins transactivate viral and cellular promoters that lead to a cascade of viral gene expression[9, 10]. Early genes that are transcribed are involved in DNA replication and metabolism, while the late genes are involved with structural proteins.

1.2 EBV INFECTION AND CLINICAL MANIFESTATIONS:

1.2.1 Primary Infection:

Transmission of EBV requires intimate contact with the saliva of an infected person[11]. Secretions from an infected person shedding virus is transferred directly to the mucosal surfaces of an uninfected individual. At this point the virus can either infect the epithelial cells in the oropharnyx crypts or a naïve circulating B cell. Once infection occurs, the virus produces proteins, maintains a latent state, and growth transformation takes place. Outgrowth of resting B-cells is controlled by EBV specific cytotoxic T cells. Periodically, cells undergo lytic replication, and can either infect nearby cells, or be shed in the saliva. (Figure 2)

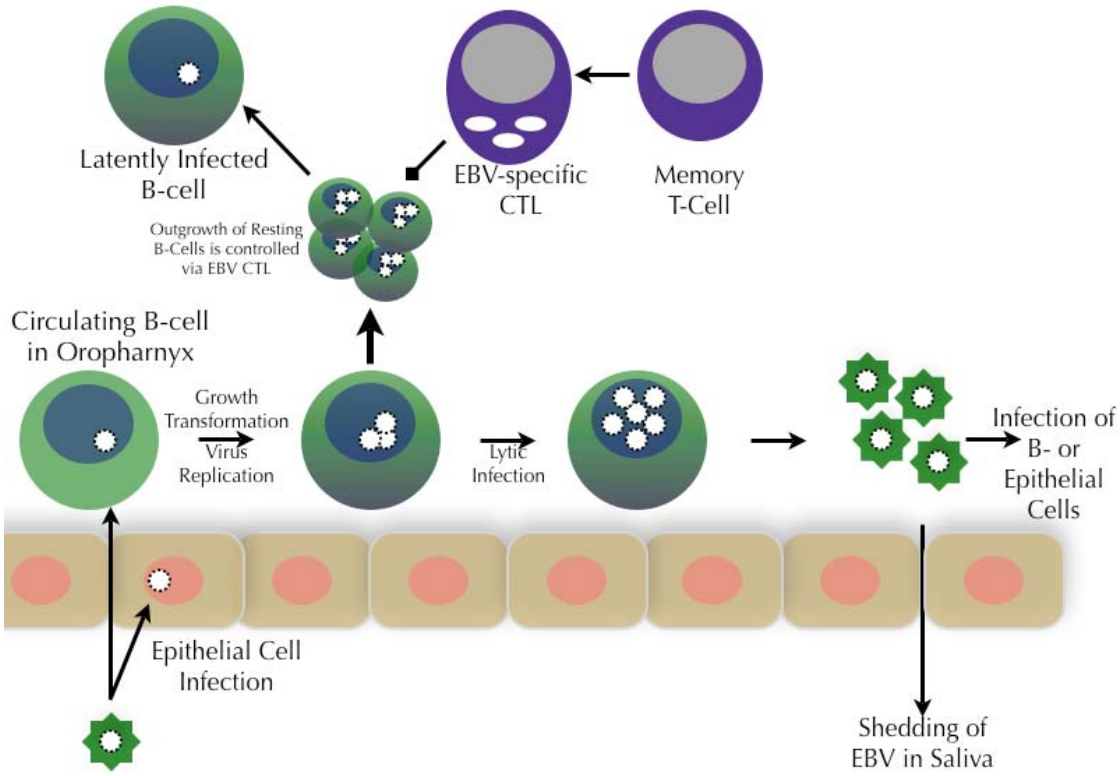

Figure 2: Pathway of EBV infection.

EBV infects 2 cellular compartments: 1) B cells, where the infection is usually latent, however can be induced to lytic infection, and 2) Epithelial cells, where the infection is predominantly lytic. The outgrowth of infected resting B-cells is controlled by EBV-specific CTL response.

Primary infection can be asymptomatic or symptomatic, determined by age and immune status. Infants become susceptible to infection when the maternal antibody protection wanes. Symptoms of primary infection worsen with increased age.

After clinical infection there is an incubation period of 30-50 days. In symptomatic primary infection sore throat, fatigue, pharyngitis, tonsillitis, and cervical lymphadenopathy are the most commonly presented symptoms.

If primary infection is postponed until adolescence, it is associated with infectious mononucleosis. In most individuals, the infection is self-limiting within 4-6 weeks, and no treatment is required. Acyclovir can decrease the load of the replicating virus, but has no effect on the course of the disease [12, 13]. Symptoms are caused by pro-inflammatory cytokines (IL-1, IFN-γ and TNF-α) that are produced by the reactive T-cells seen during IM [14]. It is unknown why primary infection causes symptomatic disease during adolescence. One theory suggests that adolescences and adults are exposed to a much higher dose of virus, thus creating more reactive T-cells.

1.2.2 Malignancies in the Immunocompetent Host:

Generally, the bulk of the EBV+ individuals are asymptomatic carriers. Several disease states exist in the immunocompetent host. These diseases most often have a genetic or environmental component that perturbs that natural course of the virus, allowing the oncogenic potential of the virus to come to fruition. (Figure 3)

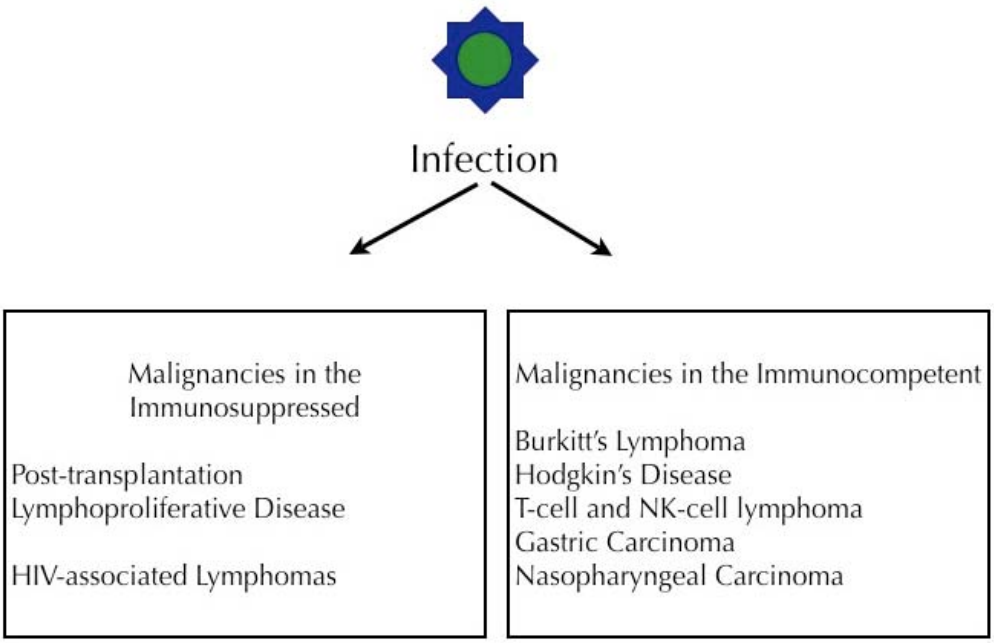

Figure 3: Malignancies associated with EBV

EBV is associated with malignancies in 2 groups of people: the immunosupressed (e.g. transplant patients and HIV positive patients) and the immunocompetent (e.g. normal population).

1.2.2.1 Burkitt's Lymphoma:

Burkitt's Lymphoma is an aggressive B-cell lymphoma found in malaria endemic regions in Sub-Saharan Africa (referred to as endemic BL). BL is the most common malignant disease in this geographic region[15]. Children under the age of 10 are the most affected[16, 17]. The lymphoma presents as extranodal tumors with a rapid growth rate, often occurring in the jaw or abdomen.

The malaria parasite contributes to BL development via production of B-cell mitogens, as well as suppression of T-cell immunity[18, 19].

Tumors have a homogeneous population of malignant cells, with occasional interceding macrophages. The cells are of B cell origin, similar to germinal center B cells, with expression of pan B-cell markers CD19 and CD20. Phenotypically, the BL cells are consistent in expression, consistently showing CD10+, CD77+, but are negative for activation markers, CD23, CD30, CD39, and CD70[20]. The cells are either low or absent for CD54, CD58, and CD80. These cells also display chromosomal translocations involving c-myc, Ig-heavy chain, and Ig-light chain[21]. Occasionally, cells also lose expression of one of the p53 alleles.

The role EBV plays in BL pathogenesis is poorly understood. It is possible that the growth transformation of B-cells amplifies the numbers of potentially malignant cells. That contribute to the clonality of the disease. EBNA1 is the only consistently detected protein in these tumors, with EBNA-LP and the EBNA-3 family of proteins being occasionally detected. EBNA1 promotes cell survival in these cells. The EBERs also enhance the apoptosis resistance and tumorgenic potential of infected cells.

EBV+ BL cells are able to escape the immune system. BL cells lack the costimulatory molecules involved in T-cell killing. BL cells are inefficient in presentation of antigens, as well as EBNA1 not being able to be presented to CD8+ CTLs.

1.2.2.2 Nasopharengeal Carcinoma:

Nasopharengeal Carcinoma (NPC) is a carcinoma of the nasopharyngeal epithelium that is seen most often in Southeast Asian populations. NPC is most often seen in middle-aged males. Tumors have mixed cellular composition, with a mix of neoplastic undifferentiated squamous cell and non-neoplastic lymphoid cells. The tumors are highly invasive, with a high rate of metastasis.

Most investigation has been done on tumor biopsies due to the inability to establish cell lines from the tumors. The NPC cellular phenotype bears a resemblance to epithelial cells that have been treated with IFN-γ and TNF-α. Cells express both class-I and -II antigens, and are efficient at processing these antigens. NPC cells express cell surface molecules involved in epithelial:lymphoid cellular interactions including: CD40, CD54, CD70, CD80, CD86 and CD95[20]. The tumor cells have also show deletions in 3p, 9p, 11q, 13q and 14q chromosomes.

Latent EBV infection is present in the neoplastic cells of NPC tumors[22]. EBV gene profiling shows that NPC cells express EBNA1, LMP1 (in ~50% of cases), LMP2a, EBERs and, the BamH1 A fragment rightward RNA transcripts (BARTs)[23-26]. The virus can be detected in pre-cancerous tissues, and this suggests that the virus has some role in the initiation of NPC[22, 27, 28]. The latent membrane proteins are implicated in disease progression. LMP1 acts as a CD40 mimic, and in epithelial cells will up-regulate genes involved with cell proliferation and motility[29, 30]. LMP2a activates the *wnt* pathway, consequently increasing the cellular growth and transformation[31, 32].

1.2.2.3 Hodgkin's Lymphoma:

Hodgkin's Lymphoma (HL) was first described by Thomas Hodgkin in 1832[33]. HL is one of the most curable cancers, with a cure rate of 93%[34].

Symptoms include the enlargement of lymph nodes, spleen or other lymph organs. HL is divided into 2 subclasses: classical and nodular lymphocyte predominate. 95% of patients have classical HL. This histology is characterized by the presence of a few rare malignant cell types among benign reactive cell types[34, 35]. The low

prevalence malignant cells are classified as either Hodgkin's (mononuclear) or Reed-Sternberg (multinuclear) cell types[36]. H-RS cells do not express conventional surface receptors, but are often positive for activation markers (CD25, CD30 and CD70)[37]. PCR analysis of RS cells indicated that classical HL is derived from a single B cell that has a vastly disrupted Ig locus.

Approximately 50% of HL is positive for EBV genomic sequences[38]. Epidemiological studies have also shown a link between primary EBV infection and HL[39]. HL shows an EBV latency profile of latency II, indicating expression of the EBERs, BamH1A transcripts, EBNA1 mRNA, and the transcripts of all the latent membrane proteins[40].

The expression of the latent membrane proteins has been given a key role in the pathogenesis of HL[41]. The LMPs may induce the proliferation of post-germinal center reaction B cells through the induction of survival signals. The survival signals will allow for the outgrowth of a population of cells for tumorgenesis.

1.2.2.4 T/NK Cell Lymphomas:

EBV appears to be linked to certain groups of T-cell or NK cell lymphomas. While, EBV does not normally infect this subset of cells, it can somehow gain access to this cell type. EBV infection of T-cells carries a high risk of lymphomagenesis. This could be due to the ability of the latently expressed proteins, LMP1, LMP2 and EBNA1 being active in T cells.

Virus-associated Hemophagocytic Syndrome T-cell lymphomas are tumors consisting of mature CD4+ or CD8+ T cells. The tumors are monoclonal, and display a Latency II pattern[42].

Nasal NK/T –cell Lymphomas are tumors that lead to bone erosion, most often in the nasal cavity. Tumors can either have a CD3+, CD56-/+ with TCR rearrangement or CD3-, CD56+ without TCR rearrangement, indicating that the malignant cells are derived from T or NK cells respectively. The expression of EBV gene transcription shows a type I/II latency pattern[43].

1.2.3 Malignancies in the Immunocompromised Host:

Malignancies in the immunocompromised are attributed to the inability of the host's immune system to clear EBV. These individuals lack the ability to clear the disease due to intentional (in transplant patients), genetic, or virus-associated immune suppression.

1.2.3.1 Post-transplantation Lymphoproliferative Disease:

Post-transplantation lymphoproliferative disease (PTLD) is the failure of the body to adequately keep EBV from uncontrollably proliferating because of immune suppressive therapies administered after transplantation. The proliferating EBV+ B-cells are not kept in check via CTL response and have the possibility to evolve to a malignant state. The risk of developing a PTLD malignancy is dependent on the type of the transplant, the age of the transplant recipient, the type of immunosuppression, and the EBV status of the recipient.

EBV has a near 100% association with PTLDs in the first year post-transplant[44], whereas EBV- PTLDs occur late (greater than 5 years post-transplant) and have an unknown cause[45]. EBV is thought to cause PTLD in a similar mechanism as Hodgkin's Lymphoma, where EBV rescues crippled germinal center reaction B-cells

by providing survival signals mediated by the latent proteins. Additionally, the effects of post-transplantation immunosuppression decrease the amount of immune surveillance and amplify the role EBV plays in lymphomagenesis.

1.2.3.2 HIV-associated lymphoma:

Lymphoma is the second leading malignancy occurring in those HIV positive patients with AIDS. EBV-mediated lymphomas in the HIV positive population are a group of disease states rather than one malignancy with one defined etiology. Two broad classes of malignancy are described: ones that could occur in the HIV negative population (e.g. BL) and lymphomas that occur almost exclusively in the HIV positive populations (e.g. Primary effusion Lymphomas). Because of the heterogeneity of the disorders, the pattern of EBV gene expression is variable, but in general holds similarities in the subcategories: where the pattern of gene expression in BL is comparable irrespective of HIV state.

1.3 EPSTEIN-BARR VIRUS: CELL INTERACTIONS:

1.3.1 B-cells:

EBV preferentially infects human B-cells. To better understand the mechanisms by which EBV manipulates cellular processes, the following section will detail B cell development and signaling.

1.3.1.1 B-cell development:

B-cell development begins in the fetal liver and continues in the bone marrow for life[46]. B-cells are generated from haemopoetic stem cells (HSCs) and develop in the bone marrow before migration into the blood and periphiral lymphoid organs[47]. A subset of HSCs, termed lymphoid progenitor cells receives signals from the bone marrow stromal cells to begin B-cell development.

Stromal cells express the chemokine CXCL12 that attracts B cell progenitors[48, 49]. These progenitor cells, called pre-pro-B-cells are negative for surface Ig expression, but do express B-cell lineage marker B220[50, 51]. This lineage marker, while common to B cells, does not indicated that these cells will exclusively become B cells.

The pre-pro-B-cells are dependant on numerous factors expressed by the bone marrow stromal cells that induce further differentiation and growth. Of these factors, FLT3L is necessary for the generation of pre-pro-B-cells and lymphoid progenitor cells[52, 53]. IL-7 is necessary for the generation of pro-B-cells and pre-B-cells[54, 55]. SCF is necessary in adults after the cells have reached the pro-B-cell stage. RANKL is involved in the generation of pre-B cells and immature B-cells[56].

Cells that have received the necessary signals and have become mitotically active are now termed pro-B-cells. Pro-B-cells initiate the rearrangement of the variable (V), diversity (D) and joining (J) regions of the B-cell receptor heavy chain locus to express the mu chain on the cell surface. Early pro-B cells perform the D-J rearrangements on both cellular chromosomes prior to initiation of the V-DJ rearrangement[57]. Once a successful mu chain is expressed at the cell surface, the

rearrangement stops[58]. If the rearrangement of the genetic material is unsuccessful, and a mu chain cannot be expressed, the pro-B cell usually dies. Cells that have a mu chain with a stabilizing surrogate light chain at the cell surface, are now termed pre-B-cells[59].

Pre-B-cells are the fraction of B-cells that are undergoing light chain rearrangement. These cells express enzymes that manipulate the expression of the kappa and lambda chains of the B-cell receptor locus. If the cell is able to produce a functional IgM molecule at the cell surface, the genetic manipulations stop[60]. Cells with a mature IgM molecule are immature B-cells and now leave the bone marrow.

Once immature B-cells leave the bone marrow, they can have several fates. If the surface IgM molecule encounters multivalent antigen, the B-cell will either undergo apoptosis or receptor editing[61]. If the surface IgM molecule binds inefficient antigen or binds antigen without the requisite T-cell help, the cell will become anergic[62]. The cells will also undergo apoptosis if they don't enter lymphoid follicles, as they have a half-life of around 3 days[63]. However, if the cells reach the follicles, they are now termed mature naïve B-cells.

Naïve B-cells are cells that have not yet encountered antigen. These cells are trolling for antigen, which is necessary for the next step in development. Naïve B-cells first encounter antigen in the T-cell rich areas of secondary lymphoid organs. They are activated by antigen-specific T-cells via recognition of the appropriate MHCII:peptide complex on the B-cell surface. The T-cells then produce CD40 ligand and IL-4. The B-cells now begin to proliferate. One fate of these cells is to produce a germinal center[64]. The germinal center is an area of high B-cell cell concentration where

proliferation takes place amidst a small percentage of T-cells that provide necessary help[58] .

The rapidly dividing B-cells that make up the germinal center, or centroblasts, undergo somatic hypermutation in which the variable portions of the immunoglobulin molecules are mutated. These mutations are in the variable region of the molecule and are responsible for determining the effectiveness of binding[65]. If the mutations increase the affinity of the antibody for antigen, the cells are induced to proliferate, thus out-competing the inefficient B-cells[64, 66]. Cytokines produced by the T-cells induce class switching in which the constant portion of the immunoglobulin changes, producing different isotypes. These changes are made in the DNA sequences in which the intervening sequence that codes for IgM are deleted and IgA/E/G can be made. Cells that have survived the germinal center will either differentiate into first into plasmablasts then plasma cells or into memory B-cells[67, 68].

Some cells differentiate into plasmablast. Plasmablasts have migratory potential, can further divide and further differentiate to plasma cells. In the process of becoming a plasma cell, the B-cell enlarges and increases the size of the organelles (Golgi apparatus and endoplasmic reticulum) that produce and transport the antibodies[69]. Plasma cells are terminally differentiated and migrate to the bone marrow, where they provide a source of long lasting antibodies[70].

Memory B-cells are long-lived products of cells that have already encountered antigen. These cells provide a rapid recall response to infection by quickly dividing and differentiating to plasma cells, while also re-seeding the memory B-cell pool.

The signals that indicate what cells mature into either memory cells or plasma cells are not yet clearly identified. It has been reported the CD40 signaling in the germinal center favors the production of memory-B cells, while blimp1 and XBP-1 mediates plasma cell differentiation[69, 71-76].

EBV is able to provide survival signals that mimic a germinal center reaction. EBV infects naïve B-cells. After infection, the virus produces 2 membrane proteins that mimic B-cell signals One of these proteins, LMP1, mimics a constitutively active CD40 receptor, which allows for survival signals in the absence of T-cell produced CD40 ligand[77]. The other protein, LMP2a, acts as a constitutively active B-cell receptor, which mimics the signal provided by the B-cell receptor undergoing antigen engagement[77].

1.3.1.2 B-cell survival signaling:

LMP1 and LMP2a provide signals in lieu of a germinal center reaction. These proteins are mimics of the BCR and CD40 signaling cascades.

The B-cell receptor (BCR) is a multimeric complex that includes a surface bound immunoglobulin that non-covalently associates with two other proteins, Ig-alpha and Ig-beta, which are necessary for expression and function of the B-cell receptor. Ig-alpha and Ig-beta have intracellular immunotyrosine activation motifs (ITAMs) that are the initiating sequences for the signaling capabilities of the B-cell receptor[78].

After antigen engagement by the immunoglobulin portion of the BCR, the BCR moves into ordered cholesterol rich domains of the membrane termed lipid rafts (Figure 4). These rafts are home to the membrane associated protein tyrosine kinases (PTKs) lyn. Lyn is responsible for the phosphorylation of the tyrosines in the ITAMs of Ig-alpha

and Ig-beta. Once these ITAMs have been phosphorylated, cytosolic PTK syk is recruited to the complex to allow for activation of downstream effectors[79]. Syk activates shc, vav and btk[80]. The shc arm of the signaling cascade initiates downstream intermediate signaling proteins, including Ras, that ultimately leads to the activation of MAP kinases including MEK1/2 and Erk1/2, which, in turn, stimulate transcriptional activators Egr-1 and Elk-1. The vav arm of the cascade activates jnk and p38, which in turn activate transcription factors CREB, ATF-2, and c-fos. The btk arm of the cascade, which stimulates PLCγ and PKC, ultimately activates NF-kB.

The larger BCR complex contains other cell surface molecules that associate with the BCR: CD19, CD81, CD9, CD21[81].

CD19 provides critical help to the signaling cascade. CD19 (and CD21) decreases the threshold for antigen dependant B-cell stimulation[82]. CD19 is phosphorylated by lyn on its ITAMs after BCR ligation (Figure 4). This activation recruits PI3K, which, in turn activates Akt. Akt activation ultimately stimulates proteins GSK-3b (for proliferation), mTor (for cell growth) and transcription factor NF-kB (for survival)[83, 84]. CD19 non-covalently associates with cell CD81 and CD9 [85]

CD9 and CD81 are members of a family of molecular organizers called tetraspanins[86]. CD81 is inducibly palmitoylated post-BCR crosslinking[87]. CD81 engagement can also activate the JNK pathway[88]. CD81 also regulates CD19 cell surface expression[89]. The role of CD9 in B-cell pathways is less well understood, and has yet to be identified[90].

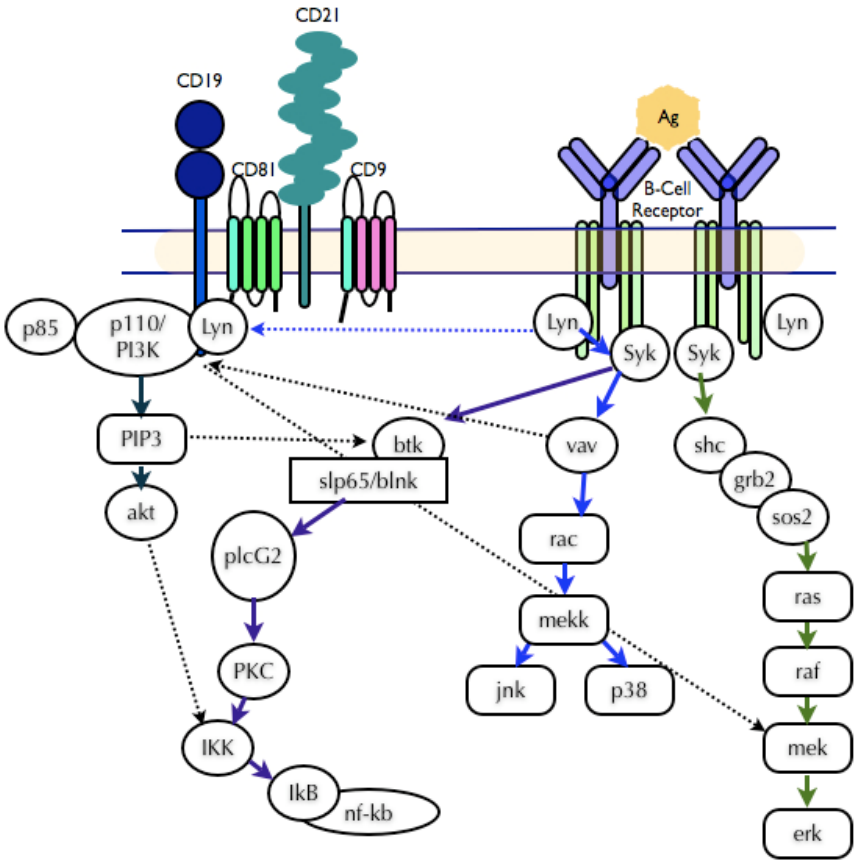

Figure 4: B-cell Receptor Signaling Pathways

Binding of antigen to the BCR (or BCR complex) promotes the activation of multiple protein tyrosine kinases (PTK) that alter the tyrosine phosphorylation in the resting B cell. Src family PTKs are activated initially and serve to phosphorylate CD79a and CD79b on ITAMs that recruit downstream signaling proteins. CD19 can also be phosphorylated on cytoplasmic ITAMs, and functions to lower the threshold needed to activate the BCR induced signal. Syk promotes phosphorylation of PLCg, Shc and Vav. Tec family member Btk is recruited to the plasma membrane where it is involved in activation of PLCg.

The endpoint of secondarily phosphorylated protein production is the regulation of several transcription factors that mediate gene transcription in the B cell.

CD40 is a member of the TNF receptor superfamily. When monomeric CD40 molecules interact with trimeric CD40 ligand, the CD40 molecules aggregate permitting association with TRAFs (Tumor necrosis factor receptor associated proteins)[91]. TRAFs 2, 3 and 5 interact with a PxQxT motif in the cytoplasmic tail of CD40, while TRAF6 interacts with a juxta-membraneous region of the protein (Figure 5)[92, 93].

TRAF2 is implicated in NF-kB and Jnk/p38 pathways. TRAF3 negatively regulates downstream molecules of TRAF2. TRAF2 and TRAF6 also may initiate the ERK, MAP kinase and PI3-K pathways, although the mechanisms of these interactions have not been dissected[94, 95]. CD40 also activates the STAT family of transcription factors, via association with Jak3[96].

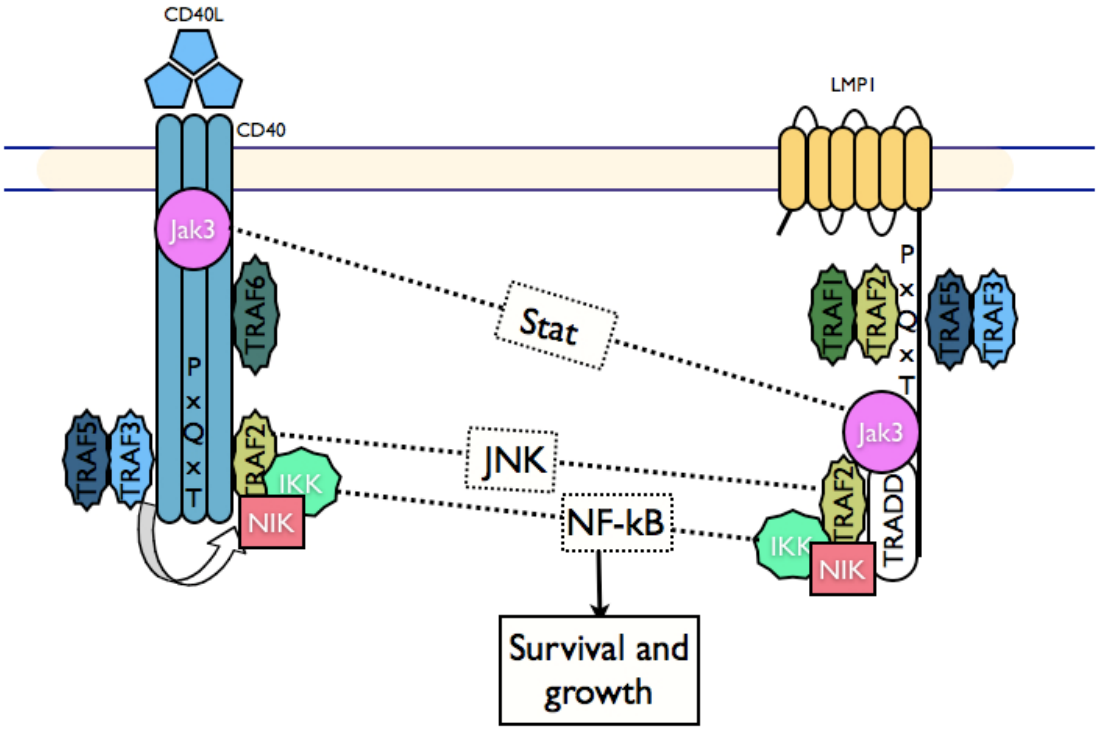

Figure 5: CD40 and LMP1 signaling.

CD40 depends on interactions with TRAFs proteins to initiate a signal in response to CD40L (CD154) binding. TRAFs bind to a region in the C-terminal cytoplasmic domain of CD40 via association with a PxQxT motif (for TFAFs 2, 3, and 5) or a proximal membrane cysteine (TRAF 6). The downstream pathway of TRAFs activates the transcription factor NF-kB through a kinase pathway involving map kinases, NIK (NF-kB inducing kinase) and I-kappa B kinases. Endpoint CD40 responses include regulation of immunoglobulin expression mediated by NF-kB transcription, and IL-6 production. EBV LMP1 also contains binding sites for TRAFs 1,2,3 and 5, and can activate the same downstream proteins as CD40.

LMP1 and LMP2a act as constitutively active mimics of CD40 and the BCR respectively. LMP1 interacts with TRAFs though regions in its C-terminal activation

regions (CTARs), initiating the same PI3K and JNK pathways as native CD40 (Figure 5)[97]. LMP2a has an N-terminal cytoplasmic signaling domain that contains an ITAM sequence that is similar to Ig-alpha and Ig-beta. On LMP2a, the ITAM is permanently phosphorylated, and consistently trigger the akt, btk and vav arms of BCR signaling[98]. The presence of LMP2a in cells, however, disallows signals originating from the BCR by blocking the movement of the BCR into lipid rafts[99, 100].

1.3.2 Latency:

The mechanism by which EBV infects primary B cells, but only makes the proteins required for the maintenance of unproductive infection is termed latency. Infection of primary B cells *in vitro* results in 10% of the cells becoming latently infected and proliferating as immortalized or transformed latently infected lymphoblastoid cell lines (LCLs).

The EBV genome encodes more than 80 gene products and of these, only 8 are expressed in transformed cells. Because of this it is thought that the presence of these proteins are critical in establishing and maintaining latency. (Figure 6)

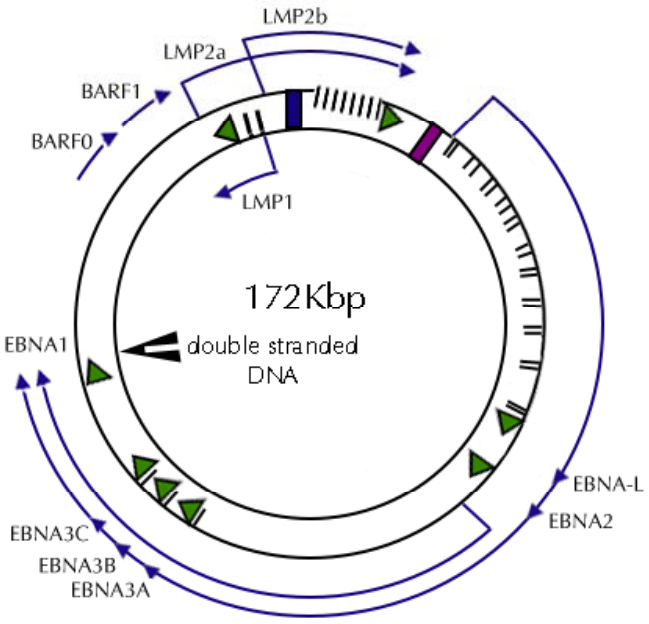

Figure 6: EBV Latent gene expression

Pictoral representation of the EBV genome. The double stranded episomal form of EBV is formed by the fusion of the terminal repeats (blue). The origin of plasmid replication is shown in purple. Black hash marks followed by green directional triangles represent the coding exons for each of the latent proteins. The arrows correspond to the direction of transcription of the latent proteins, 2 membrane proteins (LMP1 and LMP2a/b), and 6 nuclear antigens (EBNA). 2 transcripts that do not code for proteins are also produced, BamH1 A Right Fragment (BARF) 0 and 1.

Latent viral infection can be readily detected using antibodies against 8 latent proteins. Six of these proteins are nuclear (EBNA: Epstein Barr Nuclear Antigens) and 2 are membrane proteins (LMP: Latent Membrane Proteins).

1.3.3 Transformation/Immortalization:

Latently infected B-cells can become transformed. Transformation is the process by which cells are induced to be perpetually proliferating. EBV transforms cell growth via expression of viral proteins that modulate cellular gene expression at transcription or

by usurping signaling pathways. Cells that have been transformed are referred to as immortalized. Of the all the possible proteins expressed in latency, only 5 of them are required for transformation.

Different cell and tissue types associated with EBV have differentially expressed proteins[101, 102]. The protein expression, B-cell differentiation state, and disease associated with these phenotypes are used to stratify the type of latency into different programs. (Table1)

The latency-associated proteins have different mechanisms by which they maintain the viral episome or mediate survival signals. A short description of the proteins, how they work in maintaining latency, and whether or not they are necessary for immortalization is detailed in the following sections.

Table 1:Latency Programs of EBV in Human B cells

Latency	Expressed Genes	B-cell Differentiation	Disease State
0	LMP2	Memory B cell	Healthy Carrier
1	EBNA1	Germinal Center B cell	Burkitt's Lymphoma
2	EBNA1, LMP1, LMP2	Germinal Center B cell	Hodgkin's Disease
3	EBNA1-3a,-b,-c, EBNA-LP, LMP1, LMP2a/b	Naïve and Germinal Center B cell	Immunoblastic-Lymphoma

1.3.3.1 EBNA1:

EBNA-1 binds 4 recognition sites in the origin of latent DNA replication of EBV (OriP). The binding activates latent phase replication, which happens at a rate of one

round of replication per cell cycle. EBNA1 is not necessary for immortalization, but its presence helps in this process[103].

EBNA-1 is a 641 aa protein of 76 Kda. Discrete regions of this protein have been described indicating motifs for action. AA40-89 and 329-370 are the linking regions (1 and 2, respectively) that link 2 DNA elements of the viral genome that are A/T rich[104-107]. The C-terminal portion of linking region 1 is necessary for EBNA1 to initiate transcription from the family of repeats (FR)[104]. The C-terminal portion of linking region 1 is also necessary for transformation, and allows for a change in promoter usage (from Wp to Cp) in the first 5 days post-infection.

Amino acids (AA) 91-328 contains Glycine-Glycine-Alanine repeats that are used to stabilize the mature EBNA-1 protein from degradation from the proteosome. This mechanism of immune evasion allows for this protein that is expressed in all forms of latency to not produce epitopes for presentation to T-cells. However, presented epitopes are able to be isolated from EBV+ donors[108, 109]

Amino acids 378-386 are the nuclear localization sequence[110]. Nuclear localization is regulated by the phosphorylation of serine at residue 385[111]

Amino acids 458-607 encode the overlapping binding and dimerization domains [112]. AA 458-477 are responsible for the sequence specific DNA binding. EBNA-1 binds DNA as a dimer and interacts with multiple 18 bp palindromic recognition sites within 2 elements of OriP, the family of repeats (FR) and dyad symmetry (DS). FR is composed of 21 imperfect copies of a 30 bp sequence that contains 20 EBNA-1 binding sites[113]. The FR-EBNA1 complex serves as a transcriptional enhancer of promoters and also contributes to the nuclear retention and maintenance of FR-containing

plasmids. The DS region is sufficient for initiation of DNA synthesis in the presence of EBNA-1, with termination though to occur at FR where the FR-EBNA-1 complex would create a replication fork barrier[114].

EBNA-1 lacks enzymatic qualities. Cellular components necessary for replication must be recruited to the EBNA-1-DNA complex[115]. In it's latent state, EBV does not express any proteins with replication qualities.

1.3.3.2 EBNA-2:

EBNA-2 is one of the first proteins (along with EBNA-LP) to be expressed after infection. Their expression induces cell cycle transition from G0 to G1[116].

EBNA-2 does not bind DNA directly, but transactivates through interaction with cellular repressors. EBNA-2 is a transactivator that activates all of the latent viral genes in immortalized B cells via C-promoter, and is required for the initiation and maintenance of immortalization. In addition to viral genes, EBNA-2 also is able to transactivate CD21, CD23, c-fgr, c-myc, BATF, EB11/BLR2 and represses the immunoglobulin heavy chain locus[117, 118].

P3HR1 is a cell line that contains a viral genome that lacks the open reading frame of EBNA-2 and parts of EBNA-LP; this virus is unable to immortalize cells[119].

EBNA-2 mimics the intracellular portion of the Notch receptor by localizing to the nucleus, binding to the repression domain of RBP-J and relieving repression. Notch can also temporarily substitute for EBNA-2[118]. In it's native state RBP-J recruits a HDAC complex to a promoter to repress the transcription. Once either EBNA-2 or Notch IC is bound, repression is relieved, the HDAC dissociates, and RBP-J is converted to a

transcriptional activator. EBNA-2 recruits transcription factors TF11B, TF11H, TAF40, p100, P300/CBP via its acidic domain[120].

In addition to RBP-J, EBNA-2 is also able to interact with PU.1, a sequence specific DNA binding protein that binds in the promoter of LMP1[121]. The specific interaction of PU.1 and EBNA-2 is likely responsible for the expression of LMP1 in epithelial cells, thus impacting immortalization. Hyperphosphorylation of EBNA-2 during mitosis suppresses expression of LMP1.

1.3.3.3 EBNA-LP:

EBV-encoded leader protein (EBNA-LP) is a co-activator of EBNA-2 mediated transcription. EBNA-LP is created by an alternative splice product of the same pre-mRNA as EBNA-2.[122] EBNA-LP is comprised of the W1W2 multiple repeat domains which encode 22- and 44-aa repeats and the Y1 and Y2 exons which encoded 11- and 34-aa unique amino acids.

EBNA-LP and EBNA-2 are cooperative proteins. EBNA-LP stimulates EBNA-2 mediated transcription, cellular localization, phosphorylation and protein complex formation. EBNA-LP associates with many proteins: pRB, P53, Hsp70, HS1-associated X1, α- and β-tubulins, Hsp27, HA95, protein kinase A, estrogen related receptor 1 and bcl-2[123-129]. EBNA-LP localizes to ND10/PML bodies in the nucleus where the associations between EBNA-LP and cellular nuclear proteins take place[130].

EBNA-LP is a highly phosphorylated protein in which serine phosphorylation on amino acids 14 and 35 are critical for co-activation of EBNA2. A conserved region, $R_{23}RVRRR_{28}$ is necessary for LP homotypic interaction and co-activation[131]. A C-terminal 45-aa region is also responsible for co-activation and EBNA-2 association.

The interaction of EBNA-2 and EBNA-LP are responsible for transactivation of viral promoters immediately after infection, and are the first proteins detected[132]. Virus deficient in EBNA-LP is severely impaired in transformation, however it does not appear to be necessary for maintenance of transformation.

1.3.3.4 EBNA-3A, -3B &-3C:

The EBNA3 family of proteins are hydrophilic, proline rich, charged nuclear proteins. Up-regulation of transcripts from the Cp promoter by EBNA-2 and –LP leads to EBNA–3A, -3B, -3C and –1 expression. Expression of the EBNA-3's are detected at very low levels in the latently infected cell[133]. The EBNA-3's compete for binding with EBNA-2 and Notch and effectively are the regulators of their own gene expression.

Studies have indicated that EBNA-3B is not necessary for the establishment or maintenance of latency, cell survival, or lytic replication[134, 135]. Viruses lacking EBNA-3A were shown to be severely hindered in LCL survival, while, EBNA–3C is necessary for both initiation and maintenance of transformation[136, 137].

All of the EBNA-3's are able to bind and associate with RBP-Jk[138]. Because of this association, cellular expression of several genes is regulated. EBNA-3B is responsible for the up-regulation of vimentin and CD40, but the down-regulation of CD77[139]. EBNA-3C will up regulate CD21 and LMP1 and may modulate transcription of cellular genes involved in migration and invasion[140].

1.3.3.5 LMP1:

Latent membrane protein 1 (LMP1) is an integral membrane protein composed of a short cytoplasmic N-terminal domain (24aa), 6 hydrophobic transmembrane domains

(162aa), and a cytoplasmic C-terminal domain (200aa). LMP1 is a constitutively active aggregation-induced receptor that mimics the TNF family receptor CD40 (Figure 5)[141-145].

The C-terminal cytoplasmic domain of LMP1 contains 3 C-terminal NF-kB activation regions (CTAR) that are responsible for the signaling properties of LMP1. CTAR 1 interacts with TRAF trimers and initiates Nf-kB signaling analogous to CD40. CTAR 2 initiates TNFR signaling pathways via constitutive association with TRADD and RIP proteins[146]. These domains also activate JNK and p38 kinases. CTAR3 binds Janus kinase 3. Of these three signaling domains, only CTAR1 and CTAR2 are necessary for transformation[147].

1.3.3.6 LMP2:

LMP2 was initially described in 1989 as 2 mRNAs: a 2.3 and 2.0 kb message [148]. The structure of the cDNA clone revealed that the transcript was generated once the genome circularized and was across the terminal repeats of the genome [149]. The 2 mRNAs are produced by alternate promoter usage; they consist of different 5' exons and share 8 common exons. The proteins share 12 identical hydrophobic domains of at least 17 amino acids (aa) and a short 27 aa C-terminus [150, 151]

The longer of the two, LMP2a, has a 119 N-terminal cytoplasmic domain. LMP2b, whose exon 1 is non-coding, starts at the first in-frame methionine, which precedes the first hydrophobic domain [150]. These proteins are predicted to have 12 transmembrane segments connected by short loops. Both termini are cytoplasmic. Both LMP2 proteins localize to the intracellular membranes of the trans-golgi-network. The existence and

spatial features of these proteins are conserved in the rhesus lymphocryptovirus homologues [152].

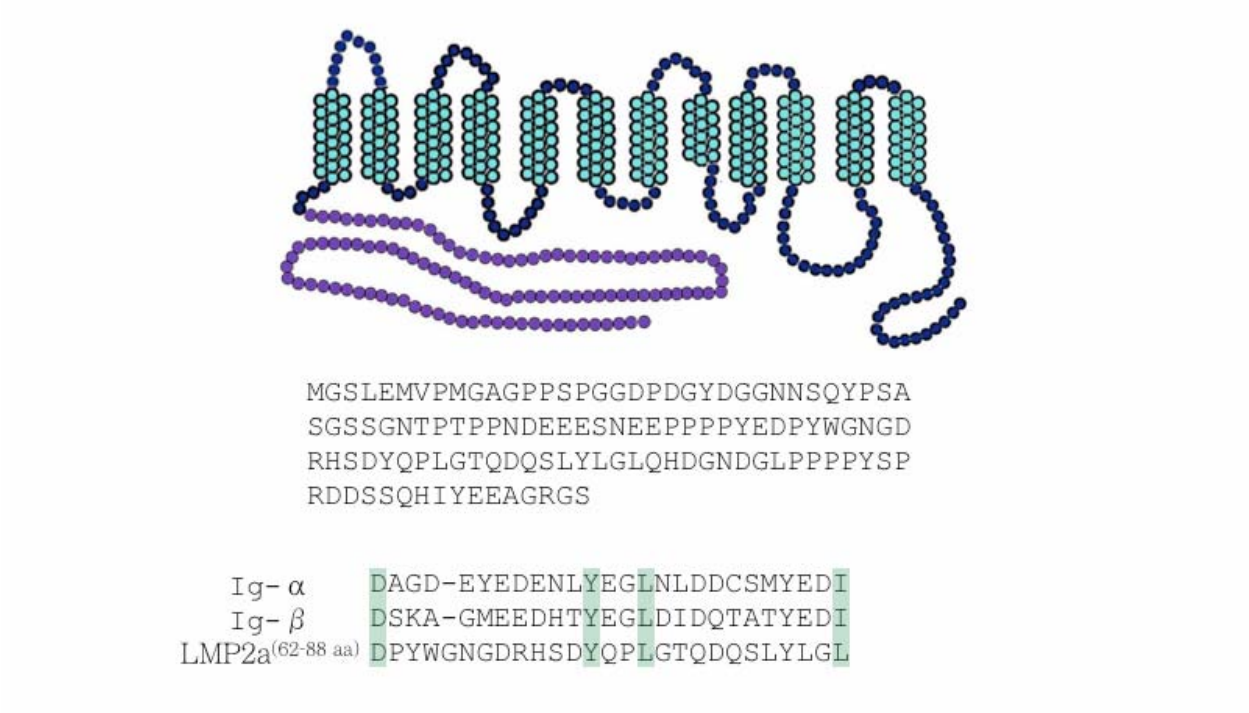

Figure 7: LMP2a Topological arrangement and structure of exon 1.

LMP2a is a 12 TM protein with a 119 aa N-terminal cytoplasmic tail. This cytoplasmic tail (purple) has sequence similarity to BCR associated alpha and beta chains (bottom). These sequence similarities resemble immunotyrosine activation motifs (ITAMs) (highlighted in green).

The cytoplasmic signaling domain (CSD) of LMP2a is largely responsible for abrogation of signal from the BCR. Tyrosines in the CSD form an ITAM (Figure 7). In LMP2a+ cells BCR activation fails to activate lyn, syk, PI3-kinase, PLCγ2, Vav, MAPK [153], and excludes the BCR from entering lipid rafts [99] (Figure 8). Once tyrosines on the LMP2a CSD are phosphorylated [153], they can interact with the same signaling molecules that the BCR does. Indeed, in transgenic mice LMP2a can act as a constitutively active proxy BCR [154, 155]. Interestingly, downstream events of the

BCR, such as calcium mobilization, can induce lytic infection in EBV positive cells [156-159] indicating that the ability of LMP2a to blocking signaling from the BCR is necessary for maintenance of latency.

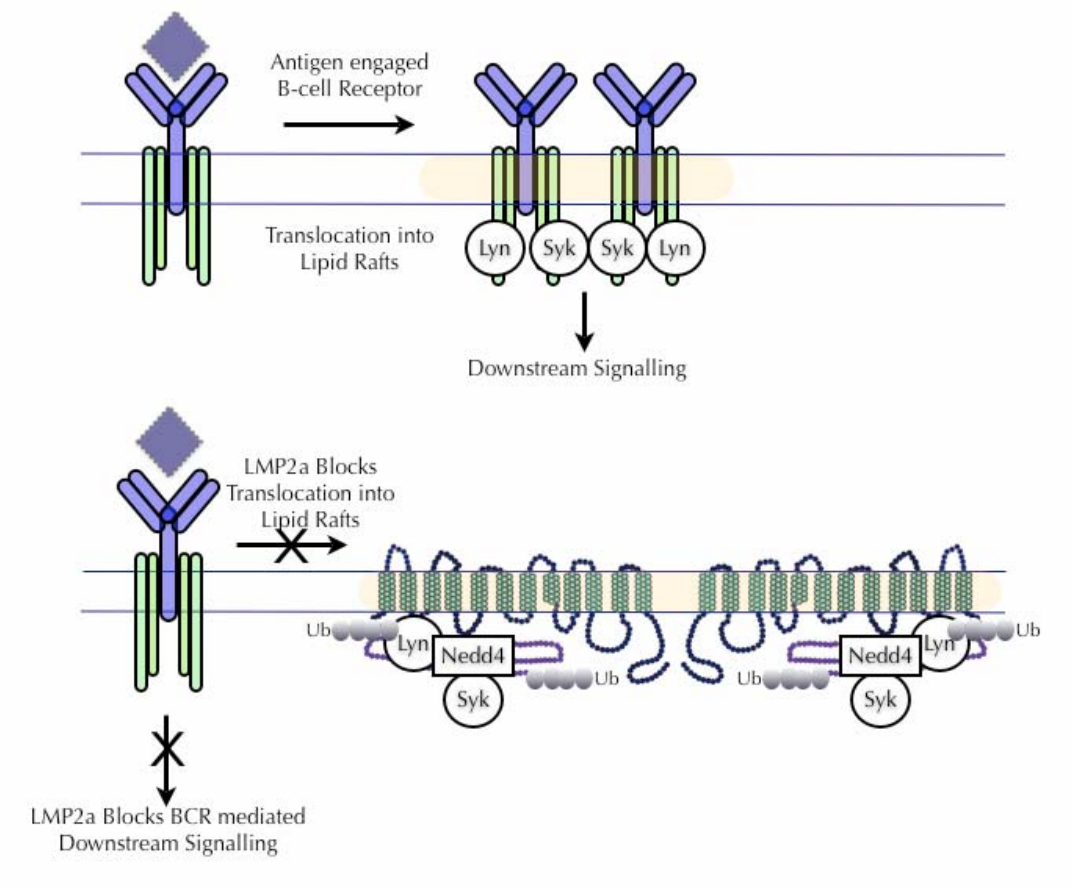

Figure 8: LMP2a blocks BCR translocation and signals in its stead.

Once the BCR binds antigen, it moves into lipid rafts on the cell surface and initiates a signal cascade. (Top) In LMP2a expressing cells, the presence of LMP2a prevents the BCR from moving into lipid rafts, thus blocking signals from being generated from the BCR. LMP2a, however, is able to signal providing required signals to the cell. (Bottom)

Few functional details have been ascribed to LMP2b. Due to the sequence homology; LMP2b has been hypothesized to be a negative regulator to LMP2a. Only recently, has any information supporting this been shown. [160]. LMP2b, but not LMP2a, was shown to influence motility in epithelial cells [161].

LMP2a has both been described at being necessary and dispensable for transformation. The contribution of LMP2b to transformation is addressed in the following work.

1.3.4 Mechanisms of Immune Evasion:

EBV persists for life in all infected hosts. This persistence happens because of the ability to establish an antigenically silent form of latent infection, and impairment of the antigen processing of cells during lytic replication. T-cell responses are detected for many EBV encoded proteins, however the virus cannot be eliminated via the immune system. Varied and duplicated systems of immune evasion mechanisms are required to maintain EBV infection.

EBNA1 is produced in all forms of latency, and is the optimal protein present to produce T-cell epitopes. The Glycine/Arginine repeats in EBNA1 stabilize the protein and disallow its degradation via the proteasome and consequent presentation to CD8+ T cells[162].

The BZLF2 gene product, gp42, associates intracellularly with HLA class II, and disrupts HLA:peptide complexes via steric hinderence[163].

EBV, like other gammaherpesviruses, encodes a viral IL-10. This viral homologue is able to augment B-cell growth and differentiation, negatively regulate inflammation, and inhibit antigen presentation.

EBV is able to shutoff host protein synthesis during the lytic replication cycle. The expression of the BGLF5 gene is able to induce shutoff of host gene expression via

enhanced mRNA turnover.[164] Targets of this turnover include HLA class I and II, indicating that this early lytic gene is partially responsible for T-cell evasion.

Another mechanism of immune evasion is blocking induction of apoptosis. Several EBV proteins are considered anti-apoptotic via mechanisms of up-regulating cellular proteins involved with preventing programmed cell death, or through checkpoint signaling. These proteins include latent proteins, LMP1 and LMP2, which provide CD40 and BCR, signals, respectively, that allow cells to undergo a mock germinal center reaction.

2.0 STATEMENT OF THE PROBLEM:

The following research is focused on the functional interactions of the Epstein-Barr virus latent membrane proteins 2a and 2b. These protein isoforms have recently been able to be detected independently, which have enabled, interactions between the isoforms and other proteins to be studied. Previous work in the Rowe laboratory has identified these proteins as trafficking to the same cellular localization. This finding, along with the conservation of the non-signaling isoform (LMP2b) in other gamma-herpesviruses, suggests a function in its existence. Understanding the protein domains responsible for aggregation and localization will help to determine a function for LMP2b as well as its role in regulating LMP2a function. Additionally, the function of LMP2 in immortalization of B cells will be addressed while looking at each isoform independently in an EBV background.

With the importance of function of LMP2a in BCR signal blocking, an equally important role for LMP2b has failed to emerge. Most studies focus on LMP2a alone, due to ability to produce an antibody against the CSD[165]. LMP2b has only recently been visualized in epithelial and lymphoblastoid cell lines, both EBV- and EBV+[166, 167]. New data has also emerged reassessing the localization of LMP2 gene products moving the localization from the plasma membrane to an intracellular Golgi-like location[166, 167]. This new localization of LMP2a and LMP2b to a perinuclear compartment puts into question previous descriptive data[165, 168]. Experimental data of LMP2b

suggests that there is a specific interaction with truncated LMP2 molecules[166, 169]. Better descriptive analysis of LMP2 is needed in order to fully assign function to LMP2b.

Lack of methods of detection and imperfect experimental design has led to the description of LMP2b function as having no real effect on latency. By determining the nature of the interaction of LMP2b with LMP2a, we could ascribe some function to this previously undescribed protein. If LMP2b should show some effect on LMP2a, then previous experiments including LMP2a need to be readdressed, as they are largely in an LMP2b negative background. Given the significance of LMP2a for the pathogenesis of EBV, the establishment and maintenance of latent infection in vivo, it is important to identify the function of LMP2b. We think that ***LMP2, which localizes intracellularly, has an affect on the establishment and maintenance of latency*****.** Towards this thought, we present the following experimental components to justify this hypothesis.

Specific Aim One: Analysis of Aggregation and Localization Domains in LMP2

Because of the contentious nature of the localization of LMP2 in different groups, we sought to determine the motifs that exist in LMP2 that are responsible for the localization and aggregative properties of protein. Using a sequence of successive deletions of LMP2 transmembrane regions in LCLs we examined what part of the proteins are responsible for localization and aggregation.

Specific Aim Two: Generation and Analysis of Recombinant EBV

Different groups have described LMP2 as alternatively necessary and dispensable for immortalization, but these groups have exclusively looked at LMP2a. In this aim, LMP2a and LMP2b deletion mutant viruses were generated. These viruses were used to determine if LMP2 proteins were necessary for EBV based immortalization. Cells generated from these assays were phenotypically analyzed to determine if there was a difference between the viruses that have been genetically modified, versus those that had a wild type phenotype.

Specific Aim Three: Assessment of LMP2b Interaction With Proteins Associated With B-cell Receptor Signaling

Due to the interactions that LMP2a has with cell signaling molecules involved with BCR signaling and the maintenance of latency, we sought to determine the involvement of LMP2b in this pathway. LMP2b cell lines were generated to exclusively look at the contribution of a protein without a signaling domain to a signaling pathway. Due to sequence and structure homology, it has always been assumed that LMP2b was merely a negative regulator of LMP2a. In this aim, we examined the possibility that LMP2b had an affect on the BCR pathway.

3.0 SPECIFIC AIM ONE:

3.1 PREFACE:

Prior work has indicated that the localization of the LMP2 proteins is contentious. LMP2 was first described as localizing to the plasma membrane in B cells. Our group, as well as others, has not been able to replicate this result. In the following, we dissected the LMP2 protein by successive deletions of the protein. We also were able to demonstrate that LMP2 contains several aggregation domains, as well as a requirement for the intact 12 TM region for localization to the trans-golgi network.

The following manuscript was submitted and accepted for publication in Virus Genes.

Minimal Protein Domain Requirements for the Intracellular Localization and Self Aggregation of Epstein Barr Virus Latent Membrane Protein 2

Monica Jo Tomaszewski-Flick[1] and David T Rowe[1]

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[1]Department of Infectious Diseases and Microbiology

Graduate School of Public Health

University of Pittsburgh

130 DeSoto St, Pittsburgh, PA 15213

*Corresponding Author +1 412 624 1529 (phone), +1 412 383 7490 (fax),

rowe1@pitt.edu

running title: LMP2b localization and interaction

keywords: Epstein-Barr Virus, LMP2, truncation mutants, membrane protein

3.2 ABSTRACT:

The EBV Latent Membrane Protein 2 (LMP2) may have a role in the establishment and maintenance of *in vivo* latency. The gene is transcribed into two mRNAs that produce two LMP2 protein isoforms. The LMP2a protein isoform has 12 transmembrane segments (TMs) and an amino terminal cytoplasmic signaling domain (CSD) while the LMP2b isoform is identical but lacks the CSD. There has not been a consensus on the cellular membrane localization being sometimes ascribed to either a plasma membrane or an intracellular location [160, 166, 167]. Fluorescent marker and epitope tagged LMP2b truncation mutants progressively removing TMs from the N and C termini were used to assess the localization and aggregation properties of LMP2b. wtLMP2b had an exclusively intracellular perinuclear localization, while all truncations of the protein resulted in localization to the cell surface. By epitope loop-tagging, all the truncated LMP2b proteins were verified to be in the predicted membrane orientation. In co-transfection experiments, the C-terminal region was implicated in the self-aggregation properties of LMP2b. Thus, an intact 12TM domain was required for intracellular localization and protein-protein interaction while a C-terminal region was responsible for auto-aggregative properties.

3.3 INTRODUCTION:

Epstein-Barr Virus (EBV) is a human gamma-herpesvirus, which is implicated in infectious mononucleosis, lymphoproliferative disease in the immunocompromised and several epithelial and lymphatic cancers [4]. In the normal host, EBV establishes a latent infection in B-lymphocytes [170]. In vitro, EBV infection of B cells induces proliferation of infected cells that can be subsequently grown as a lymphoblastoid cell line (LCL). LCLs express a limited number of viral gene products with functions associated with episome maintenance and cell survival. Of this restricted protein set, LMP2 is of particular interest due to its ability to maintain viral latency by preventing productive signaling from the B-cell receptor 6].

The LMP2 gene encodes two separate membrane protein isoforms via alternate promoter usage. These promoters, separated by approximately 3 kb, both produce transcripts that cross the fused terminal repeats[149]. The mRNAs have unique 5' first exons and 8 shared 3' exons. The messages encode LMP2a, a 497 aa protein with a 119 aa N-terminal cytoplasmic signaling domain (CSD), 12 transmembrane (TM) segments and a short 27 aa C-terminal tail and LMP2b, a 378 aa protein lacking the CSD.

LMP2a has been extensively studied with respect to protein interaction and intracellular signaling. The N-terminal CSD of LMP2a is critical to the production of survival signals and self-degradation [99, 171]. The CSD contains an immunotyrosine activation motif (ITAM), which is constitutively phosphorylated on tyrosines and interacts

with protein tyrosine kinases Syk and Lyn [98, 100, 172-175]. These interactions cause signaling that mimics B-cell receptor complex survival signals in early stages of murine B-cell development [98]. Previous reports also indicated that LMP2a excludes the BCR from detergent-insoluble lipid-enriched membranes[99]. Additionally, 2 proline rich motifs in the CSD also interact with a family of E3 ubiquitin ligases, mediating degradation of LMP2a and it's associated proteins [175, 176].

The nature of the LMP2b contribution to viral pathogenesis has not been determined. A role is suggested by the evolutionary conservation amongst primate lymphocryptoviruses of the ability to produce an LMP2b isoform [152]. Mutational analyses of the LMP2 gene in recombinant viruses have involved replacing regions of the LMP2 gene with antibiotic resistance cassettes, most of which simultaneously affect both LMP2a and LMP2b. From these studies LMP2b does not appear to contribute to immortalization [177]. Virus mutants that interrupt LMP2a exon1 and leave LMP2b intact, and presumably expressed, suggest that LMP2b has no role in the BCR signaling blockade phenotype of the LMP2 gene [178, 179]. One recent study stated that LMP2b modulates LMP2a activity, however, was unable to detect LMP2b in cells. [1]. Studies in epithelial cells demonstrate that LMP2b is able to promote cell spreading and motility, thus indicating that LMP2b does have the ability to mediate some sort of cellular signaling event independent of LMP2a [161]. We previously described the ability of LMP2b to specifically interact with endogenously produced CD19 [166]. Taken together, these studies show that LMP2b is likely to have unique effects on cells, and suggest that LMP2b is probably more than a CSD-less negative regulator of LMP2a. In this study, LMP2b truncations were made and analyzed for localization and multimerization

phenotypes. Identification of where LMP2b localizes and how the protein interacts with itself and other cellular proteins will provide important information on how the CSD-less protein could affect other cell processes.

3.4 MATERIALS AND METHODS:

3.4.1 Cell lines and cell culture:

BJAB is an EBV-negative Burkitt lymphoma cell line; 293T is a human embryonic kidney cell line containing the SV40 large T antigen. BJAB were maintained in complete RPMI -1640 medium containing 10% inactivated Foetal Bovine serum, 2mM glutamine, 60 µg ml-1, 200 µg ml-1 streptomycin at 37 ºC in 5% CO2. 293T were grown in complete Dulbecco's modified Eagle's medium (DMEM) under identical growth conditions.

3.4.2 Construction of recombinant LMP2 plasmids:

DNA fragments encoding full-length LMP2a or LMP2b incorporated with a 3X FLAG were constructed as previously described [2]. Truncation mutants of LMP2 were amplified by PCR from full-length constructs. All 5' primers contain an Xho1 restriction site. All 3' primers contain a Kpn1 restriction site. PCR products were digested with Xho1/Kpn1 and ligated into pC1EGFP or pC1DsRed2 (Clontech) for N-terminal truncations and pN1EGFP or pN1DsRed2 for C-terminal mutations. Full-length PCR products were ligated into pDSRed2 or pN1EGFP (Clontech). All constructs were sequenced for verification.

3.4.2.1 Transfections:

All DNA used for transfections were purified on $CsCl_2$ gradients. For B cell lines, 5x106 cells were washed once in 4 ºC PBS and resuspended at room temperature in 0.4 ml of serum- and phenol red- free RPMI 1640 containing 20 µg of the required plasmid. Cells were then transferred to a sterile electroporation cuvette (0.4 cm electrode gap) (BioRad) and pulsed twice. Multiporator (Eppendorf) settings were 400 V for 100 µS. Cells were allowed to settle for 10 minutes in a 37 ºC water bath. Cells were removed from the cuvette and added to 10 mls of complete phenol red-free RPMI 1640. For 293T cells, confluent T75 flasks were treated with 10x trypsin (Gibco) and washed. Cells were seeded to achieve 30 % confluency with 24 hours in a chamber slide and grown in complete DMEM. Once the cells had become 50% confluent, the media was replaced with FBS-free DMEM containing 3µl of Genejuice (Novagen) and 1µg of DNA.

3.4.3 Antibodies and immunoflourescence:

Anti-Human CD8, Syntaxin-6, GS15, GM130, & GS27 were purchased from BD Pharmingen. Anti-FLAG M2 was purchased from Sigma. Alexa-fluor secondary antibodies (488&594) were purchased from Molecular Probes. Mounting media containing DAPI was purchased from Vector Laboratories. Immunofluoresence was performed by fixing cells to polylysine coated glass slides (Fisher) using a cytospin (Shandon) set at 500 r.p.m for 5 minutes. Slides were dried for 1 hour at room

temperature in the dark. Cells were then fixed using freshly prepared 3.7% paraformaldehyde for 1 minute at room temperature. Samples were blocked for 30 minutes using SuperBlock blocking buffer in PBS (Pierce). Primary antibodies were incubated at pre-determined dilutions in blocking buffer for 30 minutes at room temperature. Secondary antibodies were diluted in blocking buffer (1:2000 dilution) for 30 minutes at room temperature. Slides were washed 3 times in cold PBS. Slides were allowed to air dry in the dark for 30 minutes. Slides were mounted with Vectashield-mounting media containing DAPI (Vector Laboratories). For cell surface immunostaining, live cells were chilled on ice for 10 minutes and incubated with anti-FLAG antibody M2 (Sigma) for 30 minutes at 4 ºC. Cells were washed in cold blocking buffer, fixed in 3.7 % paraformaldehyde and incubated with Alexa-fluor secondary antibodies (Molecular Probes).

3.4.4 Immunoprecipitation and Western Blot:

293T cells were transfected with EGFP and FLAG tagged full length LMP2b or one of the truncation mutants, and cell lines were established by G418 selection. Immunoprecipitation was performed using μMACS Protein G microbeads (Miltenyi Biotec, Inc) as per the manufactures protocol. Samples were analyzed by SDS-PAGE and transferred to nitrocellulose membranes. The proteins were detected with anti-FLAG M2 antibody (Sigma) via the Pro-Q Western Blot Stain Kit (Molecular Probes) and visualized via the FLA-2000 fluorescent image analyzer (FujiFilm, Inc).

3.4.5 Microscopy:

Slides were examined with a Nikon E600 microscope. Digital images were captured via a SPOTII CCD digital cameral and assembled using METAMORPH software (Universal Imaging). Final image montages were constructed using Adobe Photoshop.

3.4.6 Pixel Quantitation and Statistical Analysis:

Digital images of EGFP and DsRed 2 fluorescence were acquired using a cooled charge-coupled-device camera. Quantification was performed using METAMORPH software. Greyscale images were thresholded by eye to obtain pixel counts for an enriched marker on a single cell. When images were combined, the pixel counts were taken for overlap of the two single color images. The total number of pixels in each of these three single channel pictures were used to obtain a percentage of marker overlap: percentage of red overlapping with green (%R=G), and percentage of green overlapping with red (%G=R). These percentages were used in the Kruskal-Wallis rank sum test [23] via Minitab Release 14 Statistical Software (Minitab, Inc.)

Linescan histograms were generated by creating a 1 by cell width pixel region in each micrograph encompassing a cross-section of each cell. Histograms were generated via the METAMORPH software (MDS, Inc.). Each histogram measures the pixel intensity versus pixel number. Standard Deviations of mean fluorescent intensities

of 10 linescans for each mutant were compared by T-test using SPSS software (SPSS, Inc).

3.5 RESULTS:

3.5.1 Insertion of FLAG epitopes does not affect protein localization:

Due to the lack of antibodies that can independently recognize LMP2b sequences, we inserted artificial peptide (FLAG) epitopes into the loop sequences of the protein. In previous studies, we reported development of a 3xFLAG epitope tag inserted into the first extracellular loop of the protein. This epitope was shown to not interfere with localization when compared to wtLMP2b [166]. In this study, we used the same cloning strategy to insert 3xFLAG epitope tags into loop 10 connecting TM10 to TM11 (a putative intracellular loop) and into loop 11 connecting TM11 to TM12 (a putative extracellular loop). These constructs were also fused to either DsRed2 or EGFP.

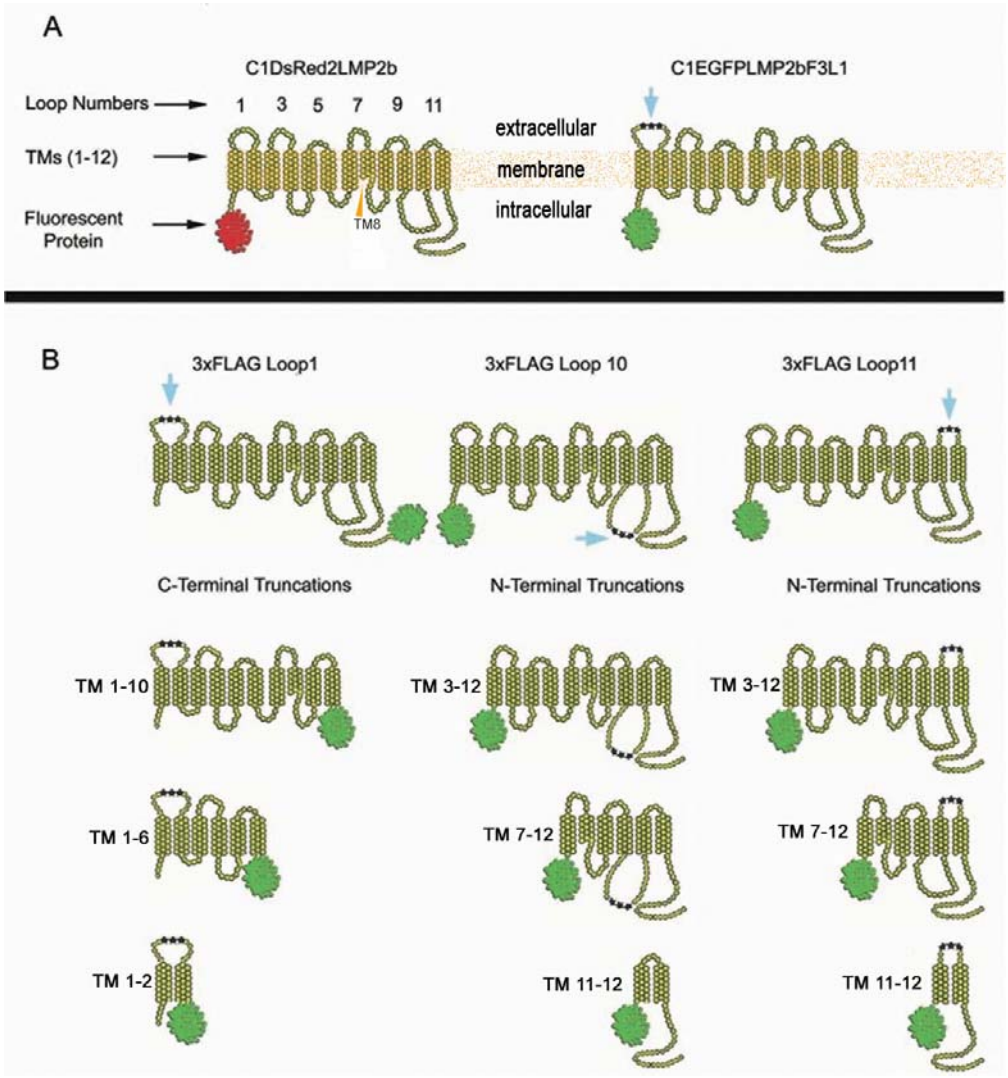

Figure 9: Modifications and Mutations of LMP2b used in this study.

(A)LMP2b is a 12 TM protein with short loops connecting the TMs. TM portions are referenced by number, 1 through 12, counting from the left. TM8 is indicated by an orange arrow. Extracellular loop are numbered as indicated. Full-length proteins were fused to a fluorescence marker (either EGFP [green] or DsRed2 [red]) and have a 3xFLAG epitope (represented by stars) in a loop of the protein (blue arrow). (B) C-terminal truncations were derived fromN1EGFPLMP2bf3L1 and have deletions of the last 2, 6, or 10 TMs. N-terminal truncations were derived from C1EGFPLMP2bf3L10 or C1EGFPLMP2bF3L11. N-terminal truncations remove the first 2, 6, or 10 TMs.

(Figure 9B, top row) Constructs with a fluorescent molecule fused to the N terminus of the full length LMP2b protein were transiently transfected into BJAB cells. (Figure 10) Protein expression was monitored over the next 3 days. In all experiments,

transiently expressed LMP2b proteins localized to a perinuclear region in live cells. This was in agreement with our previous studies that showed that LMP2 proteins with N- or C- terminal fluorescent tags co-localized with wtLMP2. In order to determine if the inserted FLAG epitopes had any affect on localization, co-transfections were performed between all three FLAG containing constructs (Figure 10). In each co-transfection, one construct was fused to DsRed2 while the other was fused to EGFP. As shown previously for the FLAG epitope insertion into loop1, FLAG epitopes inserted into loops 10 or 11 did not effect the perinuclear intracellular localization of LMP2b or the ability of differently tagged proteins to co-localize. (Figure 9) The levels of LMP2b fluorescence remained similar in all cells transfected, regardless of time post transfection, cell type or FLAG modification.

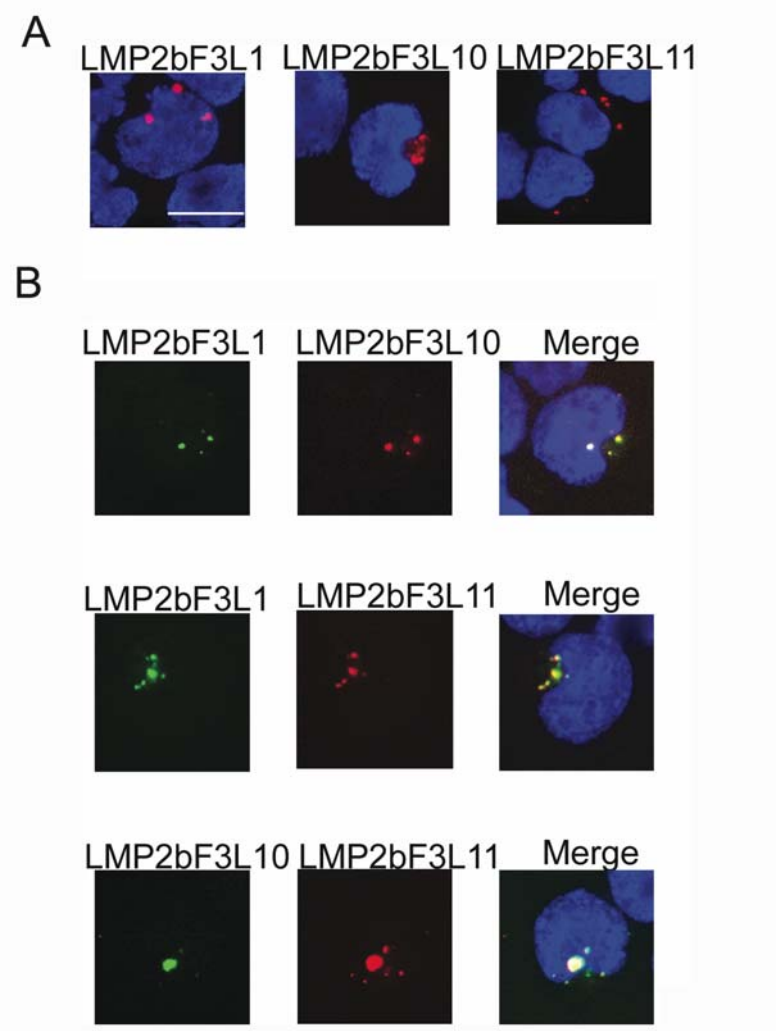

Figure 10: FLAG insertion into loops of LMP2b has no effect on protein localization.

(A)Plasmids encoding full length LMP2b fused to DsRed2 were transfected into BJAB cells. Micrographs of BJAB cells expressing single LMP2b proteins. (B) Plasmids encoding full length LMP2b fused to either EGFP or DsRed2 were transfected into BJAB cells. Micrographs are of cells co-expressing differently FLAG-tagged LMP2.

Previously, we reported that full length LMP2b is able to co-localize with γ-adaptin, a marker for the trans-golgi network. Inmmunofluorescent microscopy with antibodies detecting proteins that identify specific subregions of the Golgi were used to further refine the location of perinuclear LMP2b. Cells were probed for gm130 and GS15 (medial Golgi stack), GS27 (trans Golgi stack) and Syntaxin 6 (trans Golgi network). The largest amount of colocalization was seen between LMP2b and Syntaxin 6. Partial overlap was also observed between LMP2b and GS27 while minor overlap was detected between LMP2b and gm130 or GS15 (Figure 11, right panels).

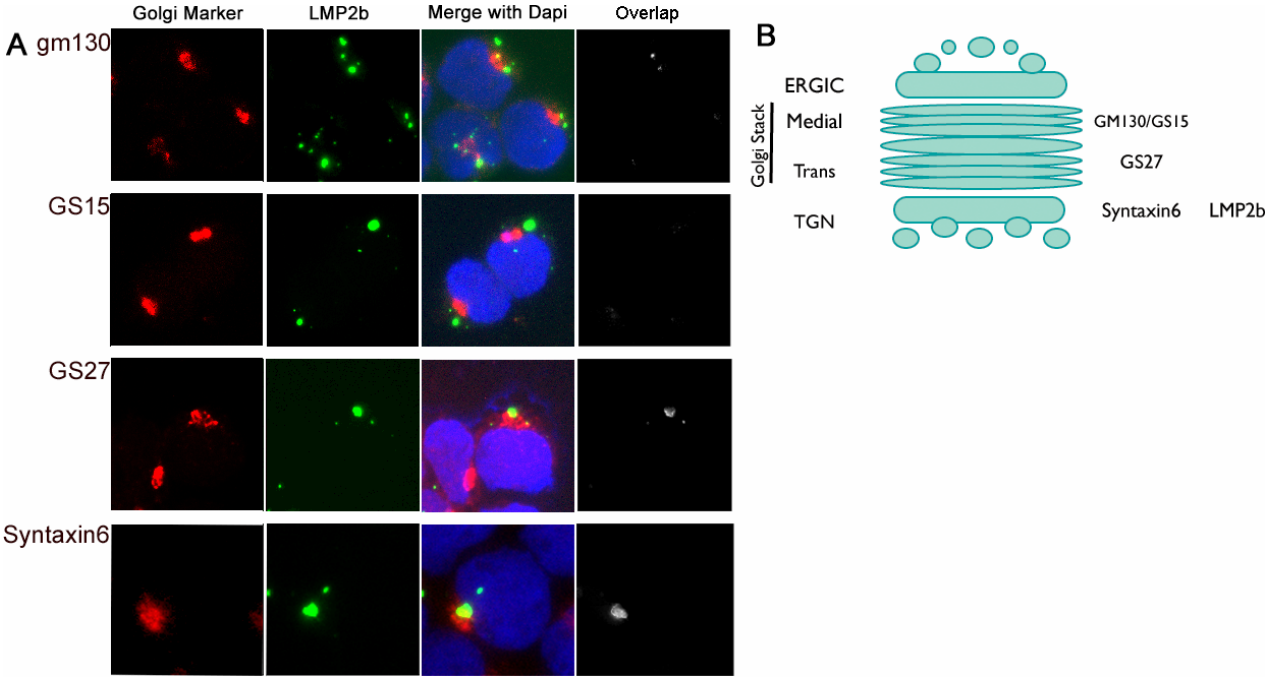

Figure 11: wtLMP2b localizes to the Trans-Golgi Network.

(A) BJAB cells expressing wt LMP2bEGFP (green) were permeablized and probed for Golgi markers (red): GM130, GS15, GS27, and Syntaxin 6. Overlapping pixels (yellow in 'Merge with Dapi') are also shown by exclusion, right. (B) Diagram of the Golgi apparatus with regions denoted on left, and markers for regions denoted on right. ERGIC: endoplasmic reticulum-Golgi intermediate compartment.

3.5.2 Removal of TMs changes the localization of LMP2b:

Truncations within the ORFs of the epitope-tagged LMP2b constructs were used to investigate the minimal number of TMs needed for intracellular localization and protein-protein interaction. Truncated proteins removing 2, 6, or 10 TMs from either the N- or C- terminus were made. C-terminal truncations contained the 3X FLAG in the sequence connecting the first and second transmembrane region (Loop1; F3L1). N-terminal truncations contained the 3X FLAG in either the loop connecting transmembrane region 10 to 11 (Loop10; F3L10) or transmembrane region 11 to 12 (Loop 11; F3L11). (Figure 9) We, and others [167], have identified the principal location of LMP2 proteins in cells as a membranous compartment in a perinuclear (golgi-like) region.

Live unpermeabilized BJABs transfected with wtLMP2b containing a loop11 3xFLAG tag were probed for surface FLAG expression. The epitope tag was not detected regardless of the time post-transfection that the cells were probed or the apparent level of EGFP fluorescence. This indicated that none of the LMP2b was trafficking to the plasma membrane. (Figure 12A) In contrast, the epitope tags were readily detected on the surface of every cell transfected with a C- terminal truncation mutant with a tag in loop 1 (TM1-2, TM1-6 & TM1-10) or an N- terminal truncation mutant with a tag in loop 11 (TM 3-12, TM 7-12, TM 11-12). (Figure 12B and data not shown) Surface expression was detected even at the earliest times post-transfection and was independent of the apparent level of EGFP fluorescence. When mutants with identical truncations carried tags in loop 10 or loop 11 (TM3-12 and TM7-12) were probed for surface expression of the FLAG epitope, proteins with loop10 tags (a

putative intracellular loop) were not detected (Figure 12C). Loop10 tags were only detected when the transfected cells were permeablized. (Figure 12D) In contrast truncated proteins with loop11 tags (a putative extracellular loop) were detected without permeablization (Figure 12B). These data showed that the truncated proteins were expressed at the cell surface and retained the conformation and orientation predicted for LMP2b. Thus, an intact 12TM protein is necessary for intracellular localization.

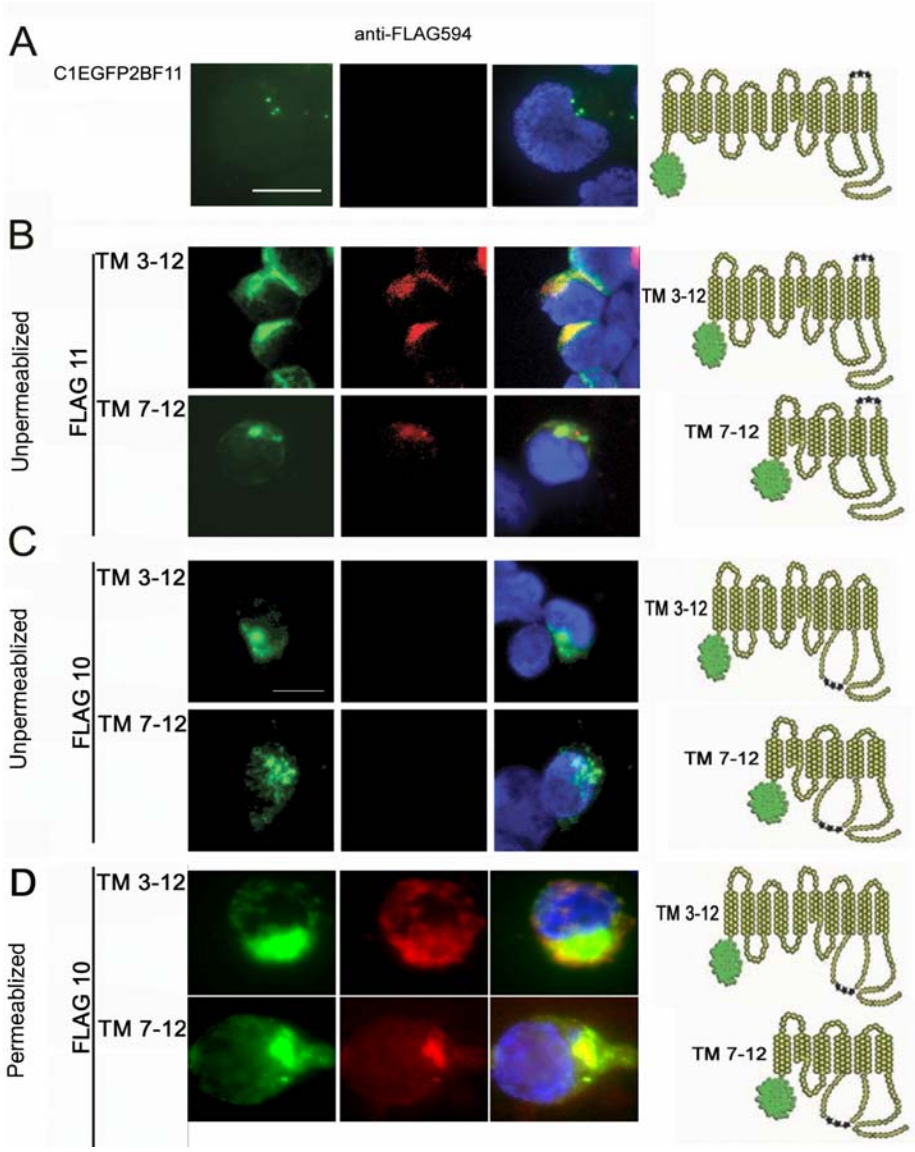

Figure 12: LMP2b truncation mutants are expressed on the cell surface.

BJAB cells transiently expressing (A)C1EGFP2BL11, (B) unpermeablized cells expressingTM 3-12, or TM 7-12 with FLAG epitopes in Loop 11, (C) unpermeablized cells expressing TM3-12, or TM7-12 with FLAG epitopes in loop 10 and (D) permeablized cells expressing TM3-12, or TM7-12 with FLAG epitopes in loop 10 were grown in coated chamber slides. Cells were chilled and probed for FLAG epitopes on the cell surface. Cells were then fixed and incubated with secondary antibodies (center, red). Bar, 5μ.

3.5.3 The C-terminal region of LMP2b is responsible for self-interaction but not localization:

Previous work [169] had shown that a portion of the C-terminus of LMP2 was responsible for 'clustering' of the protein. N- terminal truncation mutants (TM3-12, TM7-12, TM 11-12) had a distinct patchiness in the fluorescent pattern often appearing to cluster into a single patch or cap on the cell surface (Figure 13A, white arrows indicate clustered areas of protein). The effect was particularly pronounced when compared directly to the more diffuse global distribution of fluorescence observed with C-terminal truncation mutants (TM 1-2, TM 1-6, TM 1-10). A Western blot of anti-FLAG immunoprecipitates from transiently transfected cells revealed that LMP2b and all the truncation mutants except TM1-2 migrated as monomers. (Figure 13B) This confirmed that the proteins were intact and not degraded and showed that the clustering detected by immunofluorescence was easily disrupted. Linescan graphs comparing the individual pixel intensity for a cross section of the Figure 13A micrographs are shown in Figure 13C. Peaks correspond to close groupings of intensely green pixels and are an indication of clustering. As can be seen in the left column, the N-terminal truncations have more peaks than C-terminal truncations showing less fluctuation, indicating that the protein has a more uniform distribution. We also compared the means of the standard deviations of the individual mutants (Figure 13D) as well as N- versus C-

terminal mutants. We found that the mean standard deviations of the C- and N-terminal mutants were different using a T test with a p=<.001 level of significance. The differences in cell surface localization are consistent with the biochemical data that indicates the C-terminus causes LMP2 proteins to interact to form multimers. [23]

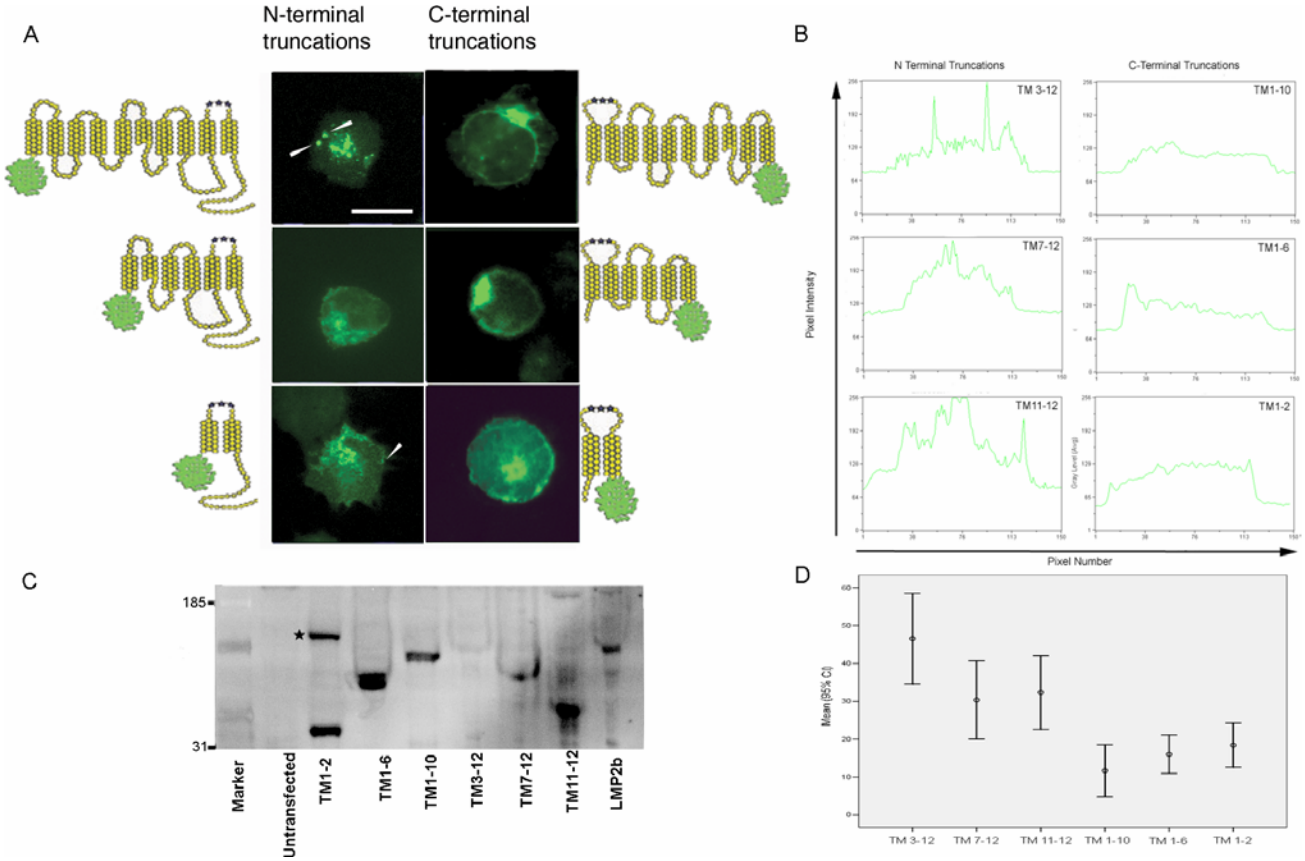

Figure 13: Truncations of LMP2b traffick to the cell surface; N-terminal truncations aggregate in the plasma membrane.

(A) BJAB cells transiently expressing individual truncation mutants were fixed 24 hours post-transfection and assessed for differences in localization patterns. Cells expressing the N-terminal truncations (green; TM 3-12, TM 7-12, TM 11-12, respectively) as indicated by the illustrations on the left. C-terminal truncations (TM 1-10, TM 1-6, TM 1-2) as indicated by the illustrations are on the right. N-terminal truncations exhibit a patch of green when compared to the more uniform green C-terminal truncations as indicated by white arrows. Bar, 5μ. (B) Linescan measurements of pixel intensity of micrographs in (A). Increased fluctuation indicates groupings of pixels with increased intensity. X-axis:

Pixel Number, Y-axis: Pixel intensity. (C) A western blot showing LMP2b truncation size. (D): Statistical analysis of linescans.

Proteins containing multiple TMs are often localized to the golgi via a phenomenon called kin-recognition that involves preferential sorting of proteins with minimal length hydrophobic stretches remaining in membranes of similar thicknesses. [180] Therefore the shortest LMP2 TM (TM8; Figure 9) might be a candidate for involvement in the perinuclear localization of LMP2 proteins. However, all the truncations that contained the eighth TM (TM1-10, TM 3-12, TM 7-12) localized to the cell surface to the same degree as truncations (TM 1-2, TM 1-6, TM 11-12) that lacked this TM. (Figures 12 and 13)

The patchy surface expression of the N-terminal mutants suggested that if a C-terminal interaction were to occur between truncated molecules and perinuclear wtLMP2b, that the wtLMP2b might alter the localization of the truncated proteins. In cells cotransfected with DsRed2-tagged wtLMP2b and EGFP-tagged truncation mutants there was always some overlapping fluorescence in the perinuclear region. (Figure 14) We quantitated red, green and overlapped pixel counts for each of the fluorescent channels. These data, generated from multiple micrographs to control for individual cell expression levels, *(Table 2)* were then used to determine the percentage of green-red overlap (% co-localization). From this image analysis the C-terminal truncations had a significantly higher percentage green-red overlap compared to N-terminal truncations (Kruskal Wallis rank sum test, P=0.035). This shows that while an LMP2b-LMP2b C-terminal interaction was capable of clustering LMP2b proteins, a different interaction appears to be required for trafficking and retention of LMP2b molecules in the

intracellular perinuclear compartment. The critical region for this phenotype maps to the N-terminal TMs. Since some truncated molecules appear on the surface, retention seems likely to require multiple protein-protein interactions involving different regions of the intact LMP2b molecule.

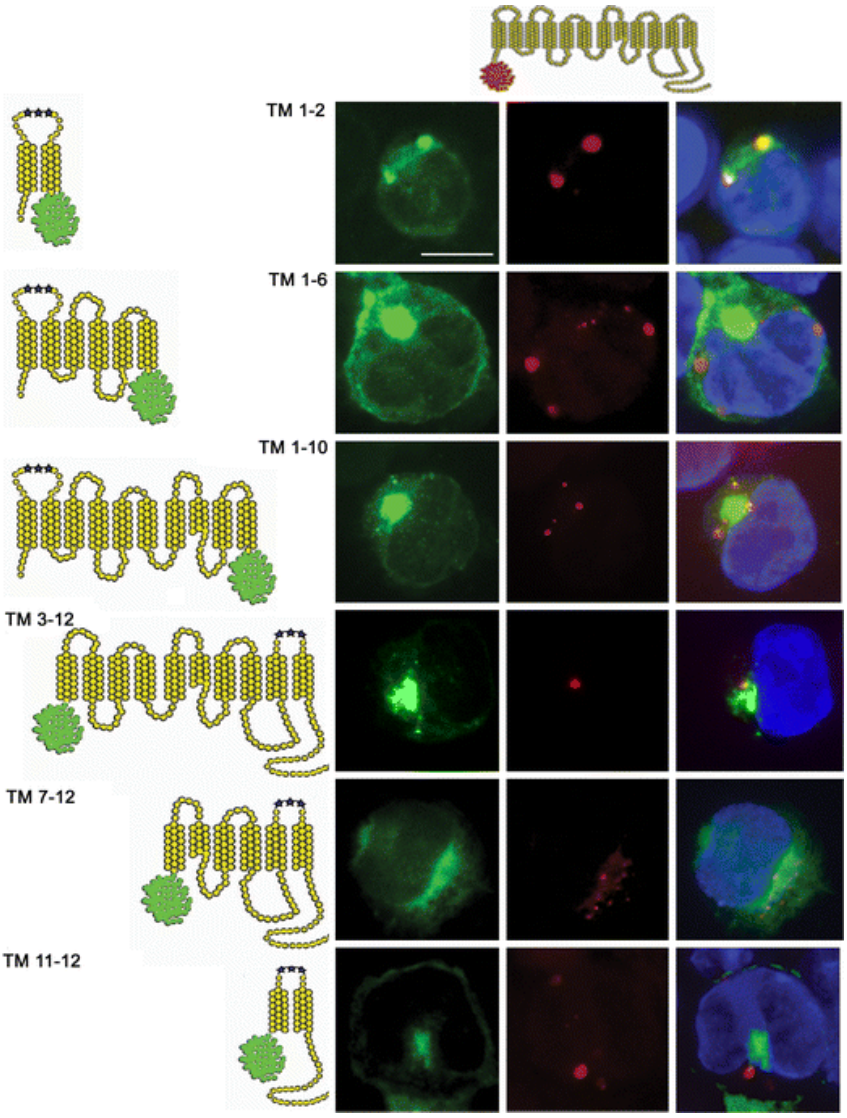

Figure 14: Co-transfection of wtLMP2b with LMP2 truncation mutants.

BJAB cells were transiently transfected with full-length DsRed2LMP2bF3 and one of the truncation mutants fused to EGFP (*green*). Diagrammatic representations of the mutant constructs are to the left of the panels. Red and Green images were merged with DAPI-stained nuclei (*blue*) in the panel on the right. Overlap between the truncated LMP2b (*green*) and full-length LMP2b (*red*) is indicated by yellow fluorescence. Bar, 5 μ

Table 2: Percentage of Green Pixels that Equal Red Pixels Base on Mutants Co-transfected.

Construct	Median (% overlap)	Ave. Rank	Z
ΔTM11-12	26.8	16.7*	1.48
ΔTM7-12	13.3	13.8*	0.77
ΔTM3-12	54.27	19.7*	2.34
ΔTM1-10	0.51	7.7	-1.49
ΔTM1-6	1.4475	6.6	-1.00
ΔTM1-2	2.75	8.0	-1.66
		Total= 11.5	
	H=11.96	DF=5	P=0.035

For each construct the number of overlapping pixels (yellow, see figure 14) was taken as a percentage of the total number of green pixels. These numbers were used in the Kruskal-Wallis mean rank sum test. Numbers above H denote a difference in mean. (*) Constructs containing the N-terminal regions of LMP2b are being sequestered more efficiently by full length LMP2.

The location of the truncation mutant proteins was compared to endogenous golgi marker proteins (Syntaxin 6) and to an exogenous control cell surface protein (CD8zeta) co-transfected into BJAB cells. (Figure 15) In contrast to the TM mutants, wtLMP2b was not observed on the surface and colocalized with Syntaxin 6 (compare Figure 11 with Figure 15). wt2b does not interfere with CD8zeta surface expression [2]. All of the truncation mutants showed some colocalization with Syntaxin 6 indicating that the proteins had some localization in the area where LMP2b is typically found. The remainder of the fluorescence was targeted to the cell surface as indicated by colocalization with CD8zeta.

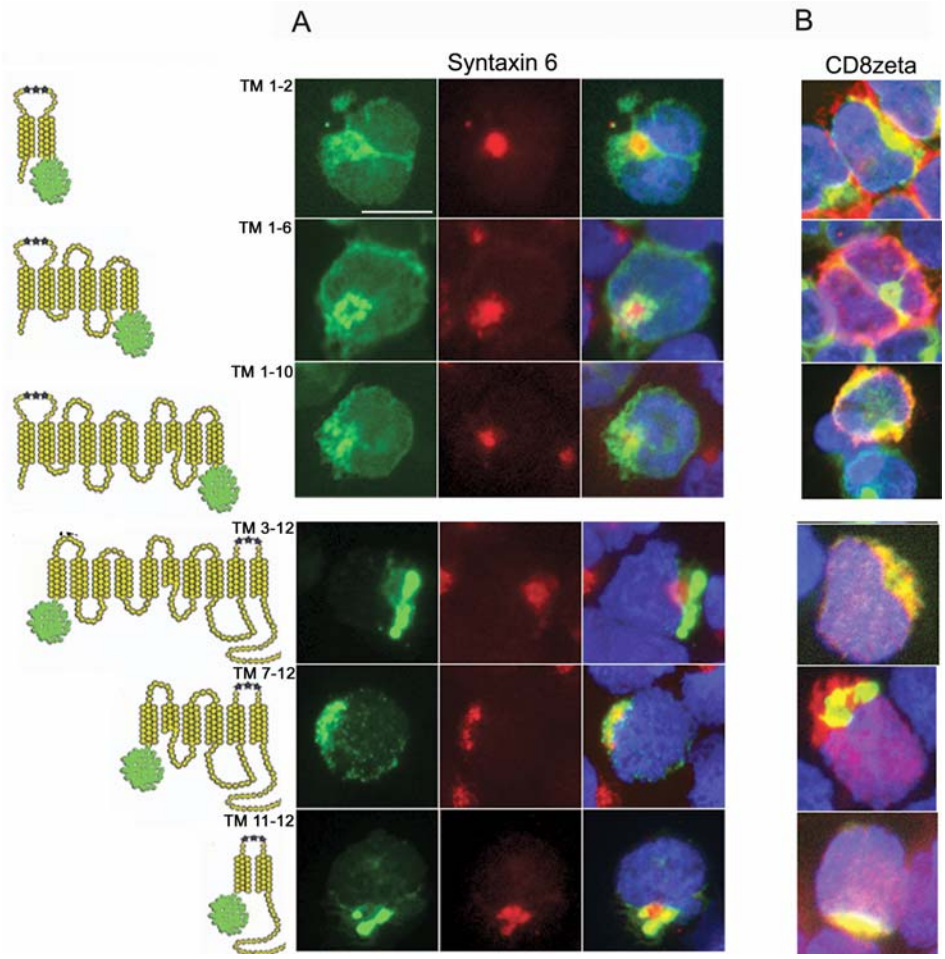

Figure 15: Colocalization of LMP2 truncation mutants with cellular compartment marker proteins

(A) Transiently transfected BJAB cells were permeablized and probed for Syntaxin 6 (a Golgi marker) to demonstrate that truncated LMP2b proteins (green, left) trafficked through the Golgi apparatus. Anti-Syntaxin 6 was detected with Alexa-fluor 594 labeled anti-mouse secondary antibody (red, center). Red and green images were merged with DAPI-stained nuclei (blue) in the panel on the right. Colocalization was indicated by yellow fluorescence. (B) BJAB cells were cotransfected with an LMP2b mutant protein (green) and CD8zeta (an exongenous cell surface marker). CD8zeta was detected with anti-CD8 and anti-mouse Alexa-fluor 594 (red). Diagramatic representation of the mutants used in each row are to the left of the panels. Bar, 5μ.

3.6 DISCUSSION:

Most studies of the LMP2 gene have focused on LMP2a isoform. Because LMP2b is essentially LMP2a without the cytoplasmic signaling domain, it has been speculated that LMP2b may act as a negative regulator of LMP2a through interference with complex formation [181]. Also, because of the molecules' similar structures, there has been no means of tracking the two LMP2 protein isoforms independently. In recent studies, we introduced fluorescent tags and peptide epitopes into the LMP2 proteins to address the issues of protein detection and interaction. Fluorescently labeled and epitope-tagged LMP2a truncation mutants were used to determine that LMP2a requires an intact 12TM region for intracellular localization [166]. The perinuclear localization of LMP2a has been independently confirmed in both B-lymphocytes and epithelial cells [167].

We have extended these studies with epitope tags in loop 10 and 11 and with N and C terminal truncations. The loop epitope tags were used to track and orient LMP2 proteins. The predicted membrane insertion structure of the LMP2 isoforms (proteins composed of 12 TM segments with alternating short extracellular and intracellular loops) has never been experimentally verified. In the studies described here, loop FLAG epitope-tagged truncation mutants all localized to the plasma membrane of live cells. All the predicted external loop tags were detected on the surface by anti-FLAG immunofluorescence while none of the internal loop tagged proteins were. All the results

were in agreement with the predicted membrane orientation for the LMP2 loops and TMs.

The localization of LMP2a to a trans-golgi network-like area might involve a retention signal. Several specific sequence motifs have been implicated in post-Golgi transport of membrane proteins [182, 183]. The best-recognized TGN retention motifs, YXXØ and DE, are not present in the LMP2 coding sequence. TGN retention may also be mediated by non-sequence related structural features such as coiled-coil motifs, which are used by resident Golgi proteins to remain in this organelle [184]. Sequence analysis and protein folding predictions for LMP2 proteins do not predict these motifs. Another feature, kin recognition, directs proteins to certain cisternae of the Golgi by the virtue of the length of the shortest TM segment [24]. Both isoforms of LMP2 contain the atypically short TM8 that could be implicated in retention via kin recognition. Our analysis of the localization of LMP2b truncations suggests that the presence of TM8 did not correlate with retention to a perinuclear location. A more specialized mutational analysis involving just alterations to TM8 will be required to determine if this TM (or any of the TMs) has a unique and critical role.

Previously, LMP2a was shown to have a ‘clustering’ domain in the 27 aa C-terminal tail [23]. This feature was analyzed by biochemical assays detecting multimerized proteins in cell lysates. Our truncation mutant study indicated that the self-interaction domains previously mapped to the C-terminus of the protein were not sufficient for intracellular localization. Truncated proteins that had intact C termini were not sequestered in an intracellular compartment. However, consistent with the presence

of a C terminal aggregation motif, only truncated proteins with intact C termini showed aggregates on the cell surface.

When the truncated proteins were expressed alone in cells, a portion of the protein was always detected in an intracellular perinuclear location suggesting that the mutants might be transiting slowly through the compartment in which wtLMP2b was localized. We performed co-transfection experiments to determine if a resident wtLMP2b could influence the localization of the truncation mutants. There was a significantly greater intracellular co-localization with LMP2b of C-terminal truncation mutants (TM1-2, TM1-6 & TM1-10) compared to N-terminal truncation mutants (TM3-12, TM7-12 & TM11-12). Thus, a previously unrecognized N-terminal region has been detected that is involved in either trafficking or clustering (or both). The appearance of readily detectable N-terminal truncation mutant proteins on the surface indicates that the C-terminal tail is not alone sufficient to mediate internal sequestration.

Aggregative properties of LMP2 proteins can be seen as having functional consequences. Because of the structural similarities between LMP2a and LMP2b, it has been suggested that LMP2b expression would have a functional effect on LMP2a. Speculation focuses on a model of LMP2b regulation of LMP2a through stochastic molecular interactions. [1, 23] In this model, the C-terminal clustering domain of LMP2b competes for clustering with LMP2a tails, decreasing LMP2a self-aggregation, phosphorylation, and downstream signaling. This clustering model of regulation is supported by our data showing that in addition to the C-terminal clustering domain, there is an N-terminal domain that is also involved in aggregation. An addition of a N-

terminal clustering domain would add a block in protein multimerization closer to the CSD of LMP2a, which could add an additional impediment to signaling by this isoform.

Full -length LMP2 proteins are detected as a discrete patch of fluorescence in the perinuclear region of cells, a location normally ascribed to the Golgi network. Previously, we reported colocalization with gamma-adaptin, a marker of the late TGN and early endosomes, here we show full length LMP2b colocalizing with another golgi marker (Syntaxin 6) but excluding others. Truncated LMP2 molecules show partial overlap with Syntaxin 6, indicating that some, possibly transient, localization to a compartment specific for wtLMP2 molecules occurs. Truncation mutants trafficked to the cell surface where co-localization with a transiently expressed exogenous control (CD8zeta) was detected. This demonstrated that neither LMP2b nor the truncation mutants interfere with the ability of other proteins to traffic to the cell surface. Since the mutants of LMP2b did not affect the distribution of CD8zeta on the surface, we found no evidence for a global reorganization of surface proteins. Thus, LMP2b appears to have a specific intracellular compartmental localization that can be disrupted by N or C terminal truncations affecting the transmembrane regions.

3.7 ACKNOWLEDGEMENTS:

The authors would like to thank Dr. L. Kingsley for expert statistical advice.

4.0 SPECIFIC AIM TWO:

One of the largest criticisms of our work is that the system we use is contrived. For instance, our system utilizes an artificial promoter in a commercial plasmid to make protein in transfected EBV negative cells. This system allows for the criticism that the localization of proteins is due to an overwhelmed protein processing system that sequesters our proteins. This system is also inefficient, as B cells, either EBV positive or not, are difficult to transfect. By creation of recombinant EBV, were able to counter most criticisms of our work thus far. In this aim, we also determined the localization of LMP2a and LMP2b that is virally generated at physiological levels.

Due to the isogenic and progressive additions to the virus that has been constructed, we were able to determine the individual contribution of LMP2a and LMP2b for the establishment and maintenance of latency after infection.

4.1 INTRODUCTION:

Early studies in the 1990's with recombinant virus production revealed that the LMP2 gene was not essential for immortalization[185-188]. These studies yielded populations of immortalized B-cell clones from infections of mixed (LMP2 positive and LMP2

negative) viruses. These investigations were ill-equipped to determine the efficiency of immortalization between LMP2 negative and LMP2 positive viruses.

These studies used quantitative analysis of immortalization rather than the use of qualitative analysis. The efficacy of quantitative analysis is also hindered by the fact that wild-type virus was present in large amounts in all of the experimental designs. The contribution of LMP2 to immortalization was determined based upon statistical analysis rather than direct experimentation.

In order to submit a more direct contribution, Breilmeier et al introduced a Mini-EBV approach[189]. This system generated a virus that contained the minimum gene content to generate a latent infection. These viruses were packaged with help of the P3HRI cell line, and thus did not have any contaminating wild type virus. Analysis of the viruses produced by this system, indicated that any of the Mini-EBV viruses lacking the LMP2 gene were limited in their ability to immortalize B cells.

The issue of LMP2 contribution to immortalization was revisited in 1999, Speck et al noted that Mini-EBV viruses were shown to acquire small mutations[177]. This data, as well as a lack of sequence data for the mini-EBV clones that were deficient in immortalization necessitated a different approach. A plasmid with an EGFP-neo cassette insertion in the block of exons 2-4 of the LMP2 gene was generated and knocked-in to the B95.8 genome in cells. Of 402 green clones, 6 had the targeted mutation. Of these 6 clones, only 1 was a high enough producer for further experimentation. The resulting virus, which was a mix of wild type and recombinant viruses (82% and 18% respectively) was used in an immortalization assay. This assay generated an expected wildtype:LMP2-:mixed ratio of 62:12:26. While this analysis

seems to show that LMP2 is not necessary for immortalization, once again, the limitations in these experiments demonstrated that this experiment was quantitative not qualitative (high backgrounds of wild type virus, low yields of recombinant virus, many cell culture manipulations and only one replicate experiment).

This work lent support to a consequential study: Konishi et al disrupted the very 5' end of LMP2a by neo insertion into the Akata virus[179]. 2 of 2880 resistant clones contained the necessary insertion. The mixed wild-type and LMP2a negative viruses were passed on EBV-negative cells and after extensive screening, 12 clones were identified that had the recombinant phenotype. Virus production from these cell lines approached wild type levels. In 2 experiments, the immortalization efficiency of the LMP2a negative virus had levels similar to wild type. Taken together, these two studies seem to indicate that LMP2a is not necessary for immortalization.

To provide a better method of virus generated not dependent on manipulation in cell lines, the Hammerschmidt group revisited their plasmid approach, this time with a complete viral genome. The B95.8 genome was manipulated in bacteria to include EGFP and a hyg resistance cassette in the B95.8 deletion. LoxP sites were introduced to flank the areas targeted for deletion. Cre-mediated recombination was then performed in E. coli. This allowed for the sequence of the virus to be verified prior to virus production.

Towards creation of LMP2a negative virus, they constructed isogenic pairs that flanked LMP2a exon1 with LoxP sites. These viruses were titered for infectivity on Raji cells, showing that comparable virus stocks can be made. Typical B cell immortalization

assays showed that virus lacking LMP2a was deficient in immortalization showing a ~40-fold decrease in immortalization efficiency[190].

The Maxi-EBV approach seems to address all the criticisms in the previous approaches (lack of contaminating wild type virus, high titers of recombinant virus, and the ability to obtain sequence information prior to virus production). However this approach still seems to demonstrate the LMP2a is needed for efficient immortalization[191].

More recently, LMP2a has shown to be necessary for outgrowth of Ig-null cells after EBV infection[191]. This demonstrates a function for LMP2a in disease progression.

The controversy over the role of LMP2 in the immortalization of B cells is not settled. Comparing isogenic pairs of LMP2a negative, LMP2b negative, and LMP2a/b negative viruses finally shows a role for LMP2 in immortalization and a measurable phenotype for LMP2.

4.2 MATERIALS AND METHODS:

4.2.1 Bacteria Manipulations:

4.2.1.1 Bacteria Culture:

E. coli (DH5α) were cultured in suspension in LB-Media or grown as single cell colonies on LB-Agar Plates. Selection of resistant bacteria was performed by adding selected antibiotics in the LB-Media. The antibiotics used were: Ampicilin (100ug/ml), Chloramphenicol (30ug/ml), Kanamycin (30ug/ml), Tetracycline (30ug/ml), Zeocin (100ug/ml) and Spectinomycin (25ug/ml). For long-term storage of bacteria, equal amounts of glycerol were added to liquid cultures, and one ml aliquots were stored at –80C.

4.2.1.2 Transformation and Electroporation of E. coli:

Transformation of plasmids being used for recombination was performed using the protocol by Hanahan[192]. Competent bacteria were stored at –80°C and thawed on ice. For each transformation, 200 ul of bacteria was used with 50 ng of ligation reaction supplemented with 7 ul of DMSO. This reaction was incubated at 42°C for 1.5 minutes in a water bath. After incubation, 1 ml of 37°C SOC media was added to the reaction. The reaction was incubated in a shaking incubator for 1.5 hours to allow for expression of the antibiotic resistance. The reaction was then plated on the appropriate LB-agar plates and incubated overnight.

Alternatively, when small plasmids were being used, the protocol from Chung was used[193]. In short, bacteria were grown to an OD of 0.3-0.4, centrifuged, and resuspended with 100 ng of plasmid DNA in 100 ul TSS buffer. This reaction was incubated on ice for 30 minutes and then shocked at 42°C for 1.5 minutes. SOC was added and then the reaction was incubated in a shaking incubator for 1.5 hours to allow for expression of the antibiotic resistance. The reaction was then plated on the appropriate LB-agar plates overnight.

Linear DNA fragments used for homologous recombination were electroporated into DH10B E. coli bacteria using a Gene-Pulser (Bio-Rad). Electrocompetent bacteria were made using a protocol from Sheng et al [194] and thawed on ice. 50 ul of competent bacteria and 3ug of plasmid DNA were added to a electroporation cuvettte (0.2 cm gap) incubated on ice for 5 minutes. The bacteria were then electroporated at 1.8 kV, 200 Ω, and 25 uF for 2 seconds. Pre-warmed SOC media was added to the bacteria, and the reaction was incubated at 30°C (for temperature sensitive recombination plasmids) for 2 hours. The reaction was then plated on LB-Agar plates, and incubated at 42°C to lose the temperature sensitive plasmid.

4.2.2 Cell Culture and Analysis of Cells:

4.2.2.1 Cell Culture:

All cells were grown in an incubator at 37C supplemented with 5% CO2. Raji cells were grown in RPMI-1640 media supplemented with 10% Fetal Bovine Serum, 100 ug/ml

Streptomycin, and 100 ug/ml Penicillin. Adherent cells were grown in DMEM media with 10% Fetal Bovine Serum, 100 ug/ml Streptomycin, and 100 ug/ml Penicillin. PBMCs were grown in RPMI-1640 media supplemented with 10% Fetal Bovine Serum, 100 ug/ml Streptomycin, 100 ug/ml Penicillin, 87 ug/ml Normocin, 1mM Sodium Pyruvate, 5 mM HEPES and 100 uM non-essential amino acids. To establish and maintain stabile Maxi-EBV cell lines, 110 ug/ml of Hygromycin was added to the media.

Adherent cell lines were routinely split every 2-3 days. The media was removed; 1ml of 10x Trypsin (Gibco) was added to the flask. The flask was then incubated at 37°C for 2 minutes. The cells were washed off the flask bottom using complete media. 20% of the total wash remained in the flask as seed for the culture.

Cells were frozen by resuspension at a concentration of 10^7cells/ml of RPMI-1640 with 30% FBS and 10%DMSO. The cells were then aliquoted into 2ml NUNC Cryotubes and stored overnight in a cryofreezer placed in a –80°C freezer. After the cells had reached –80°C, the vials were put into liquid nitrogen storage. 2 weeks after the cell vials were placed into the liquid nitrogen, one vial was tested for viability after thawing. The contents of the vial were washed with complete media at 4°C until thawed, cells were then washed with 10 ml complete media. After washing, cells were resuspended in 10 mls media, placed in a culture flask, placed in the incubator, and monitored for cell death.

4.2.2.2 Preparation of Human B cells:

Human primary B cells were harvested from blood obtained from the Pittsburgh blood bank. PBMCs were harvested from blood by ficoll gradient centrifugation. Blood

was thinned at a 1:1 ratio with PBS. 35 ml of this blood/PBS mixture was overlain on 15 ml of Histopaque and spun for 20 minutes, 2000rpm, at RT. After centrifugation, plasma was removed from the tubes, and the layer of white cells was removed. These cells were washed 2 times in PBS at 1200rpm, 5 minutes 4°C. Cells were then resuspended in the appropriate media, and used for experiments. When CD19+ cells were needed, blood was treated as above, and then purified using positive selection of cells via magnetic antibody purification with CD19 microbeads (Miltenyi Biotec).

4.2.2.3 Transfection of Cells and Selection of Stabile Cell Clones with Hygromycin:

For transfection of 293-SL cells with Maxi-EBV plasmid, cells were plated in order to achieve 30-40% confluency rate 20 hours post plating. Cells were transfected in 6 well plates. Transfection was performed using GeneJuice (Novagen) via a protocol modified from the manufacturers recommendations. 1ug of Plasmid DNA (for each well of a 6 well plate) was transferred to a 1.5 ml Eppendorf tube with 100 ul of Opti-mem serum free media (Gibco). 6 ul of GeneJuice (for each well of a 6 well plate) was also transferred to a 1.5 ml Eppendorf tube with 100 ul of Opti-mem media. The GeneJuice/Opti-mem solution was then transferred to the tube containing the DNA/Opti-mem solution. The tube was inverted twice, and then incubated at RT for 15 minutes. During this incubation, the media was removed from the cells in the 6-well plate and replaced with fresh complete RPMI-1640. At the end of the incubation, 200 ul of the combined DNA/GeneJuice/Opti-mem was added to each well dropwise. The cells were incubated overnight, and monitored for EGFP expression over the next 48 hours.

When the cells were expressing EGFP, the cells were transferred from the 6-well plate to a T75 for 48 hours. After 96 hours in culture, the cells were then transferred to a 96-well plate at a concentration of 5 green cells per well in media containing 100 ug/ml of Hygromycin. Cells were monitored for 8 weeks for outgrowth of green colonies, and moved to progressively larger culture vessels until confluency in a T-25.

4.2.2.4 EBV Production and Titering:

For induction of the EBV lytic cycle, stably EGFP expressing 293 cell lines were plated in a 2 wells of a 6 well plate (for small scale induction) or 2-100 mm round tissue culture dishes (for large scale induction) at 40% confluency. Cells were transfected with plasmids encoding BZLF (p509) and BALF4 (p2670) and incubated at 37C for 5 days. On day 5, media was harvested, passed through a filter (0.8 uM pore size), and stored at 4°C.

Virus titering was performed by infecting Raji cells and counting EGFP positive cell lines after lytic induction. $1x10^5$ Raji cells were incubated with decreasing amounts of filtered supernatants for 3 days at 37C in a 24 well plate. On day 4, lytic cycle inducers 12-O-tetra-decanoylphorbol-13-acetate (final concentration: 20 nM) and sodium butyrate (final concentration: 5 mM) were added to each well. On day 5, EGFP positive cells were counted under a fluorescence microscope. Based on the dilution of the virus supernatant, and then EGFP positive cells, a 'Green Raji Unit' (GRU) measurement was determined. This GRU was used to equalize virus titers among different Maxi-EBV cell lines in consequent experiments.

4.2.2.5 Concentration of EBV Supernatants:

To concentrate virus supernatant for use in experiments, the total supernatant was centrifuged in a Centriplus Centrifugal Filter (YM-100, Centricon) for 55 minutes at 3000xg. The flow-through was discarded, and the upper chamber of concentrated supernatant was re-titered and stored at 4°C.

4.2.2.6 Infection of Primary B cells with Maxi-EBV for the production of lymphoblastoid cell lines:

10^7 PBMCs were incubated with supernatant containing 10^6 GRU for 1 hour at 37°C. Cells were then plated at a density of 10^5 cells per well of a 96 well plate. Cyclosporin A was added at a concentration of 1ug/ml for the first 15 days of culture[195]. Wells containing EGFP expressing cell clumps were moved to 24 well plates after 4 weeks of culture. 10 wells were randomly selected for outgrowth and further characterization (§ 4.3.2)

4.2.3 DNA Manipulation and Analysis:

4.2.3.1 Extraction of Cellular DNA from Prokaryotic Cells:

3 ml of LB media were centrifuged at 14K to pellet bacteria. Bacteria was resuspended in 200 ul of TE buffer containing 3.3ng Rnase A per 1 ml. 200ul of 1% SDS, 0.2N NaOH was added to TE solution. The solution was incubated on ice for 7 minutes. 200 ul 5M Sodium Acetate was added to precipitate proteins. The solution was incubated on ice for 10 minutes, centrifuged for 10 minutes, 14K, 4°C. The supernatant was moved to a new tube, and then DNA was precipitated with 400 ul of isopropanol.

The solution was centrifuged for 10 minutes, 14K rpm, 4°C. The pellet was washed with 200 ul 80% ethanol to remove salts. The pelleted DNA was dried on the bench top for 15 minutes, and then resuspended in 50 ul water.

Large-scale purification of Plasmid DNA generated in bacteria was done out of house by MTR Scientific using proprietary methods.

4.2.3.2 Homologous Recombination in E. Coli:

Linear fragments of DNA were recombined into Maxi-EBV plasmids in a eukaryotic system via electroporation into DH10B E. coli (§4.2.1.2). The E. coli contains the Maxi-EBV plasmid p2089 (or a derivative thereof) as well as a plasmid with a temperature sensitive origin of replication and arabinose inducible promoter element that encodes the proteins for homologous recombination (pKD46). After transformation of the linear DNA fragments and phenotypic expression of the resistance cassettes, the pKD46 plasmid is lost by growth at 42°C. By double selection of the resistance markers, the Maxi-EBV (usually chloramphenicol) and the linear fragment (Zeocin, for example) double positive colonies are generated. These double positive colonies are then miniprepped (§3.1.3.1) and verified for insertion of the linear fragment into the Maxi-EBV backbone via restriction endonuclease treatment, agarose gel electrophoresis, and ethidium bromide staining as per standard protocols.

4.2.3.3 PCR Analysis:

Small insertions or deletions in the Maxi-EBV plasmids could not be detected via restriction analysis as above. These modifications were detected in individual colonies

via PCR analysis, in which the optimum primers and annealing temperatures were determined via computer algorithm (Primer Express, Applied Biosystems).

Standard Reaction for Colony PCR:

10x PCR Buffer (Applied Biosystems) 2.5ul
25 mM $MgCl_2$ (Applied Biosystems) 2.5ul
25mM dNTPs (Genetex) 4ul
Primer1 (IDT) 0.5ul
Primer2 (IDT) 0.5ul
1.5 ug/ul Lysozyme 0.5ul
Amplitaq Gold (Applied Biosystems) .25ul
Water 14.25
PCR Program: 94°C/10 minutes; 35 cycles (95°C/1minute, 50°C/1 minute, 72°C/1minute); 72°C/10 minutes; 10°C

Thermocyling was performed using a GeneAmp PCR system 9700 (Applied Biosystems). PCR products were separated by agarose gel electrophoresis and visualized via ethidium bromide staining.

4.2.3.4 Cre-Lox Recombination:

LoxP sequences consist of an 8 base pair spacer DNA sequence flanked by 2 13-base pair inverted repeats. These sequences are recognized by Cre recombinase, and can be used for deleting or inverting sequences that are flanked by the recognition sequences (floxed). (Figure 16)

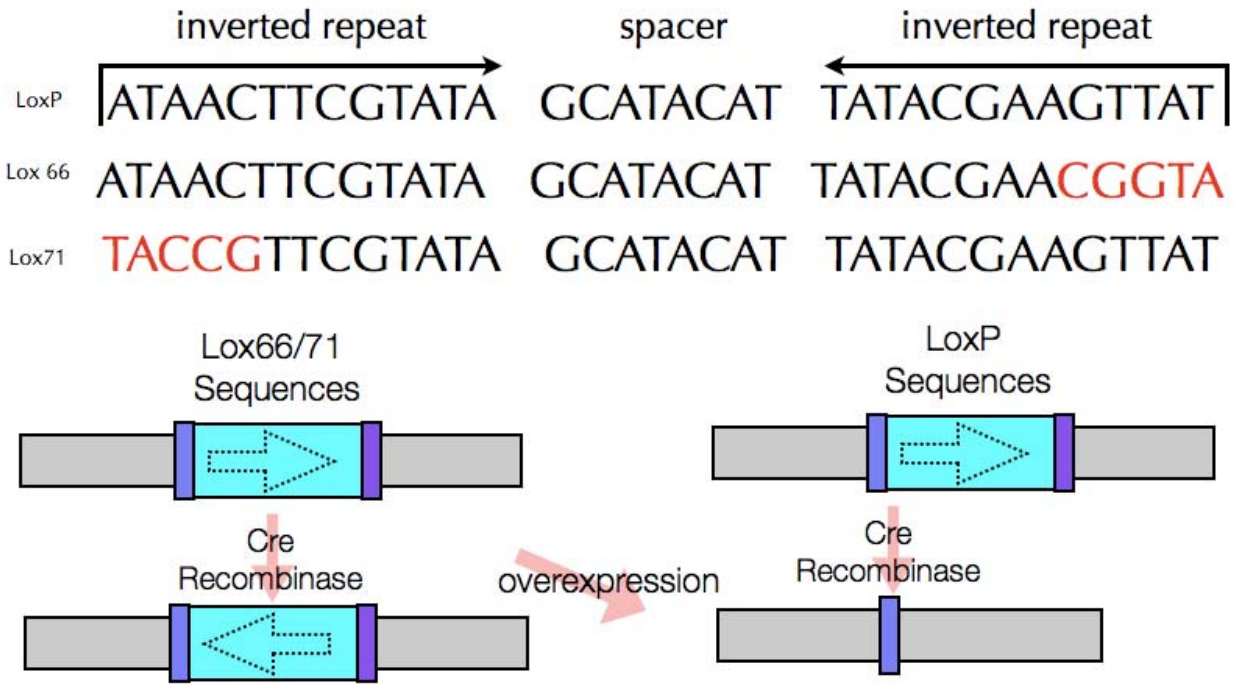

Figure 16: Sequence of wild type and mutant LoxP sequences and how they recombine.

LoxP sites consist of tandem 13-base pair inverted repeats linked by an 8-base pair spacer sequence. Cre-recombinase recognizes these sequences and either deletes the intervening sequences or inverts the sequences.

LoxP sequences were inserted into PCR primers for amplification of LMP2b exon1'. This product was inserted into a shuttle vector via integrated Bpu101 and Sand1 restriction enzyme sites and then used for recombination into BAC plasmids. The bacteria containing the BACs were transfected with an additional plasmid with a temperature sensitive origin of replication that encodes Cre recombinase. Once transfected, double positive colonies were selected based upon the resistance of both the Cre recombinase plasmid and the BAC, and incubated at 30°C for 48 hours. Once double positive colonies were selected, they were replica plated and incubated at 30°C

and 42°C. Colonies that were able to grow at 30°C, but not 42°C were assayed by PCR for recombination or inversion of the floxed sequences. Once there was positive verification, the bacteria containing the recombined BAC were induced to lose the Cre Recombinase plasmid by growth in media containing only the resistance marker for the BAC at 42°C. The resultant BACs had a deleted LMP2b exon1', with only a single mutant LoxP site in it's place.

Due to the use of multiple LoxP sequences in the BACs, occasionally the LoxP recombination was done in the shuttle vector, and then recombined into the BAC backbone. This method was used when constructing 2190.3. The shuttle vector containing the amplified LMP2b exon1' that was flanked by the LoxP sites, was introduced into bacteria, and recombined by the expression of Cre recombinase. This bacteria was then grown at the non-permissive temperature. The recombined shuttle vector (now without the genomic sequences for LMP2b exon1') was used for recombination into the BAC.

4.2.3.5 Sequencing:

2 BAC constructs were sequenced by Seqwright (Houston, TX). 2190.2 was chosen because it contained the most manipulations, but was still assumed to have a wild type phenotype. 2525.3 was chosen because it lacked both LMP2 isoforms, and was necessary to note the intactness of other genes involved with immortalization.

4.2.4 Analysis of LCLs Generated from BAC Generated EBV:

4.2.4.1 Flow Cytometry:

Anti-CD23 (Alexa 647; Biolegend), Anti-CD38 (PerCp-Cy5.5; BD Biosciences), Anti-CD69 (Pacific Blue; Biolegend), Anti-CD138 (PE; BD Biosciences), Anti-HLA-DR (PE-Cy7; BD Biosciences) and Anti-Ki67 (PE; BD Pharmingen), were used for analysis of antigens. Staining was done as previously described [196]. Staining was performed in PBS containing 2% FBS (Gibco) and 0.02% NaN_3 (with the addition of 0.5% Saponin for intracellular staining). Stained cells were fixed with 2% paraformaldehyde for flow cytometric analysis via a BD FACSAria Cytometer. The population of cells viable for analysis was determined by gating according to a typical forward and side scatter for the cells observed. Isotype-matched fluorochrome conjugated antibodies were used to as isotype controls. Compensation was done using the appropriate Compensation Particles Set (BD) and the automatic compensation calculation within FacsDiva software. Each experiment was performed a minimum of three times. Results were analyzed using FlowJo software. Cells expressing EGFP were sorted based on EGFP expression using a FACSAria (BD) and the low pressure sort profile. Cellular debris and doublets were discriminated in gating.

4.2.4.2 Immunoflorescence:

Anti-FLAG M2 Cy3 was purchased from Sigma. Mounting media containing DAPI was purchased from Vector Laboratories. Immunofluoresence was performed by fixing cells to polylysine coated glass slides (Fisher) using a cytospin (Shandon) set at 500 r.p.m for 5 minutes. Slides were dried for 1 hour at room temperature in the dark. Cells

were then fixed using freshly prepared 3.7% paraformaldehyde for 1 minute at room temperature. Samples were blocked for 30 minutes using SuperBlock blocking buffer in PBS (Pierce). Primary antibodies were incubated at pre-determined dilutions in blocking buffer for 30 minutes at room temperature. Slides were washed 3 times in cold PBS. Slides were allowed to air dry in the dark for 30 minutes. Slides were mounted with Vectashield-mounting media containing DAPI (Vector Laboratories).

4.2.4.3 Microscopy:

Slides were examined with a Nikon E600 microscope. Digital images were captured via a Nuance Multispectral Imaging system, and analyzed using Nuance 2.4.2 software (CRI, Inc, Woburn, MA). Final image montages were constructed using Adobe Photoshop.

Plates and dishes of cells were examined with a Nikon TS100 microscope fitted with a SPOT Insight 2 camera, and captured using the SPOT Advanced software 4.0.5 (Diagnostic Instruments, Inc).

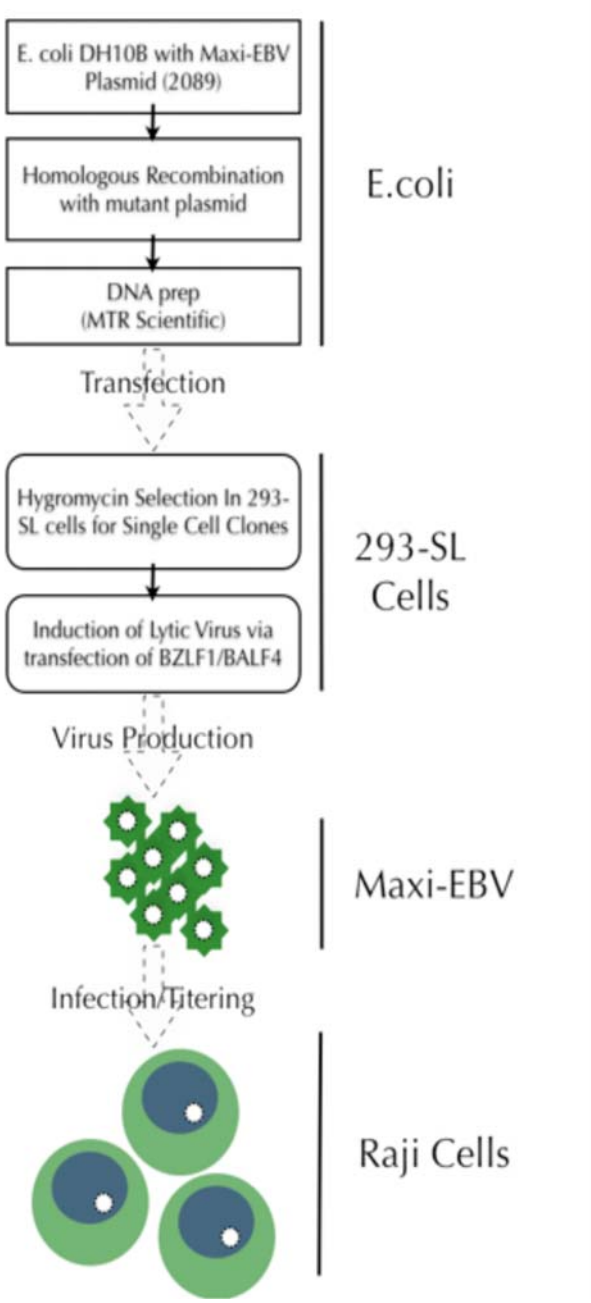

Figure 17: Overview of BAC Production Process

4.3 RESULTS:

4.3.1 BACs generated for this study:

Table 3: BAC constructs for production of recombinant EBV used for further study.

Numerical Designation	Features of Interest	Proteins Produced
2089.0	B95.8 EBV backbone with EGFP and Hygromycin resistance cassette	LMP2a/LMP2b
2089.1	2089.0 with a 3xFLAG in Loop 11 of LMP2	LMP2aF11/LMP2
2089.3	2089.1 with a deleted 2b exon1 via cre recombination around LMP2b exon 1	LMP2aF11
2190.2	2089.1, with loxP sites flanking LMP2a exon 1 and loxP sites flanking LMP2b exon 1	LMP2aF11/LMP2
2525.0	2089.0, with the intervening sequences between a loxP flanked LMP2a exon 1 deleted	LMP2b
2525.2	2525.0, with a 3xFLAG in Loop 11 of LMP2 and loxP sites flanking LMP2b exon 1	LMP2bF11
2525.3	2525.2, with a deleted 2b exon1	NO LMP2
2525.4	2525.0 with a 3xFLAG in Loop 11 of LMP2 a/b and M to C mutation at start of exon 2.	NO LMP2

Base BAC Vectors (2089.0, 2190.0, and 2525.0) and base shuttle vectors were generated by Marcus Altmann in the Hammerschmidt lab (Table 3 and appendix A).

Shuttle vector mutation and consequential versions of BAC vectors were prepared specifically for this study.

Because of the redundancy of the design, only selected BAC constructs were used for further characterization (Table 3). Other BAC vectors were created, but not used for further characterization (Appendix A)

4.3.2 The generated BACs have predictable RFLP patterns and sequences:

To determine the structure of the generated BACs, we purified the plasmid DNA from E. coli. Purified DNA was subjected to restriction digest by BamHI or HindIII and compared to the predicted sequences. HindIII was particularly useful in this analysis, because our thrice-repeated FLAG epitope has a HindIII site engineered in between the first and second repeats. This allowed us to easily determine clones containing FLAG-3 Loop-11 containing clones.

In BACs that contained insertions that added a HindIII site, or deleted sequence via Cre recombination, we were able to confirm sequences by the RFLP. For instance, the band shift in sequences from 2190.0 to 2525.0 allowed us to determine the deletion of the first exon of LMP2a by the decrease in size of a band. Additionally, regional features of EBV DNA (such as repeats at 3072bp) could be verified for intactness.

All of the purified DNA was subjected to this analysis. In cases where the band shift was too small to be identified (e.g. a decrease of ~100 bp in a 11K band) PCR analysis was used for verification.

Because of the large size of the BACs, it was prohibitive to sequence all of the clones. We chose to sequence 2190.2 and 2525.3. 2190.2 was chosen for sequencing

because it had the largest amount of manipulation (e.g. insertion of 2 sets of LoxP sites & insertion of FLAG) but was still anticipated to have a wild type phenotype in LCLs. 2525.3 was chosen because it lacked expression of both LMP2 isoforms. Rearrangements or deletions in either of these clones would have been detrimental to our results, and as such, they were sequenced.

4.3.3 Characterization of LCLs Generated from BAC-derived EBV:

An attempt was made to generate LCLs from all of the viruses produced. An initial scheme of using a virus MOI of either 3 or 5, cell number, and treatments were used to determine which viruses were able to produce LCLs.

Infected cells could be identified, via EGFP expression, in culture 2-4 days post-infection. Cells started exhibiting characteristics of LCLs (clumping and morphological changes) 5-14 days post infection, depending on virus used. Single cell clones could be used for generation of cell lines at 8-12 weeks. This information is summarized by virus in Table 4.

Table 4: Summary of LCL outgrowth characteristics

Virus	LMP2 Produced	EGFP Expression	Clumping	Cell Line
2089.0	LMP2a positive LMP2b positive	2 days	5-7 days	8 weeks
2089.1	LMP2aF11 positive LMP2bF11 positive	2 days	7-9 days	8 weeks
2089.3	LMP2aF11 positive *LMP2bF11 negative*	3 days	14-16 days	16 weeks
2190.2	LMP2aF11 positive LMP2bF11 positive	3 days	5-7 days	12 weeks
2525.2	*LMP2aF11 negative* LMP2bF11 positive	3-4 days	14 days	Did not survive
2525.3	*LMP2aF11 negative* *LMP2bF11 negative*	3-4 days	14 days	Did not survive
2525.4	*LMP2aF11 negative* *LMP2bF11 negative*	3-4 days	14 days	Did not survive

We were unable to generate LCLs from LMP2a negative viruses at this MOI. We focused on characterization of the 2089.0, 2089.1, 2190.2, and 2089.3 virus generated LCLs.

We had to initially show that in these cell lines, LMP2a and/or LMP2b was produced and able to be detected with the FLAG antibody via immunoflourescence and flow cytometry (§5.5.1). LMP2a and LMP2b, in the case of 2089.1 and 2190.2, and

LMP2b, in the case of 2089.3, could be detected in all of the clones with an integrated FLAG epitope in Loop 11 (Figure 18). Positive staining in 2089.3 shows virally produced LMP2b, as that virus lacks LMP2a exon 1 via LoxP recombination.

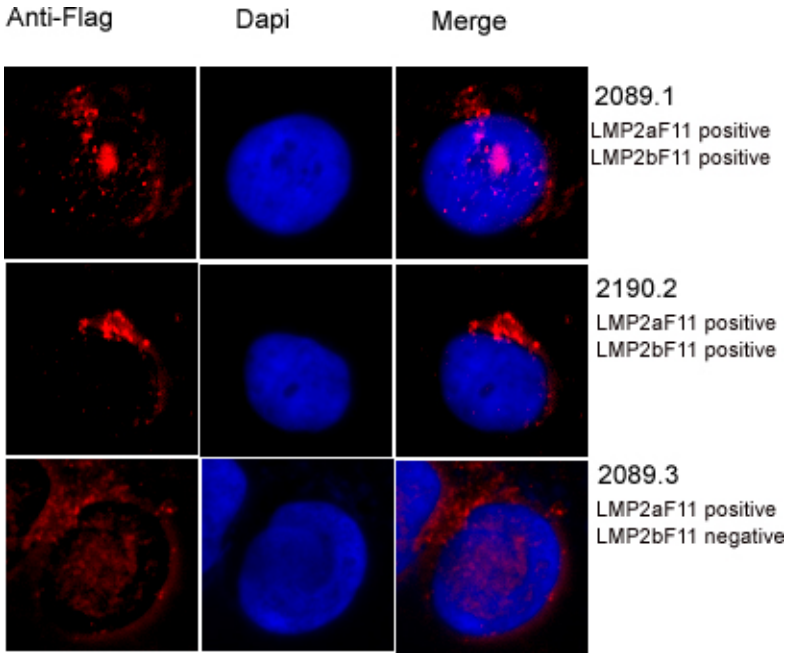

Figure 18: FLAG Staining in BAC derived LCLs

LCLs generated by infection of BAC derived virus were permeablized and stained for the expression of LMP2a and/or LMP2b via an anti-FLAG Cy3 conjugated antibody. LMP2a and/or expression is indicated by red (left and right panels), a Dapi containing mounting media was used for counterstaining of the nuclei (center and right panels). 2089.1 and 2190.2 express LMP2a and LMP2b, 2089.3 expresses LMP2b.

We then identified a panel of antibodies for immunophenotyping via flow cytometry. These antigens were selected based upon predicted qualities of the LCLs. Activation, maturity, and proliferation markers were analyzed by flow cytometry.

2089.0, 2089.1 and 2190.2 were all anticipated to be wild type in expression of these proteins. 2089.3, lacking LMP2a, showed a similar phenotype.

Table 5: Immunophenotyping of LCLs.

	2089.0	2089.1	2089.3	2190.2
	LMP2a positive	LMP2aF11 positive	LMP2aF11 positive	LMP2aF11 positive
	LMP2b positive	LMP2bF11 positive	*LMP2bF11 negative*	LMP2bF11 positive
CD23	High	High	High	High
CD38	Int	Int	Int	Int
CD69	Low	Low	Low	Low
CD138	Low	Low	Low	Low
HLA-DR	High	High	High	High

High: ≥ 4 log shift MFI from isotype; Low: ≤1 Log shift MFI from isotype; Int: log shifts between High and Low.

LCLs lacking LMP2b (2089.3 clones) grew at a substantially slower rate than other cell lines. In a comparison of doubling times, 2089.3 took 5 days to double in number, whereas cell lines expressing LMP2b have average doubling times of 2 days. The LCLs were stained for Ki-67, a cellular marker for proliferation that is preferentially expressed during G_1, S, G_2 and M phases of the cell cycle. Cells that are negative for Ki-67 are in G_0 and are not proliferating. LMP2a and LMP2b expressing cells showed a typical profile of a population of cells in different stages of proliferation (Light Grey). These cells showed 2 major peaks, non-Ki-67 expressers and Ki-67 expressing-cells, indicating that the cellular population was at varying stages of the cell cycle. 2089.3 clones, dark grey (Figure 19), showed a different profile, showing cells that are predominately Ki-67 negative, indicating that the cells were largely not proliferating. (Figure 19) Additionally, 2089.3 cells had a slightly different morphology (larger cytoplasmic volume) consistent with failure to divide.

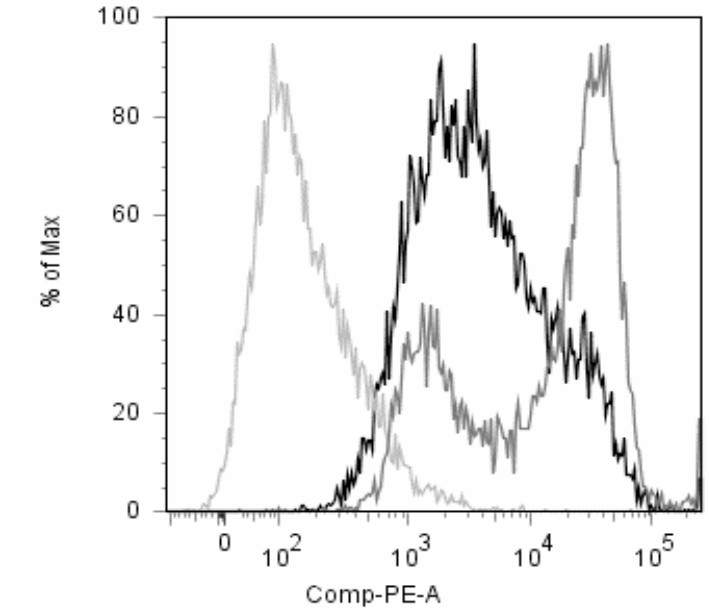

Figure 19: Ki-67 staining in BAC derived LCLs

LCLs infected with either LMP2a/b expressing virus (mid-grey), or a LMP2b negative virus (dark grey) were stained for the expression of Ki-67, a proliferation marker. LCLs generated from LMP2b negative viruses showed a marked difference of Ki-67 expression as compared to wt virus. Light grey: Isotype control.

Initial experiments used an MOI of 3 or 5 to establish LCLs. Not every virus was able to establish lines at these MOI. Because of this, we undertook a study of the immortalizing titer of virus. Most specifically, viruses lacking LMP2a (2525.2, 2525.3.and 2525.4) seemed to be unable to immortalize cells, while viruses lacking LMP2b (2089.3) seemed to be inefficient at the immortalization process. By increasing the titer, we demonstrate that LMP2a is indeed necessary for immortalization and that while LMP2b is not necessary, it confers an advantage.

PBMCs were infected with 2089.0, 2089.1, 2089.3, 2190.2, 2525.2, 2525.3, and 2525.4 at an MOI of 3, 10, 30, 50, 100 or 500 green Raji units (GRU) per estimated B cell (10% of the PBMCs). All cells used were from the same patient. 25 wells of 5000

PBMCs with cyclosporine A were used as the 'spontaneous LCL control'. At least 3 replicates were made for each virus/MOI combination.

By day three post-infection, cells had turned green in each of the experimental wells. As the MOI increased, green cells were more frequent indicating that there was a higher availability of virus. By day 5 PI, cells infected with wild type and LMP2a negative (2089.0, 2089.1, 2190.2, 2525.2, 2525.3, and 2525.4) viruses had started to proliferate. Cells infected with virus lacking LMP2b (2089.3) showed cell clumping and aggregates at day 8.

By day 14, cell lines infected with viruses 2525.3 and 2525.4 (both lacking LMP2a and LMP2b) had ceased to increase in green cell number (i.e. the proliferation of cell clumps had halted at approximately 50-75 cells per clump). Cells infected with 2089.3 (MOI 50-500) and 2525.2 (MOI 500) were substantially slower in growth than their wild type counterparts. On day 15, all cell lines had 50 ul of additional media added to each well. By day 20 all of the cells infected with LMP2a/b negative viruses (2525.3 and 2525.4) had stopped growing and their cell membranes started to rupture.

In contrast to the LMP2a and/ or LMP2b negative virus infected cells, at day 21 the cells infected with wild type virus (2089.0, 2089.1 and 2190.2) in the wells corresponding to MOIs of 100 and 500 had to be removed from their wells to be placed in a larger well.

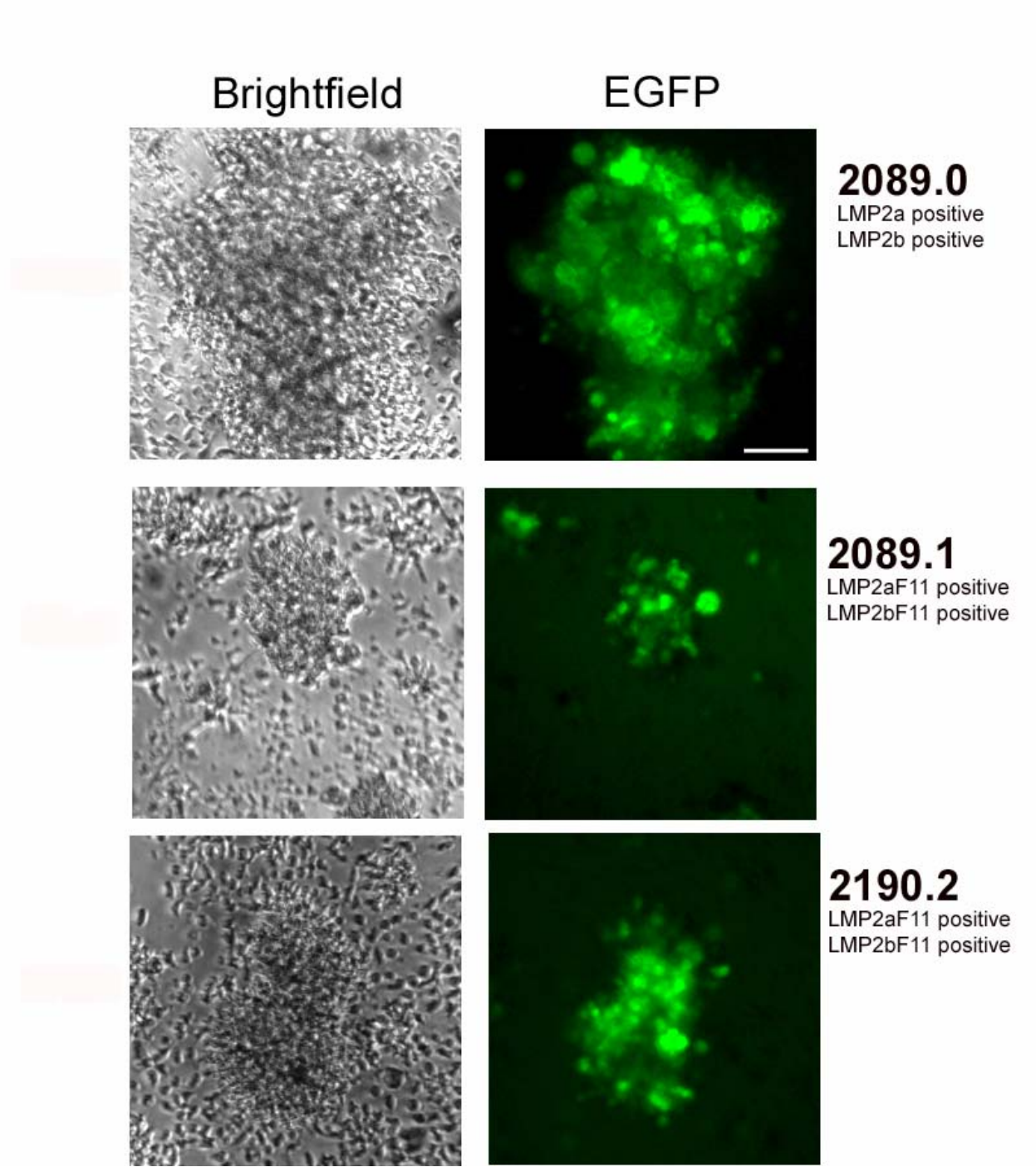

Figure 20: LCL formation with wild type virus, day 18.

Cells infected with viruses expressing both LMP2a and LMP2b show LCL formation at day 18. Cells are in wells corresponding with an MOI of 50. These micrographs show typical size and growth characteristics as in other replicates. Bar, 20μ.

At day 28, 2525.2 (LMP2a negative, LMP2b positive) started to show an increase of growth in green cells. Green cells in the wells corresponding to MOI 500, were developing increasingly larger LCL ‘clumps’. Cells infected with 2089.3 (LMP2a positive, LMP2b negative) virus were exhibiting growth in wells with MOIs 50 or greater.

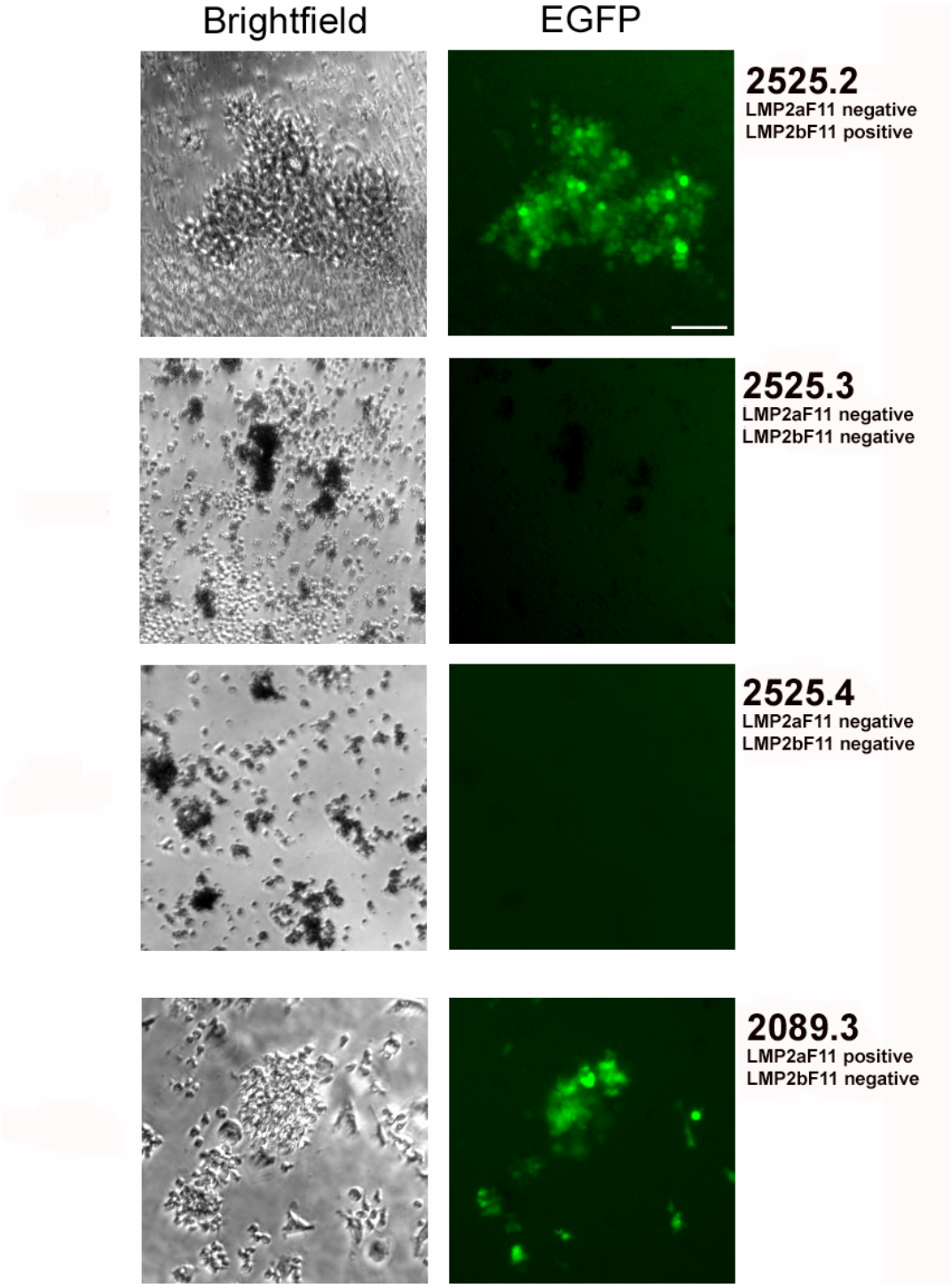

Figure 21: LCL formation in cells infected with virus lacking LMP2, day 31.

Cells infected with viruses lacking LMP2a (2525.2), LMP2b (2089.3), or both (2525.3 and 2525.4). LCL formation is only apparent in cells having at least one isoform of LMP2. Cells are in wells corresponding with an MOI of 500. These micrographs show typical size and growth characteristics as in other replicates. Bar, 20μ.

At day 35, wells with cells that had been infected with LMP2a and LMP2b negative virus (2525.3 and 2525.4) had failed to proliferate at any MOI. These wells contained exclusively cellular debris. Wells that had been infected with LMP2a negative virus (2525.2) had established LCLs in the wells corresponding to and MOI of 500. Cells that had been infected with the LMP2b negative virus (2089.3) had demonstrated outgrowth at MOIs of 50-500. All of the wild type viruses (2089.0, 2089.1 and 2190.2) were able to generate LCLs from all wells. No LCLs were generated from uninfected controls.

The results of this experiment are summarized in Table 6.

Table 6: Summary of Immortalization Titer for EBV Lacking LMP2.

Virus	Replicates	Smallest MOI at which cells are green (days post-infection)	MOI at which LCLs are generated (days post-infection)
2089.0	8	3(2)	3(35), 10 (35), 30 (30), 50 (28), 100 (20), 500 (20)
2089.1	8	3(2)	3(35), 10 (35), 30 (30), 50 (28), 100 (20), 500 (20)
2190.2	8	3(3)	3(35), 10 (35), 30 (35), 50 (31), 100 (22), 500 (22)
2089.3	8	10(5)	50 (31), 100 (22), 500 (22)
2525.2	3	3(3)	100 (35), 500 (21)
2525.3	3	3(3)	Failed to proliferate
2525.4	4	3(3)	Failed to proliferate

4.4 DISCUSSION

This work describes the contribution of both LMP2a and LMP2b to immortalization. This experimental system, based upon an already established 'Maxi-EBV system', was beneficial because it allowed for redundancies in the mutation scheme which in turn allowed us to determine the contribution of both LMP2a and LMP2b, individually and in concert, to immortalization of primary B cells.

The wild-type (2089.0) and modified, but phenotypically wild-type (2089.1 and 2190.2) viruses demonstrated that modifications to the genome did not significantly interfere with virus production, infectivity, or the process of immortalization. All of these viruses had similar kinetics of infection and were able to produce immortalized cells at similar rates and of similar phenotypes. Additionally, they produced virus from producer cell lines at similar titers.

The virus that lacked LMP2b (2089.3), via LoxP recombination of exon 1', was able to infect cells at a similar frequency as the wild-type viruses. This virus did need a slightly higher MOI for establishment of an efficient immortalization process (MOI of 3 for wild type viruses, versus a MOI of 50 for 2089.3). This virus produced infected cells that were growth deficient. The LMP2b negative cells grew at a substantially slower rate than the wild type cells, with a doubling time of almost 2x as long. The amount of virus needed for establishment of immortalized cell lines and the slow growth rate indicates that LMP2b has some function involved in the mechanism of how EBV generates transformed cell lines.

After our initial experiments, we did not think that virus lacking LMP2a would be able to generate cell lines. Our collaborators demonstrated that in a 6 week assay, they

were able to detect proliferating cells at an extrapolated MOI of 36.4 using 2525.0 virus.[190] Similar results were achieved in our immortalization assay. We saw outgrowth at MOIs of 100 and 500. We ascribe the slight increase in MOI calculations to the modified experimental set up, however we demonstrated similar results with these different designs in the wild type viruses (both experimental set-ups were able to produce proliferating cells at an MOI of 3). This increase of 34-fold virus to induce proliferating cells indicates that LMP2a is, in fact, necessary for immortalization.

The viruses lacking both LMP2a and LMP2b (either by loxP recombination, or via an M to C mutation for LMP2b) were unable to generate LCLs at any MOI. These viruses were able to efficiently infect cells, and the frequency of infection was similar to that of wild type (via EGFP expression). For the first ~2 weeks post-infection, these cells show morphological changes and proliferate, however there seems to be a critical point in which the cells cease proliferating and start dying. Proliferating cell clumps seem to reach a checkpoint around 50-75 cells, at which point cells start losing membrane integrity and start to die. No additional amount of media or virus seems to be able to counteract this phenomenon, indicating that the cells are lacking are lacking something that would normally be provided via the LMP2a/LMP2b proteins.

Our method of quantitative analysis of the virus needed to generate proliferating cells 5 weeks post-infection, and qualitative information regarding the morphological, phenotypic and growth characteristics of the resulting cells indicates that both LMP2 isoforms have a role in immortalization of B cells. While LMP2b seems to provide an signal that is perhaps more necessary for the maintenance of immortalized cell lines, LMP2a clearly provides a necessary signal for the establishment of immortalization

5.0 SPECIFIC AIM THREE:

5.1 PREFACE:

The following work describes a function for LMP2b without interference from LMP2a. This information provides initial characterization of how LMP2b interacts with exclusively cellular proteins, and how LMP2b affects these proteins.

This manuscript was submitted for peer review to Virology.

Epstein Barr Virus Latent Membrane 2b Inhibits B-cell Receptor Signaling and Promotes Pro-Apoptotic Gene Expression

Monica Jo Tomaszewski-Flick1, Sherrianne M. Gleason1,2, & David T Rowe1

1Department of Infectious Diseases and Microbiology

Graduate School of Public Health

University of Pittsburgh

130 DeSoto St, Pittsburgh, PA 15213

2Center for Vaccine Research

University of Pittsburgh,

Pittsburgh, PA, 15213

*Corresponding Author +1 412 624 1529 (phone), +1 412 383 7490 (fax), rowe1@pitt.edu

running title: LMP2b De-regulates Protein Phosphorylation

5.2 ABSTRACT:

Epstein-Barr virus (EBV) latent membrane protein 2a (LMP2a) modulates B cell receptor (BCR) signaling by excluding the BCR from lipid rafts, and by destabilizing signal transducers lyn and syk through interactions with its N-terminal ITAM domain. Latent membrane protein 2b (LMP2b), which is identical to LMP2a, but lacks the N-terminal signaling domain, has a less well-defined role in this pathway. Here we demonstrate that enforced expression of LMP2b in BJAB cells had inhibitory effects on BCR signaling in the absence of LMP2a expression. Cells that expressed LMP2b were deficient in their ability to phosphorylate signal transducers after BCR crosslinking. Serine phosphorylation levels on pro-survival proteins were constitutively elevated in LMP2b expressing cells and expression of pro-apoptosis and pro-survival genes was altered. These observations demonstrate that LMP2b possesses a unique functional role in regulating BCR signal transduction. This activity was not dependent on LMP2a co-expression; therefore it could not be the consequence of LMP2a negative regulation. In addition, the chronic pro-survival phenotype of LMP2b-expressing cells provided an insight into the instability of establish cell lines forced to persistently express LMP2b. LMP2b functions (other than negative regulation of LMP2a) may be critical for the establishment or maintenance of EBV latency.

5.3 INTRODUCTION:

Epstein-Barr Virus (EBV) is a member of the human gammaherpes family of viruses. EBV is associated with several disease states including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma and post-transplant lymphoproliferative disease[197]. EBV establishes a latent infection in circulating B cells and undergoes periodic reactivation. In vitro, EBV infection of B cells induces proliferation of the infected cells that can be subsequently grown as a lymphoblastoid cell line (LCL). LCLs express a limited number of viral gene products with functions associated with episome maintenance and cell survival. This latent state is thought to be maintained by functions of LMP2a that block induction of lytic replication in response to signals produced by BCR crosslinking.

The LMP2 gene encodes two separate membrane protein isoforms via alternate promoter usage. These promoters, separated by approximately 3 kb, both produce transcripts that cross the fused terminal repeats[198]. The mRNAs have unique 5' first exons and 8 shared 3' exons. The LMP2a open reading frame encodes a 497 aa protein with a 119 aa N-terminal cytoplasmic signaling domain (CSD), 12 transmembrane (TM) segments and a short 27 aa C-terminal tail. The LMP2b open reading frame encodes a 378 aa protein identical to LMP2a but lacking the 5' CSD [151].

Although the importance of LMP2a in BCR signal blocking has become clearer, a role for LMP2b in the process is only now starting to emerge[199]. Most studies have

focused on LMP2a only, owing to the presence of the CSD and due to the availability of an antibody that detects it[165, 200]. Recent studies have reassessed the localization of LMP2 gene products, and conceptually changed the cell location from a predominantly plasma membrane association to an intracellular Golgi-like location[166, 167]. LMP2a and LMP2b were shown to co-localize to the same compartment. Experimental data on LMP2b suggests that there is a specific interaction with truncated LMP2 molecules[166, 169]. LMP2b has been postulated to be a negative regulator of LMP2a based on sequence similarity and the lack of an obvious signaling domain. Co-expression of LMP2b with LMP2a restores the ability (i.e. unblocks the LMP2a block) of the BCR to transduce signals in transfected cells[160], and concomitantly increases the ability of the viral episome to enter the lytic replication cycle[200]. In this report we demonstrate LMP2b signaling-related functional activity in the absence of LMP2a indicating that this protein may have a significant and independent role in B cell infections.

5.4 METHODS AND MATERIALS:

5.4.1 Cell Lines, Plasmids, and Transfections:

BJAB is an EBV-negative Burkitt lymphoma cell line. BJAB were maintained in complete RPMI -1640 medium containing 10% inactivated Foetal Bovine serum, 2mM glutamine, 60 µg/ml, 200 µg/ml streptomycin at 37 ºC in 5% CO2.

DNA fragments encoding full-length LMP2b were constructed as previously described [166]. 5' primers contain an Xho1 restriction site. 3' primers contain a Kpn1 restriction site. PCR products were digested with Xho1/Kpn1 and ligated into pC1-EGFP (Clontech). Constructs were sequenced for verification. All DNA used for transfections were purified on $CsCl_2$ gradients. To establish B cell lines, $5x10^6$ cells were washed once in 4 ºC PBS and resuspended at room temperature in 0.4 ml of serum- and phenol red- free RPMI 1640 containing 20 µg of the required plasmid. Cells were then transferred to a sterile electroporation cuvette (0.4 cm electrode gap) (BioRad) and pulsed twice. Multiporator (Eppendorf) settings were 400 V for 100 µS. Cells were allowed to settle for 10 minutes in a 37 ºC water bath. Cells were removed from the cuvette and added to 10 mls of complete phenol red-free RPMI 1640. Stable cell lines were established by adding 600ug/ml G418 to the cell culture media.

5.4.2 Immunofluorescence and Microscopy:

Immunofluoresence was performed by fixing cells to polylysine coated glass slides (Fisher) using a cytospin (Shandon) set at 500 rpm for 5 minutes. Slides were dried for 1 hour at room temperature in the dark. Cells were then fixed using freshly prepared 3.7% paraformaldehyde for 1 minute at room temperature. Samples were blocked for 30 minutes using SuperBlock blocking buffer in PBS (Pierce). Primary antibodies were incubated at pre-determined dilutions in blocking buffer for 30 minutes at room temperature. Secondary antibodies (Alexa-fluor 555, Molecular Probes) were diluted in blocking buffer (1:2000 dilution) for 30 minutes at room temperature. Slides were washed 3 times in cold PBS. Slides were allowed to air dry in the dark for 30 minutes. Slides were mounted with Vectashield-mounting media containing DAPI (Vector Laboratories). Anti-Golgi 58K protein was purchased from Sigma. Anti-IgM and anti-CD 19 were purchased from BD Pharmingen.

5.4.3 Flow Cytometry:

Anti-IgM, anti-CD19 (BD Pharmingen) and anti-CD79beta (Genetex) were used for surface analysis of antigens. Staining was done as previously described [196]. Stained samples were washed and resuspended in PBS containing 2% FBS (Gibco) and 0.02% NaN_3. Stained cells were fixed with 2% paraformaldehyde for flow cytometric analysis via a Coulter EPICS XL-MCL Flow Cytometer. The population of cells viable for analysis was determined by gating according to a typical forward and side scatter for

the cells observed. Isotype-matched fluorochrome conjugated antibodies were used to as isotype controls. Each experiment was performed a minimum of three times. Results were analyzed using FlowJo software. N1-BJAB and LMP2b-BJAB cells were sorted based on EGFP expression using a FACSAria (BD) via the low-pressure sort profile. Cellular debris and doublets were discriminated in gating.

5.4.4 BCR Crosslinking:

Biotin conjugated Anti-Ig K and biotin conjugated Anti-CD19 were purchased from BD Pharmingen. Avidin was purchased from Zymed laboratories.

10^7 cells were washed 2x in cold PBS then resuspended in cold, serum-free media. Antibodies were added at a concentration of 40ug/ml and incubated on ice for 30 minutes. Cells were spun at 250xg for 3 minutes and resuspended in 37C media containing 20ug/ml avidin. Time points were taken before the avidin was added, a soon as possible after avidin addition, and every 4 minutes thereafter (max t=20 minutes). At each timepoint, cells were pelleted, resuspended in lysis buffer, and divided into aliquots for western blot and array analysis.

5.4.5 Phosphoprotein Analysis:

For phosphoprotein analysis, samples were separated by SDS-PAGE with a phosphoprotein molecular weight standard (PeppermintStick, Molecular Probes). The

gel was stained with Pro-Q Diamond Phosphoprotein Gel Stain. The gel was visualized using the pseudocolor contour capabilities of the Image Gauge software, where blue=low and red=high intensities. Bands were thresholded on efficient detection of the positive control marker (center lane, PS).

5.4.6 Antibody Arrays:

Custom antibody arrays were purchased from Hypromatrix (Boston, MA). After cell crosslinking, 10^8 cells were lysed in 15mM Tris-HCl ph7.5, 120 mM NaCl, 25 mM KCl, 2mM EDTA, 2mM EGTA, 0.1 mM DTT, 0.5% Triton x-100 and Protease Inhibitor Cocktail Tablets (Roche). Protein concentration was measured using the BCA Protein assay kit (Pierce) and concentrations were equalized for each time-point. Lysates were incubated with the antibody arrays for 2 hours with slow shaking. The membranes were washed 3x15 minutes in TBST. The levels of phosphorylation were detected using either a biotin conjugated anti-phosphotyrosine antibody (Cell Signaling Technology), or a alkaline phosphatase conjugated anti-phosphoserine antibody (Abcam) that was developed with an AP:streptavidin complex (Molecular Probes) and DDAO deposition then imaged with an FLA2000 Fuji phosphoimager. Both sets of arrayed membrane preparations were incubated for identical times with the developer and scanned at the same time. Background subtracted intensity was measured relative to negative controls. Relative light unit measurements were imported into ArrayAssist (Stratagene) and heat maps were generated.

5.4.7 Gene Profiling Arrays:

The RT2 Profiler PCR Array for Human Apoptosis was purchased from SuperArray (Frederick, MD) and performed as per manufacturers instructions on an ABI 7500 (Applied Biosystems, Inc. C(t) values were converted to relative DNA copy numbers, then normalized to the signal derived from GAPDH, a housekeeping gene. The normalized data were then compared between the different groups, and the relative expression of each gene was reported as fold change. When comparing measurements between arrays, we used a 2-fold or more increase to indicate “stringent” criteria for change. [201]

5.4.8 shRNA:

The Mission eGFP shRNA Vector (Sigma) was transfected into HEK293T cells via GeneJuice (Novagen). Stabile expression of the vector was selected for by adding 1ug/ml of Puromycin to the culture media. Virus production was performed as per the manufacturers directions. The amount of virus produced was measured using p24 counts. Infectious units were measured by infection of BJAB cell expressing EGFP. Day 2 post-infection counts of cells expressing low, or no amount of EGFP were counted. Cells were infected with the lowest amount of virus to knockdown expression of EGFP

in 90% of cells (usually an MOI of 3). These cells were sorted, collected, and subjected to flow cytometry and microscopy to determine the amount of knockdown of EGFP as compared to non-infected cells.

5.5 RESULTS:

5.5.1 LMP2b Localizes to the Golgi Network:

We hypothesized that LMP2b may have a unique functional role that is independent of LMP2a expression and function. To examine LMP2b effects on cellular behavior, we sought to establish LMP2b-expressing cell lines by transfecting an N-terminally EGFP-tagged LMP2b expression vector into the EBV-negative cell line BJAB. Attempts to establish these cell lines were frequently undermined by slow growth and rapid loss of LMP2b expression, even under stringent marker selection, indicating that the expression of LMP2b exerted a negative effect on the proliferation of transfected cells. To maintain LMP2b expression above 80% in the cultured cells, they were regularly sorted for EGFP fluorescence. EGFP-tagged LMP2b in the BJAB-LMP2b cells showed similar expression levels when compared to FLAG-tagged LMP2 produced in virus immortalized LCLs. (Figure 22A) The intracellular localization of LMP2b, which requires all 12 TMs, was examined in live (EGFP fluorescence) and fixed cells (by immunofluorescence) and by Western blotting to confirm that LMP2b remained intact (Figure 19B and data not shown). Cells were stained for IgM, CD19, and 58K Golgi Protein and imaged using confocal microscopy. When the images for IgM (surface, red) and LMP2b (green) were layered, overlap between the two markers was not detected indicating that, as expected, LMP2b was not expressed at the cell surface. In contrast,

when the images for 58K Golgi Protein (a golgi marker, red) and LMP2b (green) were layered, there was significant co-localization (indicated by yellow). These images demonstrate that the localization and interactions of EGFP-LMP2b in the sorted BJAB-LMP2b cells were identical to that previously reported for wt LMP2b.

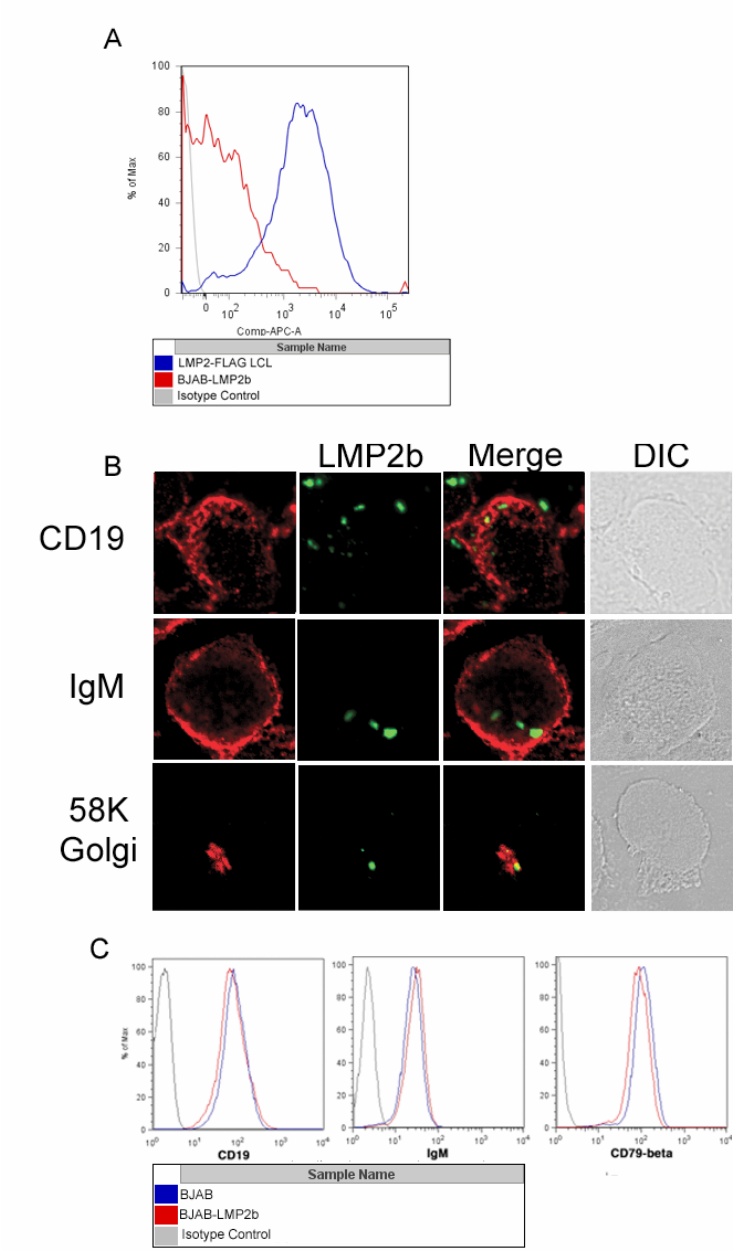

Figure 22: LMP2b localization and impact on surface expression of BCR associated proteins.

A) BJAB-LMP2b cells (red), and a LCL (blue) immortalized with a wtEBV that has a FLAG-tagged LMP2b were all stained for FLAG expression and analyzed by Flow Cytometry. B) Two color immunofluorescence of LMP2b (green) with CD19, IgM or 58K Golgi (red). In permeablized cells LMP2b shows overlap (yellow, merge) with 58K Golgi (bottom panels). C) BJAB-LMP2b cells show similar expression of proteins associated with the B-cell receptor complexes at the cell surface. Isotype (grey), BJAB cells (blue), BJAB-LMP2b cells (red).

Previously, we described an intracellular aggregation of LMP2b and CD19, suggesting that LMP2b may influence CD19-dependent signaling by decreasing the cell surface availability of CD19 via intracellular sequestration. To determine whether LMP2b/CD19 intracellular interactions influenced the surface expression of CD19 (and other molecules that form complexes with CD19 that are involved in BCR signaling) we measured cell surface expression of these proteins in LMP2b positive and LMP2b negative cell lines via flow cytometry (Figure 22C). CD19, IgM, and CD79beta all had similar surface expression levels in BJAB and BJAB-LMP2b cell lines. Therefore, none of these proteins were depleted from the cell surface by LMP2b and all were presumably available for participation in the BCR-signaling complexes indicating that signaling is probably not affected by sequestration and may be affected by some other process.

5.5.2 LMP2b inhibits signal transduction by BCR:CD19 crosslinking:

In order to determine whether LMP2b expression had any effect on B cell signal transduction, we performed a time course study of protein phosphorylation after BCR:CD19 cross-linking using the BJAB and BJAB-LMP2b cell lines. Surface receptors were cross-linked with antibodies, and equal aliquots of cells were harvested at 4 min intervals over a 20 min period. These aliquots were used for electrophoretic analysis via Western blot imaging of phosphorylated and total protein. BJAB cells (Figure 23, right) had low basal protein phosphorylation and showed a steady increase in phosphorylated proteins over time, with a maximum occurring at 16 minutes. In contrast, the BJAB-

LMP2b cells (Figure 23, left) displayed no significant phosphorylation of proteins at t=0 and no post-induction increase to a maximum. This indicated that LMP2b expression was causing a global dysregulation of phosphorylation pathways within the transfected cells, despite having no discernable effect on cell surface expression of the key components of the B cell receptor.

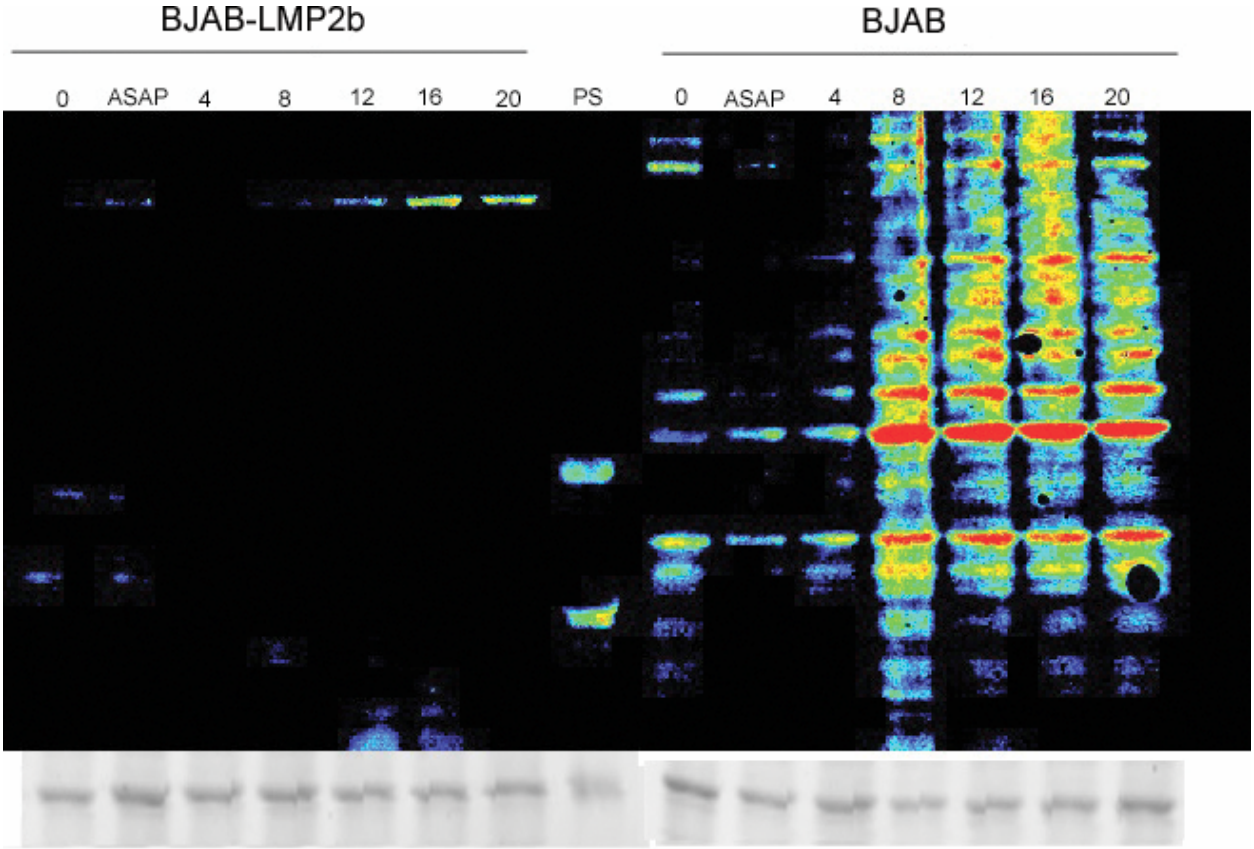

Figure 23: Impact of LMP2b expression upon post-BCR crosslinking related phosphorylation.

Cells that expressed (left) or did not express (right) LMP2b were incubated with anti-IgM-biotin and anti-CD19-biotin on ice. Cells were crosslinked by the addition of avidin for the indicated time points in minutes, and then lysed in the presence of phosphatase inhibitors. Lysates were subjected to PAGE, and then gels were treated with ProQ Phosphoprotein Gel Stain (top) or total protein stain (bottom, actin shown as loading control). A higher intensity of phospho-proteins is shown by increasingly warmer colors (blue<yellow<red). PS= PeppermintStick Phospho-protein marker was used as a marker and phosphorylation control.

5.5.3 LMP2b expression deregulates the post-BCR signal cascade:

To determine at what level LMP2b blocked the phosphorylation cascade; we examined phosphorylation of specific proteins in the BCR signal transduction pathway using antibody arrays (Hypromatrix, Inc). The amount of protein phosphorylation was detected using either an AP-conjugated anti-phosphotyrosine antibody, or a biotin-conjugated anti-phosphoserine antibody that was developed with an AP:streptavidin complex and DDAO deposition. Both sets of arrayed membrane preparations were incubated for identical times with the developer and scanned at the same time with a FLA2000 Fuji phosphoimager. Background subtracted intensity was measured relative to negative controls. Relative light unit measurements were imported into Antibody Array (Stratagene) and heat maps were generated (Figure 24). BJAB cells showed an expected pattern of tyrosine phosphorylation with proteins such as lyn and syk peaking early (t=8min and t=12min, respectively) and decreasing over time. (Figure 24B) Proteins further down the signal cascade peaked later (e.g.: akt t=16). In contrast, BJAB-LMP2b cells showed either low levels of phosphorylation (lyn) or a delayed and lower phosphorylation (syk) level compared to BJAB cells. The signaling block by LMP2b affected molecules at the very top of the cascade, suggesting that an early event was being impacted. (Figure 24B)

Serine phosphorylation patterns showed a somewhat different profile. In BJAB cells, the levels of serine phosphorylation on most phosphoproteins were initially low or undetectable and gradually increased over time after induction, reaching a maximum measured level by 20 minutes. BJAB-LMP2b cells had a higher basal phosphorylation level at t=0. After induction of the BJAB-LMP2b cell line, most proteins reached

maximum serine phosphorylation levels between 4 and 12 minutes. We verified by Western blotting that the cells contained similar amounts of the individual phosphoproteins at t=0 and during the period under observation so that the differences in phosphorylation observed were not due to an accumulation or loss of a particular protein. MEK1, akt, and lyn had no significant differences (less than 4% variation) in the level of expression between the cell types (data not shown).

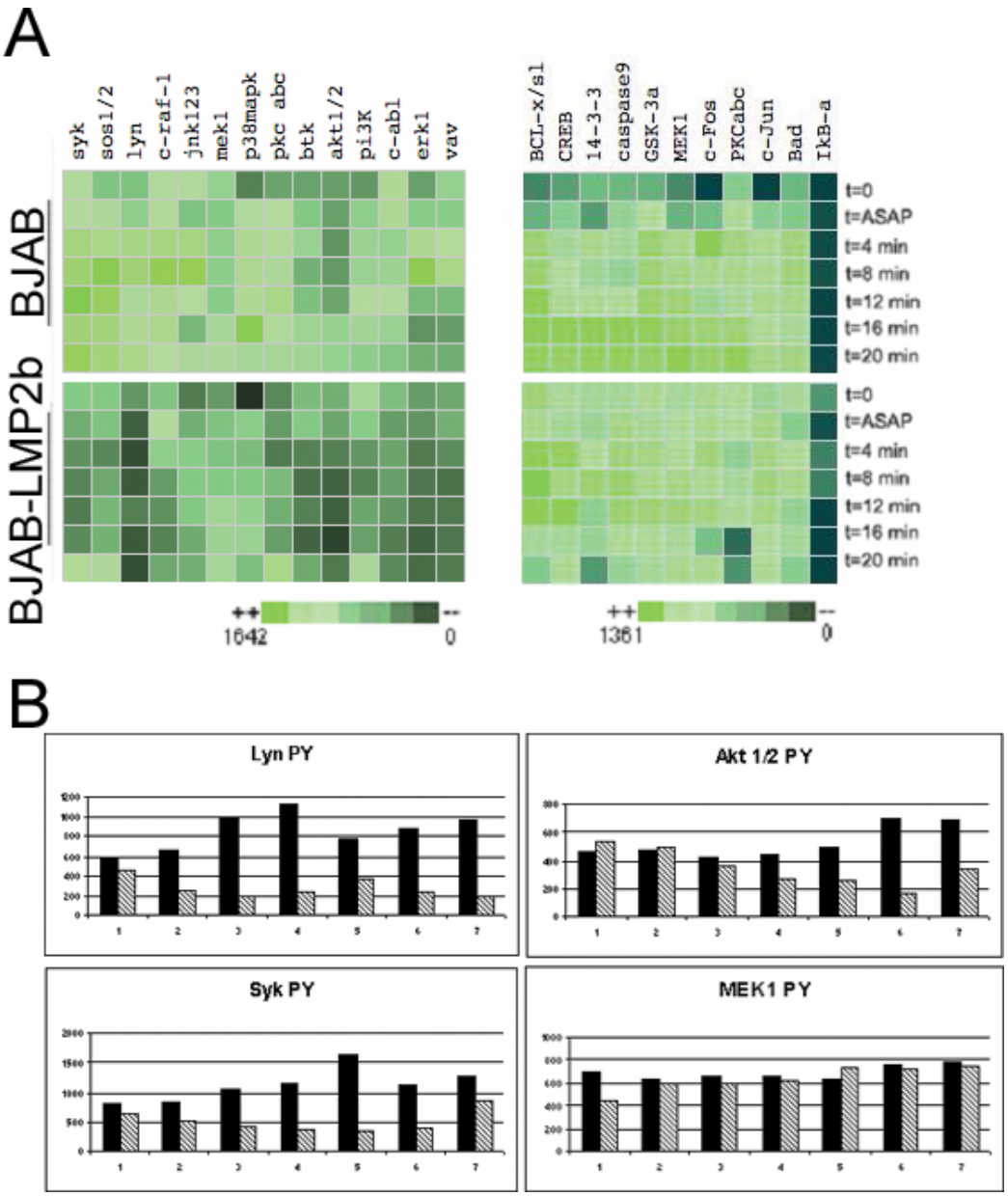

Figure 24: Impact of LMP2b expression on the phosphorylation of specific proteins after BCR crosslinking.

(A)BJAB (top) and BJAB-LMP2b cells were incubated with anti-IgM-biotin and anti-CD19-biotin. Cells were crosslinked by the addition of avidin for the indicated periods of time and then lysed in the presence of phosphatase inhibitors. Lysates were incubated with antibody arrays, and phosphorylation was detected using anti-phosphotyrosine (left) or anti-phosphoserine (right) antibodies. Membranes were scanned and thresholded, and relative light units were used for comparison. Bright green indicates more phosphorylation, progressively darker green indicates comparatively less phosphorylation. The range of relative light units is shown under each heat map. (B)Direct comparison of BJAB (solid) and BJAB-LMP2b (hatched) relative light unit measurements for selected phosphoproteins.

5.5.4 LMP2b increases expression of genes involved with apoptosis:

The high basal level of serine phosphorylation on signaling proteins in BJAB-LMP2b cells was unexpected. Phosphorylation of some of these molecules (e.g.: caspase 9 and BAD) is associated with activation of these proteins in survival pathways. Thus, for survival, LMP2b expressing cells apparently have these pathways activated. However, chronic activation of signaling molecules could potentially interfere with the normal functioning of apoptotic pathways, which suggests a mechanism by which BJAB-LMP2b cells might be prone to selective loss of LMP2b-EGFP expression during long term culture in spite of being under G418 co-selection. To determine the effect of chronically elevated basal serine phosphorylation on apoptotic pathways, we profiled gene expression in LMP2b positive cells using an apoptosis gene expression array.

RNA from equal numbers of cells from BJAB and the BJAB-LMP2b cell lines were reverse transcribed and used for gene expression profiling via real-time PCR. After normalizing for the expression levels of GAPDH, a comparison revealed that 38 genes were upregulated greater than 2-fold (*tnfsf10,bax,casp9,casp5,casp14,bag1,casp2,rpl13a,casp8,bclaf1,casp4,casp7,tnfrsf10b,casp3,traf2,ripk2,bag4,dapk1,mcl1,hprt1,tradd,tp53,tnfrsf9,bnip1,pycard,birc6,bnip2,abl1,ltbr,dffa,tp53bp2,casp6,bcl2l1,fadd,fas,cd27,cideb,cidea)* and 10 were downregulated (*cflar, casp10, birc3, lta, bcl2a1, igf1r, birc4, bnip3, tnfrsf1a, bcl2, tnfrsf25*) using 'stringent' criteria (see Materials and Methods). The remainder had less significant fold changes.

Eight of the ten most highly over-expressed genes in BJAB-LMP2b cells were pro-apoptotic genes (Table 7). Similarly, eight out of ten of the most under-expressed genes were implicated in survival (Table 8).

Table 7: Genes up regulated in BJAB-LMP2b cells

Gene	Fold Increase Relative to BJAB*	Pathway	Family
cidea	12.84	Apoptosis	CIDE Domain
cideb	12.56	Apoptosis	CIDE Domain
cd27	8.47	Survival	Survival/Apoptosis
fas	7.70	Apoptosis	Death Domain
fadd	7.64	Apoptosis	Death Domain
bcl2l1	7.12	Survival	DNA Damage
casp6	6.70	Apoptosis	Caspase
tp53bp2	6.11	Apoptosis	DNA Damage
dffa	4.98	Apoptosis	Death Domain
ltbr	4.90	Apoptosis	TNF Receptor

***Values represent fold changes in Log_2 scale**

Table 8:Genes down regulated in BJAB-LMP2b cells

Gene	Fold Increase Relative to BJAB*	Pathway	Family
cflar	-14.23	Survival	Death Effector
casp10	-8.48	Apoptosis	Caspase
birc3	-6.81	Survival	IAP
lta	-6.20	Survival	TNF Ligand
bcl2a1	-5.83	Survival	BCL-2
ifg1r	-3.98	Survival	Anti-Apoptosis
birc4	-3.88	Survival	IAP
bnip3	-2.82	Apoptosis	BCL-2
tnfrsf1a	-2.61	Survival	TNF Receptor
bcl2	-2.35	Survival	BCL-2

***Values represent fold changes in Log_2 scale**

The change in the levels of apoptosis-related genes was consistent with a shift towards an apoptotic phenotype. If the change was LMP2b-dependent, then inhibition of LMP2b expression might be expected to mitigate the apoptotic expression phenotype. To accomplish this, we infected the BJAB-LMP2b cells with a lentivirus vector expressing an shRNA to knock down expression of LMP2b. Flow cytometric and immunofluorescence profiles were used to monitor the decrease in LMP2b expression (Figure 22). Maximal decrease was observed 48 hours post infection. Mean fluorescence intensity decreased 90% although 12.6 % of cells remained at the pre-infection level of fluorescence. We opted not to re-sort an already fragile cell line

(possibly selecting for cells that weren't ‘knocked down’ but were instead 'shut off' as occurs in extended culture conditions) and collected all the cells in the infected culture for RNA analysis.

In comparing the shRNA infected and uninfected BJAB-LMP2b cells, the greatest fraction of tested genes (61genes) showed no significant change in the level of expression (including some members of the group of genes that had been upregulated in BJAB-LMP2b cells). We did observe increased expression of 3 genes (*bnip, bnip3l, and casp3*) and a decrease in expression of 2 genes (*tnfrsf1a* and *cd40*). Several genes that were highly dysregulated in BJAB-LMP2b cells showed lowered expression levels and two (*tp53bp2* and *cd27*) were markedly decreased (Figure 22C). There was no significant upregulation within the group of genes downregulated in BJAB-LMP2b cells.

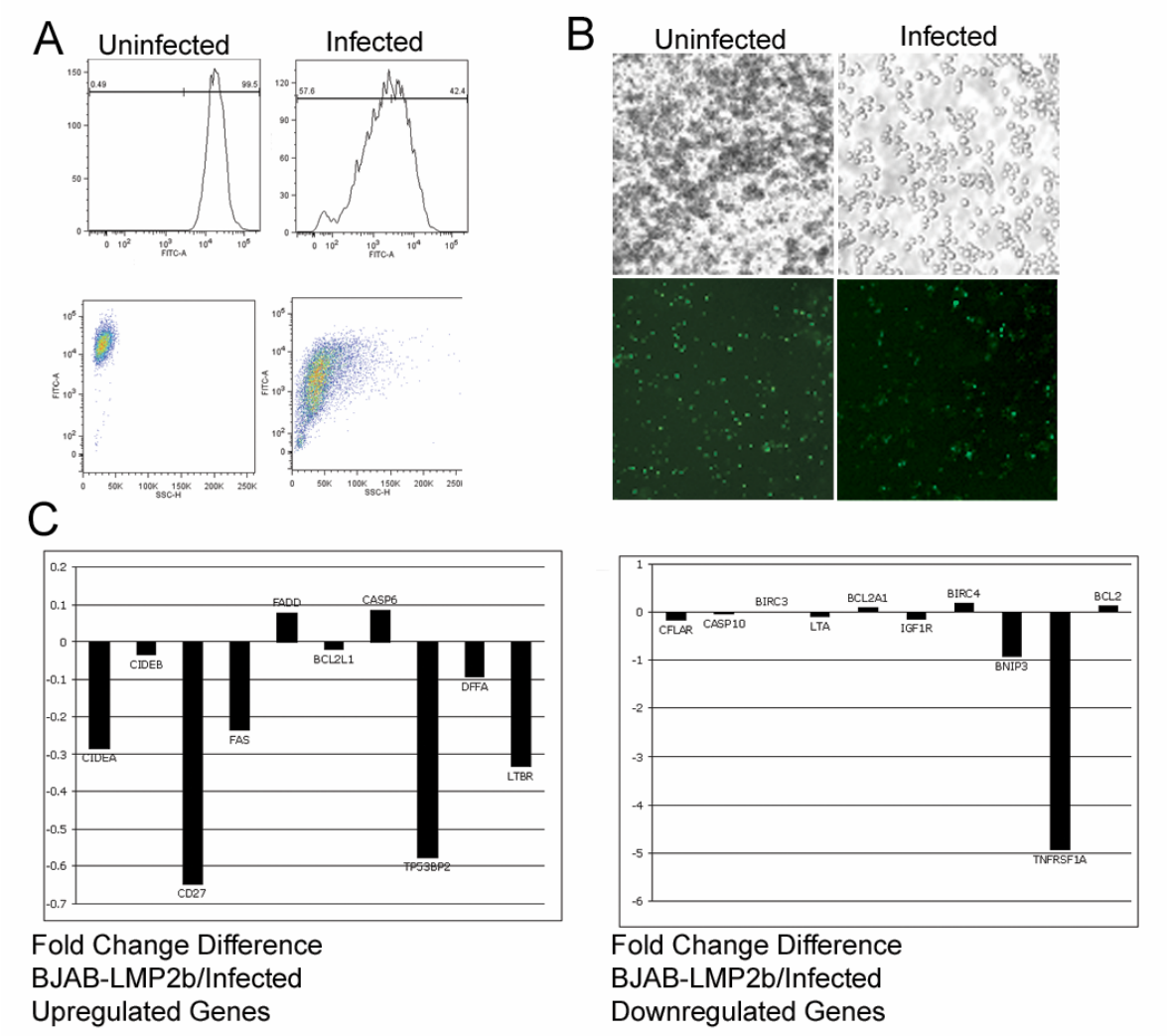

Figure 25: Lentivirus encoding shRNA for EGFP was able to knockdown LMP2b expression

LMP2b expressing cells were sorted for EGFP expression, and then infected with a lentivirus BJAB-LMP2b cells were sorted for EGFP expression and then infected with a lentivirus encoding shRNA for EGFP. A) Flow cytometric analysis 2 days post-infection showed a decrease of LMP2b-EGFP expression B) fluorescent microscopy of lentivirus-infected cells. C) Relative fold changes between uninfected and infected BJAB-LMP2b cells for the 20 genes most affected by LMP2b expression as listed in Tables 7 and 8.

5.6 DISCUSSION:

Although regulation of LMP2 expression has not been studied rigorously, the messages for LMP2a and LMP2b appear to be coordinately regulated in B cells. In virus immortalized LCLs, whenever LMP2a is expressed, LMP2b is also made[148, 149]. Because LMP2b lacks the signaling domain of LMP2a and differential expression levels between the two proteins, the prevailing hypothesis has been that LMP2b serves as a negative regulator of LMP2a. The ability of these proteins to aggregate and co-localize within the same intracellular compartment suggested both a mechanism for LMP2a-mediated signaling and for LMP2b interference in the process[161, 202]. Over-expression of LMP2b in the Akata BL model cell line increased the magnitude of lytic virus production after BCR cross-linking and decreased the degree of BCR stimulation required to induce lytic EBV production[200].

That LMP2b might have a phenotypic role independent of LMP2a was first indicated by the ability of LMP2b, but not LMP2a, to co-localize with CD19 in intracellular compartments[166]. Since CD19, and the CD19:BCR interaction might be compromised by LMP2b expression, we initially investigated the distribution of signaling molecules to determine if they were available on the surface for cross linking and signaling. Levels of IgM, CD79beta (Igbeta) and CD19 all showed similar cell surface expression in BJAB and BJAB-LMP2b cell lines and, as described previously, the LMP2b had an intracellular perinuclear localization. Nonetheless, cross linking the BCR on BJAB-LMP2b had no signaling effect, suggesting that the cells were deficient in BCR signaling. There was a marked absence of phosphorylated proteins at times when the control BJAB was clearly engaged in signaling. LMP2b disrupted BCR signaling.

Since the knockdown of phosphorylation was so complete, it was likely that a step high in the signaling cascade would be implicated in the global dysregulation. Using an antibody array format, several specific proteins that were candidates for this mechanism were examined. This format also allowed the phosphorylation kinetics of many proteins to be followed in the same cellular lysates harvested after BCR crosslinking. Levels of protein phosphorylation rose and fell in cross-linked BJAB cells while the BJAB-LMP2b cells displayed no change in the phosphotyrosine and minimal changes in the phosphoserine time course studies. Among the proteins examined were molecules lyn and syk, whose phosphorylation are one of the first the cascade-initiating event. Our data indicates the LMP2b block was complete and involved the highest levels of the signaling cascade because these two proteins failed to initiate signals. The phosphoprotein array also revealed that the baseline phosphoserine levels of signaling proteins in the BJAB-LMP2b cells were higher than BJAB. Some phosphorylated molecules (e.g.: caspase 9 and BAD) were associated with activation of pro-survival, anti-apoptotic pathways. Phenotypically, transfected cells under drug selection to enforce LMP2b-expression plasmids are characteristically unstable and prone to lose LMP2b expression. Cell lines that express an EGFP-tagged LMP2b must frequently be sorted by flow cytometry to maintain EGFP expression levels. This phenomenon had led us to suspect that LMP2b expression had a deleterious affect on proliferation. Western blot and immunofluorescence analysis indicated that this was not toxicity due to over expression as the levels of LMP2b were similar to those detected in proliferating LCLs. The apoptosis-related gene expression analysis showed that there was an increase in the expression of pro-apoptotic genes, and a decrease in the levels of anti-

apoptotic genes in LMP2b expressing cells relative to BJAB cells. These results indicated that LMP2b exerted some influence on the regulation of apoptotic pathways.

Up-regulated genes were largely involved in the mediation of apoptosis via the extrinsic process. The extrinsic process initiates apoptosis from a ligand:receptor interaction or receptor oligiomerization on the cell surface. This, taken together with the ability of LMP2b to mulitimerize with other proteins intracellularly, suggests that LMP2b could be chronically stimulating survival signals via a mechanism similar to receptor oligiomerization. High levels of expression of pro-apoptosis genes would be viewed as a cellular response to redress the chronic stimulation. When LMP2b expression was inhibited by the shRNA lentivirus, levels of expression of most of the pro-apoptotic genes (and CD27 and TP53BP2 in particular) declined within 48 hrs. Due to an incomplete LMP2b knockdown (a decrease of EGFP expression by ~90%) there was still a positive differential expression between infected BJAB-LMP2b and BJAB cells. Nevertheless, the reduction clearly indicated that the altered gene expression was an LMP2b-related process. Genes whose LMP2b-altered expression was not affected by the knockdown might be considered to be genes not directly targeted by LMP2b. Their expression levels perhaps were a response to the effects of long-term selection that were not reversed in the 48hrs incubation with shRNA virus.

From amongst the genes whose expression is linked to LMP2b expression, CD27 appears to stand out because of its sensitivity to LMP2b expression and its role in B cell ontogeny. In humans, CD27 is expressed in a high percentage of germinal center B cells, throughout B cell differentiation, and on a subset of memory cells where it has commonly been used as a marker for the peripheral memory B cell compartment. CD27

binds to the adaptor proteins Traf-2 and Traf-5 and signals through the NF-KB and c-Jun kinase pathways, promoting immunoglobulin production and plasma cell differentiation[203]. In Burkitt lymphoma cells, CD27 protects against B cell receptor-induced apoptosis and upregulates Bcl-2 and Bcl-xL. However, some studies suggest that CD27 can induce apoptosis by binding and signaling through Siva[204]. CD27 function is further complicated because B cells also express CD70, whose signaling role has been suggested to oppose that of CD27 on B cells[205]. An understanding of the effects of LMP2b expression on CD27 and CD70 signaling deserves further study.

The *TP53BP2* gene, another LMP2b regulated gene, encodes the Apoptosis Simulating Protein of P53 2 (ASPP2). ASPP2 is a pro-apoptotic protein and the best studied of this family of apoptosis stimulating proteins. It plays a critical role in the regulation of cell growth and apoptosis via its interactions with P53 family of proteins[206]. ASPP2 bound to P53 stimulates the DNA binding and transactivation function of P53 on the promoters of apoptotic genes in vivo (e.g. *BAX* and *PIG3*)[207]. This protein also halts cell cycle progression at G2/M[208]. The comparatively high level of this gene expression in the BJAB-LMP2b cells seems to correlate with phenotypic observations. The growth inhibition and loss of LMP2b in BJAB-LMP2b cells could be explained by over expression of the ASPP2 protein. The contribution of this protein to the LMP2b mediated block to BCR signaling needs to be more clearly defined.

Overall, the effects of LMP2b on apoptotic pathways appear to mimic rather than oppose LMP2a. LMP2a has a constitutively phosphorylated signaling domain that is responsible for initiating downstream signaling pathways via lyn, syk, MAPK, and Nedd4[173, 174, 209, 210]. It has been previously demonstrated the LMP2a activates

the Bcl-2/BCL-xL and Ras/PI3K/Akt pathways mediating survival [211, 212]. LMP2a has also been shown to be involved in the inhibition of BCR induced signaling and apoptosis in Burkitt Lymphoma cells[213]. It remains to be determined whether isolated LMP2a expression also alters expression of pro-apoptotic genes in a manner similar to LMP2b. The work presented in this study provides additional evidence and a potential mechanism (differences in the way apoptotic pathways are affected) for how LMP2b negatively regulates LMP2a. If LMP2b is a negative regulator of signals generated by LMP2a, perhaps the mechanism is not as simple as a stochastic 1:1 LMP2a/LMP2b hetero-dimer interaction that disrupts homodimeric LMP2a signaling complexes.

6.0 OVERALL DISCUSSION:

The LMP2 is a protein can only be formed after the fusion of the terminal repeats on the end of the EBV genome. Since the circular form of the genome only exists during latency, it is convenient to say that these LMP2 proteins are formed for and by the latent state of EBV.

The 2 isoforms of LMP2 suggest that there is a probable relationship between what both of these proteins do for latency. LMP2a, has an obvious signaling domain, which mimics B-cell receptor signaling from an internal location of the cell. Somehow, this protein is able to restrict movement of other membrane-associated molecules into lipid rafts (e.g. the B-cell receptor). These raft-like structures of organized membrane components are the stages of the initiation of signaling. The ability of LMP2a to both disrupt and signal from a protected location in the intracellular membranes is advantageous. How or why LMP2b plays into this scenario is less understood.

LMP2b exists as a 12-TM protein with alternating extra- and intracellular loops connecting the TMs. This protein lacks the 119aa C-terminal signaling domain of LMP2a and is essentially a N-terminally truncated form of LMP2a expressed by a different promoter. LMP2b lacks any obvious signaling or localization domains. It has been reported previously that the shared LMP2 N-terminal domain contains a clustering signal [169]. LMP2 proteins are also palmitoylated on juxtamembraneous cysteine residues, but palmitoylation is not necessary for localization, function, or association

with lipid rafts [214]. The bulk of knowledge of LMP2b is inferred through what LMP2a does. LMP2b is postulated to be a negative regulator of LMP2a, however until recently, little investigation has dealt with LMP2b either alone, or in concert with LMP2a.

Protein localization is a critical clue to the function of a protein. Because of the deficient knowledge of LMP2b, we undertook studies of its localization and multimerization domains in order to further our knowledge of how the protein might function. Initial studies of LMP2a demonstrated that LMP2a was a plasma membrane proteins. These studies were performed before the advent of confocal microscopy, and while the staining was specific, the lack of a counter stain or reference stain hindered the determination of localization[151] LMP2a was also shown to localize to membranes via biochemical studies [99] This localization was not contested until the Dawson group demonstrated human antibodies detecting endogenously produced LMP2b in an intracellular location[167]. Our lab also showed that LMP2a and LMP2b localized to an intracellular location using fluorescently labeled and tagged LMP2a and LMP2b. Additionally, we showed that our LMP2a and LMP2b could co-localize with virally produced LMP2a detected by the antibodies in the original study. In the studies presented here, we elucidated the domains of the protein that are responsible for localization to the intracellular compartment.

Our initial investigation was concerned with the analysis of aggregation and localization domains in LMP2. Successive deletions of the transmembrane regions were used to determine areas of the proteins that were needed for self-aggregation. We were able to verify that a previously identified region of the N-terminal tail was needed for self-aggregation. This region showed especially pronounced effects at the cell surface.

We were then able to demonstrate that this N-terminal region was not responsible for the intracellular localization as truncation mutants containing this tail were just as likely to traffic to the cell surface. We showed that a region encompassing the C-terminal portion of the protein was associated with intracellular localization. This was interesting because while all of the mutants localized to the cell surface when alone, when co-expressed with a full-length protein, they could be sequestered intracellularly. This aggregation was specific to LMP2 proteins and did not affect other co-transfected proteins.

The aggregative qualities of these proteins point to a possible function. Our lab previously demonstrated that LMP2b (but not LMP2a) was able to co-localize with CD19[166]. CD19 is an important cell surface molecule that interacts as a rheostat for signals generated from the B-cell receptor[215]. LMP2a acts as a constitutively active B-cell receptor consequently driving downstream signaling[216]. LMP2b seems to interfere with this mechanism, but the process is unknown[200]. It is also unknown if CD19 processively amplifies signals from LMP2a, or if its function is exclusively BCR related. It is feasible that LMP2b is necessary to relocate CD19 to the LMP2a containing intracellular compartment to facilitate the BCR mimic's signals.

The initial characterization of viral proteins has generally taken place in cells that express the protein at high levels and without any other viral proteins that the protein may interact with. These studies are valuable and provide information about the exclusive action of these proteins, especially interactions that are exclusive to cellular proteins. Towards this aim, we began studies with an EBV- Burkitt's Lymphoma cell line (BJAB) that expressed an LMP2b protein that was fluorescently labeled and epitope

tagged. We were interested in any functional characteristics that LMP2b could show us in this system. Without any clues from sequence homology, we focused on what function this protein could provide via it's aggregative properties. Since there is an obvious LMP2b-CD19 interaction, we started to investigate the LMP2b contribution to impeding BCR signaling.

A model for investigating LMP2b function involved crosslinking the BCR with CD19 and determining what cellular changes took place. When BJAB cells are crosslinked, we see a typical expression of proteins that initialize a signaling cascade via phosphorylation over a defined time period. In cells expressing the LMP2b protein, it appeared that this cascade was never established. Phosphoproteins were only weakly induced, if at all, and did not seem to increase with time. This lack of function would have been easily been explained by the lack of available cell surface CD19, but CD19 along with other members of the B cell receptor complex (CD79β, CD9, CD81, and IgM) showed no difference in surface expression between BJAB and LMP2b expressing cells. These surprising results indicated to us that LMP2b was having some sort of influence on the cellular signals even without an inherent signaling domain.

For the most part, signaling cascades follow a predictable directional pattern. Because of the vast differences between phosphoproteins in these compared groups, we chose to investigate the phosphorylation status of several proteins at the beginning of these cascades. Through the use of nitrocellulose membranes spotted with antibodies against several signaling proteins, we crosslinked the surface receptors of these cells and looked over a time course for differential phosphorylation status. If LMP2b were able to specifically block the phosphorylation of a critical protein high in the

pathway consequential signaling would be blocked. In contrast to our hypothesis, LMP2b did not block one specific protein; instead it showed a widespread dysregulation of all of the proteins investigated. These data agreed with our previous result indicating that LMP2b has a global repressing of signaling.

One interesting phenomena the antibody array showed was a slight increase in the baseline levels of serine phosphorylation. Many of the proteins that we investigated were implicated in programmed cell death signaling, but the increased levels of the phosphorylation would block these apoptotic signals, thus becoming pro-survival. BJAB cells that express LMP2b have a growth disadvantage that may be the consequence of these competing signals. In culture we can see a loss of expression of LMP2b even with strict selection criteria. Frequently, the cells must be sorted for EGFP expression to allow for a high enough level of expression for experiments. It was unknown if the cells that were expressing LMP2b were dying from LMP2b expression, or if the cells were losing LMP2b expression and the selection process was killing the negative cells. These two characteristics of LMP2b expressing cells lead us to investigate the expression of genes involved with apoptosis.

Using an array format for real time PCR we looked at equal numbers of LMP2b positive and negative cells. The results demonstrated that cells expressing LMP2b were prone to express genes that were pro-apoptotic, and decrease expression of pro-survival genes. This was in agreement with our observations that LMP2b expressing cells died in culture.

BJAB cells are not necessarily the ideal situation for the expression of proteins. The c-myc rearrangement is what is responsible for the creation of this lymphoblastoid

cell line. This may have some affect on our observations, so we infected the LMP2b expressing cell line with a retrovirus that expressed an shRNA that consequentially degraded the LMP2b message. This allowed us to determine what genes were restored upon the removal of LMP2b. We demonstrated partial restoration of several genes, even though the retroviral infection was not at 100% efficiency.

The previous set of experiments demonstrates that LMP2b does have an affect on BCR signaling and consequential expression of apoptotic proteins. LMP2a exists as a way for EBV to provide survival signals by blocking BCR induced apoptosis. If LMP2b were indeed a negative regulator of LMP2a, then 'unblocking the block' would be a necessary function. In latency, LMP2a blocks BCR signaling and consequential progression into the lytic phase of infection. We submit that LMP2b exists as a negative regulator of LMP2a by critical regulation of it's signaling capabilities. In this model, LMP2b would act as an inhibitor of LMP2a signal transduction and interrupt the constant tonic signaling from the LMP2a CSD thus inducing lytic replication of the virus. Indeed, it has been recently shown that LMP2b over expressed in LMP2a containing cells will push the virus into lytic replication[200].

While it is convenient to study proteins in the absence of other viral proteins, the genuine function of any viral protein must be observed in the native background. In the ideal situation, the mutation is made in the single gene within the viral genome. This allows for a direct comparison between viruses with and without the mutation.

The ability to look at the effect of both LMP2a and LMP2b in concert and singly in an EBV background was the advantage to augmenting the BAC systems. This system has been shown as a valuable resource for the generation of virus mutants. In our

hands, it provided a way to finally determine the effect of LMP2a upon immortalization, and to provide an idea of what role LMP2b plays in this process.

We demonstrated that LMP2 has a role in virus immortalization. Cells that are infected with virus that is lacking one of the isoforms of LMP2 is partly deficient in the immortalization process, needing an MOI of 33-150x more virus to establish an LCL. Viruses lacking both isoforms are unable to establish cells lines at any of the MOIs in our experimental conditions. This is in agreement with previous work[189]. The new information presented is that LMP2b clearly provides some type of help to cells during the immortalization process as LCLs lacking LMP2b exhibit growth retardation.

The work presented here demonstrates the LMP2b has a function independent of LMP2a and is more than a simple negative regulator of LMP2a.

7.0 FUTURE DIRECTIONS:

Since the discovery of the proteins that are produced via circularization of the EBV episome in 1989 [149], most of the knowledge has been focused on the LMP2a isoform. Over the years, LMP2b function has been inferred to be a negative regulator of LMP2a by sequence similarity alone. In recent years, the focus has switched to LMP2b and how this protein, without any obvious signaling or interaction domains, can interfere with LMP2a.

The work presented here does not definitively describe the nature of interactions within the LMP2 12-TM domain. While we are readily able to describe which TMs are responsible for self-interaction, the minimal necessary domain is not described. In order to determine the minimal domains needed to mediate interaction between the LMP2 isoforms, sequential replacement of TM regions with generic alanine, LMP1, or non-sequential LMP2 transmembrane regions can be used to ascertain the TM-TM interactions. An intact 12-TM domain is required for intracellular localization, however it is unknown if this requirement is a function of the intra-molecular requirements within the protein that directs the protein to the trans-golgi network, or if it is just a non-specific organization of the protein in the membrane. Trans-membrane replacement studies that selectively integrate different transmembrane sequences into this protein may be able to

determine if and what specific domain is required for localization and multimerization of this protein.

There is usually a critical link between the localization of a protein and its function. Some proteins do not become functional until they have translocated into the nucleus. For others, this localization is a less specific phenomenon. We have demonstrated that the intact LMP2b has a specific localization. Based upon this location, we have shown that LMP2b is able to interact with other proteins. The next logical step is to determine it this localization is critical to the function of both LMP2a and LMP2b. As LMP2a has a signaling domain, the signal it generates may have different consequences if the signal was initiated from the plasma membrane (in case of a truncation mutant) versus an intact protein localizing to the trans-golgi network. The localization function relationship of LMP2b will be equally as interesting. Will the negative regulating capabilities of LMP2b hold up if it cannot mulitimerize with LMP2a? The investigation of the relationship of LMP2 localization and function will shed light on how these proteins integrate into the larger scope of B cell signaling.

The nature of how LMP2b regulates signaling will be a critical determinate of how this protein fits into the BCR signal transduction cascade to regulate latency. Our experiments demonstrate that LMP2b, even lacking an obvious signaling domain, is able to disrupt this signaling pathway via non-phosphorylation of proteins. Analysis indicated that this disruption is global, and disrupts even primary signal proteins such as lyn and syk. While this is an important finding, the mechanism of this process will shed more light on the interaction of LMP2 proteins. This result may indicate the LMP2b disrupts the cascade by not allowing the BCR complex to enter organized lipid raft

domains, which is also a feature of LMP2a [99]. The ability of LMP2b to co-localize with CD19 in the trans-golgi network may be critical in this process, as lipid rafts are first assembled in this organelle. Looking at the proteins resident in lipid rafts in LMP2b+ cell lines would determine if LMP2b is able disrupt the BCR signalsome post cross-linking.

The study of LMP2 has frequently been hampered due to poor experimental requirements. LCLs are difficult to transfect and are unable to be infected by common viral transducing systems. There is not a commercial antibody available that detects LMP2b because it lacks the epitope that the LMP2a antibody recognizes, consequently it must have an artificial epitope inserted into its sequence. The studies of an isolated gene can give interesting results and clues to its in vivo functions, but to achieve a better understanding of what the protein does is to study the mutation in the context of the entire viral genome.

We used an established system of Maxi-EBV to determine the contribution of LMP2a andLMP2b to immortalization. Using this system we can determine at what point the isoforms of LMP2 are necessary for infection, and how immortalization is aborted if the virus lacks these proteins. Future studies should include inducible expression of the LMP2 proteins at different time points post infection to rescue the immortalization pathway for long enough to generate LMP2 negative LCLs. Finally, this BAC system should be modified to allow for independent tracking of LMP2a and LMP2b via alternative epitope usage.

APPENDIX A:

PLASMIDS FOR CONSTRUCTION OF RECOMBINANT EBV

Name	Feature	Reference
P706	Cre-Expression Plasmid, Tetracycline resistant	GeneBridges
P705	Cre-Expression Plasmid, Cam resistant	GeneBridges
P509	PCMV-BZLF1	[217]
P2670	PCMV-BALF1	Hammerschmidt Lab
PKD46	Recombination; arabinose inducible; amp resistant	[218]
P2766	EBV #163473-172281, #1-960, Mun1 partial EcoRV from p925	[219]
P925	EBV #163473-172281, #1-3960	[219]
P2768.5	Intermediate construct for 3xFLAG insertion in MaxiEBV, MCS for ex2-8 of LMP2	Hammerschmidt Lab
PSV40Zeo2	Commercial construct	Clontech
P2167.1	EBV #1-3644, #163473-172281, PCP15, Kan; MCS of p2144.1	Hammerschmidt Lab

P2089.0	Wildtype Maxi-EBV, F-Plasmid, EGFP, Hygromycin resistance in B95.8 DNA	Created for this study
P2089.1	2089.0, 3x Flag insertion in exon 7, zeocin gene insertion prior to BNRF1	Created for this study
P2089.2	2089.1+ lox66/71 loxP flanked LMP2b exon1 via homologous recombination of p2089.1	Created for this study
P2089.3	P2089.2 + floxed LMP2b	Created for this study
P2089.4	P2089.1 with MtoC mutation at start codon of LMP2 exon2	Created for this study
P2190.0	2089.0 +Floxed LMP2a exon 1	Created for this study
P2190.1	2190.0, 3x Flag insertion in exon 7, zeocin gene insertion prior to BNRF1	Created for this study
P2190.2	2190.1+ lox66/71 loxP flanked LMP2b exon1 via homologous recombination of p2190.1	Created for this study
P2190.3	P2190.2 + floxed LMP2b	Created for this study
P2190.4	P2190.1 with MtoC mutation at start codon of LMP2 exon2	Created for this study
P2525.0	P2190.0, Floxed LMP2a exon 1	Created for this study

P2525.1	2525.0, 3x Flag insertion in exon 7, zeocin gene insertion prior to BNRF1	Created for this study
P2525.2	2525.1+ lox66/71 loxP flanked LMP2b exon1 via homologous recombination of p2525.1 and p	Created for this study
P2525.3	P2525.2 + floxed LMP2b	Created for this study
P2525.4	P2525.1 with MtoC mutation at start codon of LMP2 exon2	Created for this study

APPENDIX B:

LIST OF ABBREVIATIONS

AA	Amino Acid
AP	Alkaline Phosphatase
BAC	Bacterial Artificial Chromosomes
BARTs	BamHI A Rightward Transcripts
BCA	Bicinchoninic Acid Assay
BCR	B-cell Receptor
BL	Burkitt's Lymphoma
C	Celsius
CCD	Charge coupled device
CD	Cluster of Differentiation
CTAR	C-terminal Activation Region
CSD	Cytoplasmic Signaling domain
CTL	Cytotoxic T-Lymphocyte
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Media

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DS	Dyad Symmetry Element
DTT	Dithiothreitol
EA	Early Antigen
EBER	Epstein-Barr Virus Encoded RNA
EBNA	Epstein-Barr Virus Nuclear Antigen
EBNA-LP	Epstein-Barr Virus Nuclear Antigen- Leader Protein
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetra-acetic-acid
EGFP	Enhanced Green Fluorescent Protein
EGTA	ethylene glycol tetraacetic acid
FACS	Fluorescence Activated Cell Sorting
FITC	Fluorescein isothiocyanate
FR	Family of Repeats
Gp	Glycoprotein
GRU	Green Raji Units
HD	Hodgkin's Disease
HDAC	Histone Deacytylase Complex
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
HL	Hodgkin's Lymphoma
HRS	Hodgkin and Reed-Sternberg Cells

IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Infectious Mononucleosis
IR	Internal Repeat
ITAM	Immunotyrosine Activation Motif
K	1000
Kb	Kilobase
kDa	Kilodalton
LB	Luria-Bertrani
LCL	Lymphoblastoid Cell Line
LMP	Latent Membrane Protein
MACS	Magnet associated cell sorting
mls	Milliliters
MOI	Multiplicity of Infection
mRNA	Messenger Ribonucleic Acid
NK	Natural Killer Cell
NPC	Nasopharyngeal carcinoma
OD	Optical Density
ORF	Open Reading Frame
OriP	Origin of Latent DNA Replication
PAGE	Polyacrylamide Gel Electrophoresis

PBMCs	peripheral blood mononuclear cells
PBS	Phosphate buffered Saline
PCR	Polymerase chain Reaction
PE	Phycoerythrin
PS	PeppermintStick Phospho-protein Marker
PTLD	Post-transplant Lymphoproliferative Disease
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
shRNA	Short Hairpin Ribonucleic Acid
SV40	Simian Virus 40
t	Time
TBST	Tris buffered Saline with Tween
TCR	T-cell Receptor
TE	Tris- Ethylenediaminetetra-acetic-acid
TGN	Trans-Golgi Network
TMs	Trans-membrane Segment
TNF	Tumor Necrosis Factor
TR	Terminal Repeats
TRADD	TNF-receptor 1-associated death domain
TSS	Transfection Storage Solution
UL	Unique Long
US	Unique Short

VSV-G	Vesicular Stomatitis Virus-Glycoprotein
wt	Wild Type

BIBLIOGRAPHY

1. Knipe, D., Howley, PM, ed. *Fields Virology*. 4th Edition ed. 2001, Lippincott Williams & Wilkins. 3280.
2. Epstein, M.A., B.G. Achong, and Y.M. Barr, *Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma.* Lancet, 1964. **1**: p. 702-3.
3. Gerper, P., J. Whang-Peng, and J.H. Monroe, *Transformation and chromosome changes induced by Epstein-Barr virus in normal human leukocyte cultures.* Proc Natl Acad Sci U S A, 1969. **63**(3): p. 740-7.
4. Johannsen, E., et al., *Proteins of purified Epstein-Barr virus.* Proc Natl Acad Sci U S A, 2004. **101**(46): p. 16286-91.
5. Miyamoto, K., T. Sairenji, and Y. Hinuma, *Electron microscopic studies of tumor viruses. II. Entry and uncoating of Epstein-Barr virus.* Microbiol Immunol, 1978. **22**(8): p. 463-74.
6. Kintner, C.R. and B. Sugden, *The structure of the termini of the DNA of Epstein-Barr virus.* Cell, 1979. **17**(3): p. 661-71.
7. Hurley, E.A. and D.A. Thorley-Lawson, *B cell activation and the establishment of Epstein-Barr virus latency.* J Exp Med, 1988. **168**(6): p. 2059-75.
8. Aiyar, A., C. Tyree, and B. Sugden, *The plasmid replicon of EBV consists of multiple cis-acting elements that facilitate DNA synthesis by the cell and a viral maintenance element.* Embo J, 1998. **17**(21): p. 6394-403.
9. Marschall, M., et al., *The lytic transition of Epstein-Barr virus is imitated by recombinant B-cells.* Arch Virol, 1993. **129**(1-4): p. 23-33.
10. Miller, G., *The switch between EBV latency and replication.* Yale J Biol Med, 1989. **62**(2): p. 205-13.
11. Seigneurin, J.M., et al., *[Epstein-Barr virus and infectious mononucleosis. A clinical study of Epstein-Barr antibodies (author's transl)].* Nouv Presse Med, 1978. **7**(44): p. 4005-10.
12. Yao, Q.Y., et al., *The Epstein-Barr virus:host balance in acute infectious mononucleosis patients receiving acyclovir anti-viral therapy.* Int J Cancer, 1989. **43**(1): p. 61-6.
13. Andersson, J., et al., *Effect of acyclovir on infectious mononucleosis: a double-blind, placebo-controlled study.* J Infect Dis, 1986. **153**(2): p. 283-90.

14. Wright-Browne, V., et al., *Serum cytokine levels in infectious mononucleosis at diagnosis and convalescence.* Leuk Lymphoma, 1998. **30**(5-6): p. 583-9.
15. van den Bosch, C.A., *Is endemic Burkitt's lymphoma an alliance between three infections and a tumour promoter?* Lancet Oncol, 2004. **5**(12): p. 738-46.
16. van den Bosch, C., et al., *Time-space case clusters of Burkitt's lymphoma in Malawi.* Leukemia, 1993. **7**(11): p. 1875-8.
17. Burkitt, D., *A children's cancer dependent on climatic factors.* Nature, 1962. **194**: p. 232-4.
18. Moss, D.J., et al., *A comparison of Epstein-Barr virus-specific T-cell immunity in malaria-endemic and -nonendemic regions of Papua New Guinea.* Int J Cancer, 1983. **31**(6): p. 727-32.
19. Lam, K.M., et al., *Circulating Epstein-Barr virus-carrying B cells in acute malaria.* Lancet, 1991. **337**(8746): p. 876-8.
20. Khanna, R., D. Moss, and M. Gandhi, *Technology insight: Applications of emerging immunotherapeutic strategies for Epstein-Barr virus-associated malignancies.* Nat Clin Pract Oncol, 2005. **2**(3): p. 138-49.
21. Roschke, V., et al., *Chromosomal translocations deregulating c-myc are associated with normal immune responses.* Oncogene, 1997. **14**(25): p. 3011-6.
22. Raab-Traub, N. and K. Flynn, *The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation.* Cell, 1986. **47**(6): p. 883-9.
23. Brooks, L., et al., *Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts.* J Virol, 1992. **66**(5): p. 2689-97.
24. Busson, P., et al., *Consistent transcription of the Epstein-Barr virus LMP2 gene in nasopharyngeal carcinoma.* J Virol, 1992. **66**(5): p. 3257-62.
25. Brooks, L.A., et al., *Transcripts from the Epstein-Barr virus BamHI A fragment are detectable in all three forms of virus latency.* J Virol, 1993. **67**(6): p. 3182-90.
26. Niedobitek, G., et al., *Expression of Epstein-Barr virus genes and of lymphocyte activation molecules in undifferentiated nasopharyngeal carcinomas.* Am J Pathol, 1992. **140**(4): p. 879-87.
27. Pathmanathan, R., et al., *Undifferentiated, nonkeratinizing, and squamous cell carcinoma of the nasopharynx. Variants of Epstein-Barr virus-infected neoplasia.* Am J Pathol, 1995. **146**(6): p. 1355-67.
28. Pathmanathan, R., et al., *Clonal proliferations of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma.* N Engl J Med, 1995. **333**(11): p. 693-8.
29. Thornburg, N.J., R. Pathmanathan, and N. Raab-Traub, *Activation of nuclear factor-kappaB p50 homodimer/Bcl-3 complexes in nasopharyngeal carcinoma.* Cancer Res, 2003. **63**(23): p. 8293-301.
30. Paine, E., et al., *Expression of LMP1 in epithelial cells leads to the activation of a select subset of NF-kappa B/Rel family proteins.* J Virol, 1995. **69**(7): p. 4572-6.
31. Morrison, J.A. and N. Raab-Traub, *Roles of the ITAM and PY motifs of Epstein-Barr virus latent membrane protein 2A in the inhibition of epithelial cell differentiation and activation of {beta}-catenin signaling.* J Virol, 2005. **79**(4): p. 2375-82.

32. Morrison, J.A., A.J. Klingelhutz, and N. Raab-Traub, *Epstein-Barr virus latent membrane protein 2A activates beta-catenin signaling in epithelial cells.* J Virol, 2003. **77**(22): p. 12276-84.
33. *Hodgkin's Disease*. [cited 2008 February 6]; Available from: http://www.mayoclinic.com/health/hodgkins-disease/DS00186.
34. Yung, L. and D. Linch, *Hodgkin's lymphoma.* Lancet, 2003. **361**(9361): p. 943-51.
35. Skinnider, B.F. and T.W. Mak, *The role of cytokines in classical Hodgkin lymphoma.* Blood, 2002. **99**(12): p. 4283-97.
36. Pattle, S.B. and P.J. Farrell, *The role of Epstein-Barr virus in cancer.* Expert Opin Biol Ther, 2006. **6**(11): p. 1193-205.
37. Schwering, I., et al., *Loss of the B-lineage-specific gene expression program in Hodgkin and Reed-Sternberg cells of Hodgkin lymphoma.* Blood, 2003. **101**(4): p. 1505-12.
38. Jarrett, R.F., *Risk factors for Hodgkin's lymphoma by EBV status and significance of detection of EBV genomes in serum of patients with EBV-associated Hodgkin's lymphoma.* Leuk Lymphoma, 2003. **44 Suppl 3**: p. S27-32.
39. Glaser, S.L. and R.F. Jarrett, *The epidemiology of Hodgkin's disease.* Baillieres Clin Haematol, 1996. **9**(3): p. 401-16.
40. Deacon, E.M., et al., *Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells.* J Exp Med, 1993. **177**(2): p. 339-49.
41. Mehl, A.M., et al., *Isolation and analysis of two strongly transforming isoforms of the Epstein-Barr-Virus(EBV)-encoded latent membrane protein-1 (LMP1) from a single Hodgkin's lymphoma.* Int J Cancer, 1998. **76**(2): p. 194-200.
42. Kawaguchi, H., et al., *Epstein-Barr virus-infected T lymphocytes in Epstein-Barr virus-associated hemophagocytic syndrome.* J Clin Invest, 1993. **92**(3): p. 1444-50.
43. Chiang, A.K., et al., *Nasal NK- and T-cell lymphomas share the same type of Epstein-Barr virus latency as nasopharyngeal carcinoma and Hodgkin's disease.* Int J Cancer, 1996. **68**(3): p. 285-90.
44. Thompson, M.P. and R. Kurzrock, *Epstein-Barr virus and cancer.* Clin Cancer Res, 2004. **10**(3): p. 803-21.
45. Jaffe ES, H.N., Stein N, Wardiman JW, ed. *World Health Organization classification of tumors. Pathology and genetics of tumors of haemopoietic and lymphoid tissues.* 2001, IARC Press: Lyon.
46. Rajewsky, K., *Clonal selection and learning in the antibody system.* Nature, 1996. **381**(6585): p. 751-8.
47. Kiel, M.J., et al., *SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells.* Cell, 2005. **121**(7): p. 1109-21.
48. Hirose, J., et al., *A developing picture of lymphopoiesis in bone marrow.* Immunol Rev, 2002. **189**: p. 28-40.
49. Tokoyoda, K., et al., *Cellular niches controlling B lymphocyte behavior within bone marrow during development.* Immunity, 2004. **20**(6): p. 707-18.
50. Hardy, R.R., et al., *Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow.* J Exp Med, 1991. **173**(5): p. 1213-25.

51. Li, Y.S., et al., *Identification of the earliest B lineage stage in mouse bone marrow.* Immunity, 1996. **5**(6): p. 527-35.
52. Sitnicka, E., et al., *Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool.* Immunity, 2002. **17**(4): p. 463-72.
53. Sitnicka, E., et al., *Complementary signaling through flt3 and interleukin-7 receptor alpha is indispensable for fetal and adult B cell genesis.* J Exp Med, 2003. **198**(10): p. 1495-506.
54. Peschon, J.J., et al., *Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice.* J Exp Med, 1994. **180**(5): p. 1955-60.
55. von Freeden-Jeffry, U., et al., *Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine.* J Exp Med, 1995. **181**(4): p. 1519-26.
56. Kong, Y.Y., et al., *OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis.* Nature, 1999. **397**(6717): p. 315-23.
57. Ehlich, A., et al., *Analysis of the B-cell progenitor compartment at the level of single cells.* Curr Biol, 1994. **4**(7): p. 573-83.
58. Murphy, K.M., P. Travers, and M. Walport, *Janeway's Immunobiology.* 2007.
59. Ohnishi, K. and T. Takemori, *Molecular components and assembly of mu.surrogate light chain complexes in pre-B cell lines.* J Biol Chem, 1994. **269**(45): p. 28347-53.
60. Manz, R.A., et al., *Survival of long-lived plasma cells is independent of antigen.* Int Immunol, 1998. **10**(11): p. 1703-11.
61. Melamed, D., et al., *Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor selection.* Cell, 1998. **92**(2): p. 173-82.
62. Bretscher, P.A. and M. Cohn, *Minimal model for the mechanism of antibody induction and paralysis by antigen.* Nature, 1968. **220**(5166): p. 444-8.
63. Melchers, F., *Anergic B cells caught in the act.* Immunity, 2006. **25**(6): p. 864-7.
64. Liu, Y.J. and C. Arpin, *Germinal center development.* Immunol Rev, 1997. **156**: p. 111-26.
65. Reynaud, C.A., et al., *Mismatch repair and immunoglobulin gene hypermutation: did we learn something?* Immunol Today, 1999. **20**(11): p. 522-7.
66. MacLennan, I.C., *Germinal centers.* Annu Rev Immunol, 1994. **12**: p. 117-39.
67. Nossal, G.J., *Genetic control of lymphopoiesis, plasma cell formation, and antibody production.* Int Rev Exp Pathol, 1962. **1**: p. 1-72.
68. Schittek, B. and K. Rajewsky, *Maintenance of B-cell memory by long-lived cells generated from proliferating precursors.* Nature, 1990. **346**(6286): p. 749-51.
69. Reimold, A.M., et al., *Plasma cell differentiation requires the transcription factor XBP-1.* Nature, 2001. **412**(6844): p. 300-7.
70. Radbruch, A., et al., *Competence and competition: the challenge of becoming a long-lived plasma cell.* Nat Rev Immunol, 2006. **6**(10): p. 741-50.
71. Gray, D., et al., *Observations on memory B-cell development.* Semin Immunol, 1997. **9**(4): p. 249-54.

72. Endres, R., et al., *Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells.* J Exp Med, 1999. **189**(1): p. 159-68.
73. Smith, K.G., et al., *The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response.* Embo J, 1997. **16**(11): p. 2996-3006.
74. Ozaki, K., et al., *Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6.* J Immunol, 2004. **173**(9): p. 5361-71.
75. Lin, K.I., et al., *Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells.* Mol Cell Biol, 2002. **22**(13): p. 4771-80.
76. Nera, K.P., et al., *Loss of Pax5 promotes plasma cell differentiation.* Immunity, 2006. **24**(3): p. 283-93.
77. Thorley-Lawson, D.A., *Epstein-Barr virus: exploiting the immune system.* Nat Rev Immunol, 2001. **1**(1): p. 75-82.
78. Monroe, J.G., *ITAM-mediated tonic signalling through pre-BCR and BCR complexes.* Nat Rev Immunol, 2006. **6**(4): p. 283-94.
79. Takata, M., et al., *Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca2+ mobilization through distinct pathways.* Embo J, 1994. **13**(6): p. 1341-9.
80. Tamir, I. and J.C. Cambier, *Antigen receptor signaling: integration of protein tyrosine kinase functions.* Oncogene, 1998. **17**(11 Reviews): p. 1353-64.
81. Ulivieri, C. and C.T. Baldari, *The BCR signalosome: where cell fate is decided.* J Biol Regul Homeost Agents, 2005. **19**(1-2): p. 1-16.
82. Fujimoto, M., et al., *CD19 regulates intrinsic B lymphocyte signal transduction and activation through a novel mechanism of processive amplification.* Immunol Res, 2000. **22**(2-3): p. 281-98.
83. Elghazi, L., et al., *Regulation of beta-cell mass and function by the Akt/protein kinase B signalling pathway.* Diabetes Obes Metab, 2007. **9 Suppl 2**: p. 147-57.
84. Brown, E.J., et al., *A mammalian protein targeted by G1-arresting rapamycin-receptor complex.* Nature, 1994. **369**(6483): p. 756-8.
85. Horvath, G., et al., *CD19 is linked to the integrin-associated tetraspans CD9, CD81, and CD82.* J Biol Chem, 1998. **273**(46): p. 30537-43.
86. Maecker, H.T., S.C. Todd, and S. Levy, *The tetraspanin superfamily: molecular facilitators.* Faseb J, 1997. **11**(6): p. 428-42.
87. Cherukuri, A., et al., *B cell signaling is regulated by induced palmitoylation of CD81.* J Biol Chem, 2004. **279**(30): p. 31973-82.
88. Rosa, D., et al., *Activation of naive B lymphocytes via CD81, a pathogenetic mechanism for hepatitis C virus-associated B lymphocyte disorders.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18544-9.
89. Cherukuri, A., et al., *The tetraspanin CD81 is necessary for partitioning of coligated CD19/CD21-B cell antigen receptor complexes into signaling-active lipid rafts.* J Immunol, 2004. **172**(1): p. 370-80.
90. Cariappa, A., et al., *The CD9 tetraspanin is not required for the development of peripheral B cells or for humoral immunity.* J Immunol, 2005. **175**(5): p. 2925-30.

91. Bishop, G.A., B.S. Hostager, and K.D. Brown, *Mechanisms of TNF receptor-associated factor (TRAF) regulation in B lymphocytes.* J Leukoc Biol, 2002. **72**(1): p. 19-23.
92. Devergne, O., et al., *Association of TRAF1, TRAF2, and TRAF3 with an Epstein-Barr virus LMP1 domain important for B-lymphocyte transformation: role in NF-kappaB activation.* Mol Cell Biol, 1996. **16**(12): p. 7098-108.
93. Darnay, B.G., et al., *Activation of NF-kappaB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-kappaB-inducing kinase. Identification of a novel TRAF6 interaction motif.* J Biol Chem, 1999. **274**(12): p. 7724-31.
94. Hostager, B.S., et al., *Tumor necrosis factor receptor-associated factor 2 (TRAF2)-deficient B lymphocytes reveal novel roles for TRAF2 in CD40 signaling.* J Biol Chem, 2003. **278**(46): p. 45382-90.
95. Rowland, S.L., et al., *A novel mechanism for TNFR-associated factor 6-dependent CD40 signaling.* J Immunol, 2007. **179**(7): p. 4645-53.
96. Briscoe, J., F. Kohlhuber, and M. Muller, *JAKs and STATs branch out.* Trends Cell Biol, 1996. **6**(9): p. 336-40.
97. Huen, D.S., et al., *The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-kappa B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain.* Oncogene, 1995. **10**(3): p. 549-60.
98. Merchant, M., R.G. Caldwell, and R. Longnecker, *The LMP2A ITAM is essential for providing B cells with development and survival signals in vivo.* J Virol, 2000. **74**(19): p. 9115-24.
99. Dykstra, M.L., R. Longnecker, and S.K. Pierce, *Epstein-Barr virus coopts lipid rafts to block the signaling and antigen transport functions of the BCR.* Immunity, 2001. **14**(1): p. 57-67.
100. Fruehling, S. and R. Longnecker, *The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction.* Virology, 1997. **235**(2): p. 241-51.
101. Kerr, B.M., et al., *Three transcriptionally distinct forms of Epstein-Barr virus latency in somatic cell hybrids: cell phenotype dependence of virus promoter usage.* Virology, 1992. **187**(1): p. 189-201.
102. Rowe, M., et al., *Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes.* J Virol, 1992. **66**(1): p. 122-31.
103. Humme, S., et al., *The EBV nuclear antigen 1 (EBNA1) enhances B cell immortalization several thousandfold.* Proc Natl Acad Sci U S A, 2003. **100**(19): p. 10989-94.
104. Mackey, D. and B. Sugden, *The linking regions of EBNA1 are essential for its support of replication and transcription.* Mol Cell Biol, 1999. **19**(5): p. 3349-59.
105. Avolio-Hunter, T.M. and L. Frappier, *Mechanistic studies on the DNA linking activity of Epstein-Barr nuclear antigen 1.* Nucleic Acids Res, 1998. **26**(19): p. 4462-70.
106. Mackey, D., T. Middleton, and B. Sugden, *Multiple regions within EBNA1 can link DNAs.* J Virol, 1995. **69**(10): p. 6199-208.

107. Wu, H., D.F. Ceccarelli, and L. Frappier, *The DNA segregation mechanism of Epstein-Barr virus nuclear antigen 1.* EMBO Rep, 2000. **1**(2): p. 140-4.
108. Blake, N., et al., *Human CD8+ T cell responses to EBV EBNA1: HLA class I presentation of the (Gly-Ala)-containing protein requires exogenous processing.* Immunity, 1997. **7**(6): p. 791-802.
109. Khanna, R., et al., *Isolation of cytotoxic T lymphocytes from healthy seropositive individuals specific for peptide epitopes from Epstein-Barr virus nuclear antigen 1: implications for viral persistence and tumor surveillance.* Virology, 1995. **214**(2): p. 633-7.
110. Kim, A.L., et al., *An imperfect correlation between DNA replication activity of Epstein-Barr virus nuclear antigen 1 (EBNA1) and binding to the nuclear import receptor, Rch1/importin alpha.* Virology, 1997. **239**(2): p. 340-51.
111. Kitamura, R., et al., *Nuclear import of Epstein-Barr virus nuclear antigen 1 mediated by NPI-1 (Importin alpha5) is up- and down-regulated by phosphorylation of the nuclear localization signal for which Lys379 and Arg380 are essential.* J Virol, 2006. **80**(4): p. 1979-91.
112. Summers, H., et al., *Cooperative assembly of EBNA1 on the Epstein-Barr virus latent origin of replication.* J Virol, 1996. **70**(2): p. 1228-31.
113. Wysokenski, D.A. and J.L. Yates, *Multiple EBNA1-binding sites are required to form an EBNA1-dependent enhancer and to activate a minimal replicative origin within oriP of Epstein-Barr virus.* J Virol, 1989. **63**(6): p. 2657-66.
114. Frappier, L., K. Goldsmith, and L. Bendell, *Stabilization of the EBNA1 protein on the Epstein-Barr virus latent origin of DNA replication by a DNA looping mechanism.* J Biol Chem, 1994. **269**(2): p. 1057-62.
115. Ruf, I.K. and J. Sample, *Repression of Epstein-Barr virus EBNA-1 gene transcription by pRb during restricted latency.* J Virol, 1999. **73**(10): p. 7943-51.
116. Kempkes, B., et al., *B-cell proliferation and induction of early G1-regulating proteins by Epstein-Barr virus mutants conditional for EBNA2.* Embo J, 1995. **14**(1): p. 88-96.
117. Kaiser, C., et al., *The proto-oncogene c-myc is a direct target gene of Epstein-Barr virus nuclear antigen 2.* J Virol, 1999. **73**(5): p. 4481-4.
118. Johansen, L.M., et al., *EBNA2 and activated Notch induce expression of BATF.* J Virol, 2003. **77**(10): p. 6029-40.
119. Murray, R.J., et al., *Different patterns of Epstein-Barr virus gene expression and of cytotoxic T-cell recognition in B-cell lines infected with transforming (B95.8) or nontransforming (P3HR1) virus strains.* J Virol, 1988. **62**(3): p. 894-901.
120. Wang, L., S.R. Grossman, and E. Kieff, *Epstein-Barr virus nuclear protein 2 interacts with p300, CBP, and PCAF histone acetyltransferases in activation of the LMP1 promoter.* Proc Natl Acad Sci U S A, 2000. **97**(1): p. 430-5.
121. Johannsen, E., et al., *Epstein-Barr virus nuclear protein 2 transactivation of the latent membrane protein 1 promoter is mediated by J kappa and PU.1.* J Virol, 1995. **69**(1): p. 253-62.
122. Wang, F., et al., *A bicistronic Epstein-Barr virus mRNA encodes two nuclear proteins in latently infected, growth-transformed lymphocytes.* J Virol, 1987. **61**(4): p. 945-54.

123. Szekely, L., et al., *EBNA-5, an Epstein-Barr virus-encoded nuclear antigen, binds to the retinoblastoma and p53 proteins.* Proc Natl Acad Sci U S A, 1993. **90**(12): p. 5455-9.
124. Inman, G.J. and P.J. Farrell, *Epstein-Barr virus EBNA-LP and transcription regulation properties of pRB, p107 and p53 in transfection assays.* J Gen Virol, 1995. **76 (Pt 9)**: p. 2141-9.
125. Mannick, J.B., et al., *The Epstein-Barr virus nuclear antigen leader protein associates with hsp72/hsc73.* J Virol, 1995. **69**(12): p. 8169-72.
126. Kitay, M.K. and D.T. Rowe, *Protein-protein interactions between Epstein-Barr virus nuclear antigen-LP and cellular gene products: binding of 70-kilodalton heat shock proteins.* Virology, 1996. **220**(1): p. 91-9.
127. Han, I., et al., *EBNA-LP associates with cellular proteins including DNA-PK and HA95.* J Virol, 2001. **75**(5): p. 2475-81.
128. Matsuda, G., et al., *Epstein-Barr virus (EBV) nuclear antigen leader protein (EBNA-LP) forms complexes with a cellular anti-apoptosis protein Bcl-2 or its EBV counterpart BHRF1 through HS1-associated protein X-1.* Microbiol Immunol, 2003. **47**(1): p. 91-9.
129. Igarashi, M., et al., *Physical interaction of Epstein-Barr virus (EBV) nuclear antigen leader protein (EBNA-LP) with human oestrogen-related receptor 1 (hERR1): hERR1 interacts with a conserved domain of EBNA-LP that is critical for EBV-induced B-cell immortalization.* J Gen Virol, 2003. **84**(Pt 2): p. 319-27.
130. Peng, R., J. Tan, and P.D. Ling, *Conserved regions in the Epstein-Barr virus leader protein define distinct domains required for nuclear localization and transcriptional cooperation with EBNA2.* J Virol, 2000. **74**(21): p. 9953-63.
131. Tanaka, M., et al., *Conserved region CR2 of Epstein-Barr virus nuclear antigen leader protein is a multifunctional domain that mediates self-association as well as nuclear localization and nuclear matrix association.* J Virol, 2002. **76**(3): p. 1025-32.
132. Harada, S. and E. Kieff, *Epstein-Barr virus nuclear protein LP stimulates EBNA-2 acidic domain-mediated transcriptional activation.* J Virol, 1997. **71**(9): p. 6611-8.
133. Sawada, K., et al., *Expression of EBNA-3 family in fresh B lymphocytes infected with Epstein-Barr virus.* Virology, 1989. **168**(1): p. 22-30.
134. Tomkinson, B. and E. Kieff, *Use of second-site homologous recombination to demonstrate that Epstein-Barr virus nuclear protein 3B is not important for lymphocyte infection or growth transformation in vitro.* J Virol, 1992. **66**(5): p. 2893-903.
135. Chen, A., et al., *Epstein-Barr virus with the latent infection nuclear antigen 3B completely deleted is still competent for B-cell growth transformation in vitro.* J Virol, 2005. **79**(7): p. 4506-9.
136. Tomkinson, B., E. Robertson, and E. Kieff, *Epstein-Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation.* J Virol, 1993. **67**(4): p. 2014-25.
137. Maruo, S., et al., *Epstein-Barr virus nuclear protein EBNA3C is required for cell cycle progression and growth maintenance of lymphoblastoid cells.* Proc Natl Acad Sci U S A, 2006. **103**(51): p. 19500-5.

138. Zhao, B., D.R. Marshall, and C.E. Sample, *A conserved domain of the Epstein-Barr virus nuclear antigens 3A and 3C binds to a discrete domain of Jkappa.* J Virol, 1996. **70**(7): p. 4228-36.
139. Silins, S.L. and T.B. Sculley, *Modulation of vimentin, the CD40 activation antigen and Burkitt's lymphoma antigen (CD77) by the Epstein-Barr virus nuclear antigen EBNA-4.* Virology, 1994. **202**(1): p. 16-24.
140. Sample, C. and B. Parker, *Biochemical characterization of Epstein-Barr virus nuclear antigen 3A and 3C proteins.* Virology, 1994. **205**(2): p. 534-9.
141. Gires, O., et al., *Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule.* Embo J, 1997. **16**(20): p. 6131-40.
142. Mosialos, G., et al., *The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family.* Cell, 1995. **80**(3): p. 389-99.
143. Eliopoulos, A.G. and A.B. Rickinson, *Epstein-Barr virus: LMP1 masquerades as an active receptor.* Curr Biol, 1998. **8**(6): p. R196-8.
144. Floettmann, J.E., et al., *Epstein-Barr virus latent membrane protein-1 (LMP1) signalling is distinct from CD40 and involves physical cooperation of its two C-terminus functional regions.* Oncogene, 1998. **17**(18): p. 2383-92.
145. Kilger, E., et al., *Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor.* Embo J, 1998. **17**(6): p. 1700-9.
146. Floettmann, J.E. and M. Rowe, *Epstein-Barr virus latent membrane protein-1 (LMP1) C-terminus activation region 2 (CTAR2) maps to the far C-terminus and requires oligomerisation for NF-kappaB activation.* Oncogene, 1997. **15**(15): p. 1851-8.
147. Hatzivassiliou, E. and G. Mosialos, *Cellular signaling pathways engaged by the Epstein-Barr virus transforming protein LMP1.* Front Biosci, 2002. **7**: p. d319-29.
148. Sample, J., D. Liebowitz, and E. Kieff, *Two related Epstein-Barr virus membrane proteins are encoded by separate genes.* J Virol, 1989. **63**(2): p. 933-7.
149. Laux, G., A. Economou, and P.J. Farrell, *The terminal protein gene 2 of Epstein-Barr virus is transcribed from a bidirectional latent promoter region.* J Gen Virol, 1989. **70 (Pt 11)**: p. 3079-84.
150. Fennewald, S., V. van Santen, and E. Kieff, *Nucleotide sequence of an mRNA transcribed in latent growth-transforming virus infection indicates that it may encode a membrane protein.* J Virol, 1984. **51**(2): p. 411-9.
151. Longnecker, R. and E. Kieff, *A second Epstein-Barr virus membrane protein (LMP2) is expressed in latent infection and colocalizes with LMP1.* J Virol, 1990. **64**(5): p. 2319-26.
152. Rivailler, P., C. Quink, and F. Wang, *Strong selective pressure for evolution of an Epstein-Barr virus LMP2B homologue in the rhesus lymphocryptovirus.* J Virol, 1999. **73**(10): p. 8867-72.
153. Bornkamm, G.W. and W. Hammerschmidt, *Molecular virology of Epstein-Barr virus.* Philos Trans R Soc Lond B Biol Sci, 2001. **356**(1408): p. 437-59.
154. Caldwell, R.G., et al., *Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals.* Immunity, 1998. **9**(3): p. 405-11.

155. Caldwell, R.G., R.C. Brown, and R. Longnecker, *Epstein-Barr virus LMP2A-induced B-cell survival in two unique classes of EmuLMP2A transgenic mice.* J Virol, 2000. **74**(3): p. 1101-13.
156. Miller, C.L., et al., *Integral membrane protein 2 of Epstein-Barr virus regulates reactivation from latency through dominant negative effects on protein-tyrosine kinases.* Immunity, 1995. **2**(2): p. 155-66.
157. Miller, C.L., et al., *Epstein-Barr virus protein LMP2A regulates reactivation from latency by negatively regulating tyrosine kinases involved in sIg-mediated signal transduction.* Infect Agents Dis, 1994. **3**(2-3): p. 128-36.
158. Miller, C.L., et al., *An integral membrane protein (LMP2) blocks reactivation of Epstein-Barr virus from latency following surface immunoglobulin crosslinking.* Proc Natl Acad Sci U S A, 1994. **91**(2): p. 772-6.
159. Miller, C.L., R. Longnecker, and E. Kieff, *Epstein-Barr virus latent membrane protein 2A blocks calcium mobilization in B lymphocytes.* J Virol, 1993. **67**(6): p. 3087-94.
160. Rovedo, M. and R. Longnecker, *Epstein-barr virus latent membrane protein 2B (LMP2B) modulates LMP2A activity.* J Virol, 2007. **81**(1): p. 84-94.
161. Allen, M.D., L.S. Young, and C.W. Dawson, *The Epstein-Barr virus-encoded LMP2A and LMP2B proteins promote epithelial cell spreading and motility.* J Virol, 2005. **79**(3): p. 1789-802.
162. Sharipo, A., et al., *cis-Inhibition of proteasomal degradation by viral repeats: impact of length and amino acid composition.* FEBS Lett, 2001. **499**(1-2): p. 137-42.
163. Spriggs, M.K., et al., *The extracellular domain of the Epstein-Barr virus BZLF2 protein binds the HLA-DR beta chain and inhibits antigen presentation.* J Virol, 1996. **70**(8): p. 5557-63.
164. Rowe, M., et al., *Host shutoff during productive Epstein-Barr virus infection is mediated by BGLF5 and may contribute to immune evasion.* Proc Natl Acad Sci U S A, 2007. **104**(9): p. 3366-71.
165. Swart, R., S. Fruehling, and R. Longnecker, *Tyrosines 60, 64, and 101 of Epstein-Barr virus LMP2A are not essential for blocking B cell signal transduction.* Virology, 1999. **263**(2): p. 485-95.
166. Lynch, D.T., J.S. Zimmerman, and D.T. Rowe, *Epstein-Barr virus latent membrane protein 2B (LMP2B) co-localizes with LMP2A in perinuclear regions in transiently transfected cells.* J Gen Virol, 2002. **83**(Pt 5): p. 1025-35.
167. Dawson, C.W., et al., *The Epstein-Barr virus encoded latent membrane protein 2A augments signaling from latent membrane protein 1.* Virology, 2001. **289**(2): p. 192-207.
168. Frech, B., et al., *Characterization of the antibody response to the latent infection terminal proteins of Epstein-Barr virus in patients with nasopharyngeal carcinoma.* J Gen Virol, 1993. **74 (Pt 5)**: p. 811-8.
169. Matskova, L., et al., *C-terminal domain of the Epstein-Barr virus LMP2A membrane protein contains a clustering signal.* J Virol, 2001. **75**(22): p. 10941-9.
170. Miyashita, E.M., et al., *Identification of the site of Epstein-Barr virus persistence in vivo as a resting B cell.* J Virol, 1997. **71**(7): p. 4882-91.

171. Ikeda, A., et al., *Itchy, a Nedd4 ubiquitin ligase, downregulates latent membrane protein 2A activity in B-cell signaling.* J Virol, 2003. **77**(9): p. 5529-34.
172. Longnecker, R., et al., *An Epstein-Barr virus protein associated with cell growth transformation interacts with a tyrosine kinase.* J Virol, 1991. **65**(7): p. 3681-92.
173. Burkhardt, A.L., et al., *An Epstein-Barr virus transformation-associated membrane protein interacts with src family tyrosine kinases.* J Virol, 1992. **66**(8): p. 5161-7.
174. Fruehling, S., et al., *Tyrosine 112 of latent membrane protein 2A is essential for protein tyrosine kinase loading and regulation of Epstein-Barr virus latency.* J Virol, 1998. **72**(10): p. 7796-806.
175. Ikeda, M., A. Ikeda, and R. Longnecker, *Lysine-independent ubiquitination of Epstein-Barr virus LMP2A.* Virology, 2002. **300**(1): p. 153-9.
176. Winberg, G., et al., *Latent membrane protein 2A of Epstein-Barr virus binds WW domain E3 protein-ubiquitin ligases that ubiquitinate B-cell tyrosine kinases.* Mol Cell Biol, 2000. **20**(22): p. 8526-35.
177. Speck, P., et al., *Epstein-Barr virus lacking latent membrane protein 2 immortalizes B cells with efficiency indistinguishable from that of wild-type virus.* J Gen Virol, 1999. **80 (Pt 8)**: p. 2193-203.
178. Rochford, R., et al., *In vivo growth of Epstein-Barr virus transformed B cells with mutations in latent membrane protein 2 (LMP2).* Arch Virol, 1997. **142**(4): p. 707-20.
179. Konishi, K., et al., *Role of Epstein-Barr virus-encoded latent membrane protein 2A on virus-induced immortalization and virus activation.* J Gen Virol, 2001. **82**(Pt 6): p. 1451-6.
180. Colley, K.J., *Golgi localization of glycosyltransferases: more questions than answers.* Glycobiology, 1997. **7**(1): p. 1-13.
181. Longnecker, R., *Epstein-Barr virus latency: LMP2, a regulator or means for Epstein-Barr virus persistence?* Adv Cancer Res, 2000. **79**: p. 175-200.
182. Kirchhausen, T., et al., *Membranes and sorting. Membrane permeability.* Curr Opin Cell Biol, 1997. **9**(4): p. 473.
183. Stanley, K.K., *Regulation of targeting signals in membrane proteins. [review].* Mol Membr Biol, 1996. **13**(1): p. 19-27.
184. Luke, M.R., et al., *The trans-Golgi network GRIP-domain proteins form alpha-helical homodimers.* Biochem J, 2005. **388**(Pt 3): p. 835-41.
185. Kim, O.J. and J.L. Yates, *Mutants of Epstein-Barr virus with a selective marker disrupting the TP gene transform B cells and replicate normally in culture.* J Virol, 1993. **67**(12): p. 7634-40.
186. Longnecker, R., et al., *The only domain which distinguishes Epstein-Barr virus latent membrane protein 2A (LMP2A) from LMP2B is dispensable for lymphocyte infection and growth transformation in vitro; LMP2A is therefore nonessential.* J Virol, 1992. **66**(11): p. 6461-9.
187. Longnecker, R., et al., *The last seven transmembrane and carboxy-terminal cytoplasmic domains of Epstein-Barr virus latent membrane protein 2 (LMP2) are dispensable for lymphocyte infection and growth transformation in vitro.* J Virol, 1993. **67**(4): p. 2006-13.

188. Longnecker, R., et al., *Deletion of DNA encoding the first five transmembrane domains of Epstein-Barr virus latent membrane proteins 2A and 2B.* J Virol, 1993. **67**(8): p. 5068-74.
189. Brielmeier, M., et al., *The latent membrane protein 2 gene of Epstein-Barr virus is important for efficient B cell immortalization.* J Gen Virol, 1996. **77 (Pt 11)**: p. 2807-18.
190. Altmann, M., *Funktion fruher Gene des Epstein-Barr Virus bei der Immortalisierung humaner primarer B-Lymphozyten.* 2003.
191. Mancao, C. and W. Hammerschmidt, *Epstein-Barr virus latent membrane protein 2A is a B-cell receptor mimic and essential for B-cell survival.* Blood, 2007. **110**(10): p. 3715-21.
192. Hanahan, D., *Studies on transformation of Escherichia coli with plasmids.* J Mol Biol, 1983. **166**(4): p. 557-80.
193. Chung, C.T., S.L. Niemela, and R.H. Miller, *One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution.* Proc Natl Acad Sci U S A, 1989. **86**(7): p. 2172-5.
194. Sheng, Y., V. Mancino, and B. Birren, *Transformation of Escherichia coli with large DNA molecules by electroporation.* Nucleic Acids Res, 1995. **23**(11): p. 1990-6.
195. Pelloquin, F., J.P. Lamelin, and G.M. Lenoir, *Human B lymphocytes immortalization by Epstein-Barr virus in the presence of cyclosporin A.* In Vitro Cell Dev Biol, 1986. **22**(12): p. 689-94.
196. Tohami, T., et al., *Expression of tetraspanins in peripheral blood leukocytes: a comparison between normal and infectious conditions.* Tissue Antigens, 2004. **64**(3): p. 235-42.
197. Murray, P.G. and L.S. Young, *The Role of the Epstein-Barr virus in human disease.* Front Biosci, 2002. **7**: p. d519-40.
198. Laux, G., M. Perricaudet, and P.J. Farrell, *A spliced Epstein-Barr virus gene expressed in immortalized lymphocytes is created by circularization of the linear viral genome.* Embo J, 1988. **7**(3): p. 769-74.
199. Rechsteiner, M.P., et al., *Silencing of latent membrane protein 2B reduces susceptibility to activation of lytic Epstein-Barr virus in Burkitt's lymphoma Akata cells.* J Gen Virol, 2007. **88**(Pt 5): p. 1454-9.
200. Rechsteiner, M.P., et al., *Latent membrane protein 2B regulates susceptibility to induction of lytic Epstein-Barr virus infection.* J Virol, 2008. **82**(4): p. 1739-47.
201. Aravindan, N., et al., *Alteration of apoptotic signaling molecules as a function of time after radiation in human neuroblastoma cells.* Mol Cell Biochem, 2008. **310**(1-2): p. 167-79.
202. Tomaszewski-Flick, M.J. and D.T. Rowe, *Minimal protein domain requirements for the intracellular localization and self-aggregation of Epstein-Barr virus latent membrane protein 2.* Virus Genes, 2007. **35**(2): p. 225-34.
203. Borst, J., J. Hendriks, and Y. Xiao, *CD27 and CD70 in T cell and B cell activation.* Curr Opin Immunol, 2005. **17**(3): p. 275-81.
204. Prasad, K.V., et al., *CD27, a member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein.* Proc Natl Acad Sci U S A, 1997. **94**(12): p. 6346-51.

205. Swanson-Mungerson, M., R. Bultema, and R. Longnecker, *Epstein-Barr virus LMP2A enhances B-cell responses in vivo and in vitro.* J Virol, 2006. **80**(14): p. 6764-70.
206. Robinson, R.A., et al., *Biochemical and structural studies of ASPP proteins reveal differential binding to p53, p63, and p73.* Structure, 2008. **16**(2): p. 259-68.
207. Samuels-Lev, Y., et al., *ASPP proteins specifically stimulate the apoptotic function of p53.* Mol Cell, 2001. **8**(4): p. 781-94.
208. Naumovski, L. and M.L. Cleary, *The p53-binding protein 53BP2 also interacts with Bc12 and impedes cell cycle progression at G2/M.* Mol Cell Biol, 1996. **16**(7): p. 3884-92.
209. Ikeda, M., A. Ikeda, and R. Longnecker, *PY motifs of Epstein-Barr virus LMP2A regulate protein stability and phosphorylation of LMP2A-associated proteins.* J Virol, 2001. **75**(12): p. 5711-8.
210. Ikeda, M., et al., *The Epstein-Barr virus latent membrane protein 2A PY motif recruits WW domain-containing ubiquitin-protein ligases.* Virology, 2000. **268**(1): p. 178-91.
211. Portis, T. and R. Longnecker, *Epstein-Barr virus (EBV) LMP2A mediates B-lymphocyte survival through constitutive activation of the Ras/PI3K/Akt pathway.* Oncogene, 2004. **23**(53): p. 8619-28.
212. Fukuda, M. and R. Longnecker, *Latent membrane protein 2A inhibits transforming growth factor-beta 1-induced apoptosis through the phosphatidylinositol 3-kinase/Akt pathway.* J Virol, 2004. **78**(4): p. 1697-705.
213. Fukuda, M. and R. Longnecker, *Epstein-Barr virus (EBV) latent membrane protein 2A regulates B-cell receptor-induced apoptosis and EBV reactivation through tyrosine phosphorylation.* J Virol, 2005. **79**(13): p. 8655-60.
214. Katzman, R.B. and R. Longnecker, *LMP2A does not require palmitoylation to localize to buoyant complexes or for function.* J Virol, 2004. **78**(20): p. 10878-87.
215. Doody, G.M., P.W. Dempsey, and D.T. Fearon, *Activation of B lymphocytes: integrating signals from CD19, CD22 and Fc gamma RIIb1.* Curr Opin Immunol, 1996. **8**(3): p. 378-82.
216. Merchant, M., et al., *The effects of the Epstein-Barr virus latent membrane protein 2A on B cell function.* Int Rev Immunol, 2001. **20**(6): p. 805-35.
217. Hammerschmidt, W. and B. Sugden, *Identification and characterization of oriLyt, a lytic origin of DNA replication of Epstein-Barr virus.* Cell, 1988. **55**(3): p. 427-33.
218. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* Proc Natl Acad Sci U S A, 2000. **97**(12): p. 6640-5.
219. Baer, R., et al., *DNA sequence and expression of the B95-8 Epstein-Barr virus genome.* Nature, 1984. **310**(5974): p. 207-11.