THE EFFECT OF EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN 2 EXPRESSION ON THE KINETICS OF EARLY B CELL INFECTION AND IMMORTALIZATION

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Epstein-Barr virus (EBV) has been associated with the development of several human malignancies. Infection of B cells with wild-type EBV *in vitro* leads to activation and proliferation that result in efficient production of lymphoblastoid cell lines (LCLs). The majority of latent genes are expressed during early infection, including latent membrane protein 2 (LMP2). Currently, the role of LMP2 in B cell proliferation is controversial; some studies have shown that LMP2 is dispensable, while others report it is important role for this process. However, each of these experimental systems were limited by either wild-type virus contamination or use of incomplete viral genomes (mini-EBV), which precluded clear assessments of the effects of LMP2 during early infection.

In this study I investigated the effect of LMP2 on early B cell infection and subsequent immortalization via complete recombinant EBV with knockouts of either or both isoforms of the LMP2 gene, LMP2A and LMP2B (Δ2A, Δ2B and Δ2A/Δ2B). Infection of B cells with LMP2A knockout viruses resulted in marked decreases in activation and proliferation relative to wild-type, and higher percentages of apoptotic B cells. Δ2B virus infection exhibited activation levels comparable to wild-type, but with fewer numbers of proliferating B cells. The stability of viral latency was determined for early B cell infection by evaluating latent and lytic gene expression with or without lytic stimulation via the B cell receptor (BCR). Infection with wild-type, Δ2A and Δ2B viruses with or without BCR stimulation did not result in changes in viral latency,

whereas stimulation of BCR signaling in Δ2A/Δ2B-infected cells resulted in decreased LMP1 expression, suggesting loss of stability in viral latency. The long-term effects of LMP2 deletion on B cell outgrowth were investigated using LCL establishment assays, which revealed that LMP2A, but not LMP2B, is critical for efficient immortalization of B cells *in vitro*. Loss of both isoforms promoted the least activation, proliferation and LCL formation. This study enhances our knowledge of events required for B cell transformation by EBV, and clearly shows the public health relevance of understanding genes involved in tumorigenesis for the future pursuit of more effective treatments for EBV-associated malignancies.

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1.0 INTRODUCTION

1.1 EBV CLASSIFICATION

Epstein-Barr virus (EBV), or Human Herpesvirus 4 (HHV-4), is a ubiquitous member of the gammaherpesvirus subfamily that has infected approximately 95% of the global adult population. EBV has a specific tropism for B cells and epithelial cells, but has recently been shown to infect other cell types, such as T cells and Natural Killer (NK) cells. Similar to other herpesviruses, initial infection with EBV can be asymptomatic or symptomatic. EBV infection in children generally results in asymptomatic or mild infection. However, infection in adolescents and adults can lead to development of infectious mononucleosis (IM) in ~30-50% of cases. Following initial infection the virus establishes latency within the B cell population that persists for the lifetime of the host. Although complications of EBV infection are rare, EBV has been associated with the development of malignant diseases [1-3]. A unique attribute of EBV is its ability to induce the proliferation and transformation of infected B cells *in vitro.*

The virus was first identified by a collaboration of scientists that included Anthony Epstein, Yvonne Barr and Bert Achong in 1964, when a herpesvirus-like particle was identified in electron micrographs of tumor cells derived from Burkitt's lymphomas (BL) obtained from children in equatorial Africa [4,5]. Unlike previously discovered herpesviruses, this new herpesvirus could not be grown in established culture conditions or identified with antibodies

specific to known herpesviruses. Following its initial discovery, EBV was found to induce B cell proliferation and immortalization in cell culture [6]. Though there was considerable evidence to indicate a possible role for viruses in human cancers, it was not until the identification of BL and EBV that the association of viruses with human cancers became clear. Therefore, EBV was identified as the first human tumor virus.

EBV is the only human virus associated with the Lymphocryptoviridae (LCV) genus, while Kaposi sarcoma-associated herpesvirus (KSHV), or HHV-8, is a member of the Rhadinoviridae (RDV) genus of the gammaherpesvirus subfamily [7]. The gammaherpesvirus subfamily was initially established based on shared biological properties of the viruses that included oncogenic associations, host range in cell culture, and episome persistence in dividing cells. The LCV are thought to have evolved from RDV; both have genomes that contain substantial homology. However, LCV can be distinguished from RDV by their genome size, as well as by differences in glycoproteins, cytokine homologues and anti-apoptotic proteins [8]. Specifically, the two genomes vary by approximately 25kb, with the size of the EBV genome measuring at 184kb and the KSHV genome at 160kb. Also, EBV expresses a viral homologue for the cytokine Interleukin (IL)-10, while KSHV expresses a viral homologue for IL-6. EBV infection is specific to humans, but there are many simian homologues that infect other primates, such as the marmoset LCV known as Callitrichine herpesvirus 3 (CalHV-3), baboon LCV called Herpesvirus papio, and rhesus LCV 1 and 2 [9,10]. The RDV family, in addition to the human virus KSHV, also includes simian homologues, such as Herpesvirus saimiri and Herpesvirus ateles [11].

There are two distinct strains of EBV, EBV1 and EBV2, which are closely related. Sequencing of both viruses determined that sequence variability is most significant in the nuclear

protein genes EBNA-LP, EBNA2 and EBNA3 (EBNA3A, EBNA3B and EBNA3C), which are all expressed during growth transformation of infected B cells. Epidemiologic studies of EBV in global populations has determined that EBV2 is most commonly found in equatorial Africa and Papua New Guinea, but has been identified in certain Western populations, such as HIV+ males. EBV1 does not appear to be specific to any one population, and represents the dominant subtype of EBV found throughout the world. EBV1 has been shown to induce B cell immortalization in culture more efficiently than EBV2, and is most commonly used for research studies even though EBV2 is found in various commonly used cell lines such as JiJoye BL cells. The prototype EBV virus, B95.8, is classified as EBV1 subtype, and was used to create the BAC-derived viruses for this study. B95.8 differs from naturally occurring EBV due to an approximately 12kb deletion in the region of the genome that contains the two BamHI A rightward transcript (BART) miRNA clusters [12,13].

The structure of EBV virions resembles other herpesviruses. The virion is approximately 200nm in diameter and contains one copy of linear double-stranded DNA, which circularizes following cell entry. This single genome is surrounded by an icosahedral capsid that is composed of 162 capsomeres, specifically 150 hexons and 12 pentons. The capsid is surrounded by a complex layer of tegument proteins, which, in turn, is surrounded by an outermost layer of lipid envelope that contains various viral glycoproteins. Several of these glycoproteins are homologous to other herpesvirus glycoproteins (gH, gL, gM and gN), while others are specific to EBV (gp350/220, the major EBV glycoprotein, and gp110). The structure of the EBV genome has several unique features, which includes two to five identical 0.5kb tandem terminal repeats (TR) at both termini, six to twelve tandem repeats of 3kb internal sequence, and 5 largely unique

sequences of varying length. The TRs are duplicated during virus replication and may vary in number between different EBV viruses [7].

The EBV genome is in linear form within the virus particles, but circularizes at the TR following uncoating in the infected cells. Circularization of the genome allows for transcription of the LMP2 isoforms, LMP2A and LMP2B, which must cross the fused TR for complete transcription, and results in establishment of latency **(Figure 1)**. EBNA1 tethers the viral episome to the host cell chromosome, which ensures that the EBV genome will be replicated during S phase of the cell cycle and distributed into the new daughter cells [14,15]. Switch to the lytic cycle will induce replication of the viral episome by the rolling-circle mechanism, which creates multiple concatemers that are cut at the TR for packaging into the newly produced virions [16]. The observed differences in numbers of TRs are a contributing factor to the variability between EBV strains, and can be calculated by an inverse correlation to LMP1 mRNA expression [17]. This is possible because one of the LMP1 promoters is in close proximity to the TR region. The LMP2 promoters are also in close proximity to the TR region, but expression of these two isoforms has not been correlated with terminal repeat number.

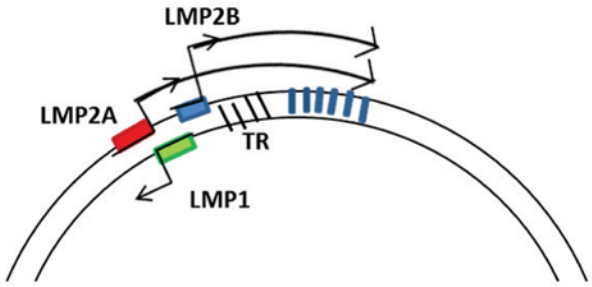

Figure 1. LMP2A and LMP2B transcripts cross terminal repeat region.

EBV genome circularizes following uncoating in the nucleus. This allows for transcription of the terminal transcripts, LMP2A and LMP2B, which must cross the terminal repeat (TR) region for complete transcription.

1.2 NATURAL HISTORY OF EBV INFECTION AND CLINICAL MANIFESTATIONS

1.2.1 Primary Infection and viral persistence

Initial infection with EBV occurs when the new host comes into contact with the saliva of an infected person. The infectious EBV in the saliva infects the mucosal epithelial cells of the oropharynx. In these epithelial cells, EBV can undergo lytic replication and move into local secondary lymphoid organs, such as the tonsil, where resident B cells become infected by the invading virus. The expression of EBV latent genes leads to activation and proliferation of infected B cells, which leads to induction of a strong cellular immune response, especially against EBNA3 family epitopes expressed in these B cells. Elimination of large numbers of EBV-infected B cells is carried out by cytotoxic T killer (CTL) cells and Natural Killer (NK) cells [18,19].

Primary infection in children leads to asymptomatic or mild infection that is indistinguishable from other infections. However, primary infection in adolescence or young adulthood can lead to development of mononucleosis in 35-50% of cases. The symptoms of mononucleosis include fever, fatigue, cervical lymphadenopathy, and sore throat that can last for 1-2 months. In some cases hepatosplenomegaly and jaundice can occur, and heart problems or involvement of the central nervous system are extremely rare [18,19]. When infectious mononucleosis illness lasts for more than 6 months, it is referred to as chronic active EBV infection (CAEBV). High antibody levels against lytic antigens or low antibody levels against EBNAs can be used to diagnose CAEBV [20]. Originally, CAEBV was characterized by ongoing proliferation of B cells, but many cases in Asia have determined that the proliferation

occurs in T cells and NK cells, which were previously not considered targets of the virus [21]. There is limited understanding as to how these cells become infected by EBV because they do not express the main viral receptor, CD21. EBV is not known to express viral glycoproteins specific for heparin sulfate, as is the case for most herpesviruses, however it is possible that combinations of existing glycoproteins may be involved in viral entry into these particular cell types.

Following primary infection, EBV establishes latency in a small percentage of memory B cells within the host [22,23]. However, another subset of B cells has been shown to harbor the EBV episome, and was identified as CD10+ CD77+ germinal center B cells that are found in the follicles of lymph nodes and other lymphoid organs. Healthy carriers of the virus shed infectious EBV into the saliva, which allows for easy distribution to susceptible hosts [24]. The shedding of virus takes place when infected B cells in the tonsils differentiate into plasma cells, the cell type in which lytic induction is activated *in vivo* [25]. The newly produced EBV then infects local epithelial cells, where the majority of lytic induction most likely occurs [26,27].

1.2.2 EBV entry and life cycle

Most herpesviruses have surface glycoproteins that interact with heparin sulfate, which allows for a very diverse pool of target cells [28,29]. However, EBV does not express a glycoprotein with specificity to heparin sulfate, and, therefore, has a much more restrictive pool of target cells. Instead, EBV utilizes the CD21 receptor, which is also known as complement receptor 2 (CR2) [30], and is a major component of the B cell receptor (BCR) co-receptor complex that also contains CD19 and CD81.

EBV attaches to B cells via interaction of CD21 with the major viral glycoprotein gp350/220, which is an abundant, highly glycosylated type 1 membrane protein that exists in two alternatively spliced forms. The splice maintains the reading frame and results in deletion of amino acid residues 500-757 of the 907 amino acid protein, but retains the CD21-binding site [31,32]. The interaction with CD21 induces tyrosine phosphorylation of CD19 and activation of phosphatidylinositol 3-kinase (PI3-K) and subsequent downstream activation of NF-κB [33]. Stimulation of CD21 transduced signaling does not affect EBV entry, but is required for efficient transformation of infected B cells by EBV [34]. Following attachment of EBV to B cells, endocytosis of the virus into thin-walled non-clathrin coated vesicles occurs [35,36].

After EBV entry into endosomes, fusion of the viral envelope with the endosomal membrane allows for release of the EBV capsid into the cytoplasm. In B cells fusion occurs in low pH environment, though low pH is not essential for the process. Conversely, fusion in epithelial cells occurs at neutral pH [37]. This process requires the actions of the viral glycoproteins gB, gH, gL and gp42, of which gH, gL and gp42 form a non-covalently linked complex in the virus [38]. Interaction between gp42 and the major histocompatibility complex (MHC) class II molecule is required for fusion between the virus envelope and the B cell membrane [39], and may also play a role in blocking MHC Class II antigen presentation to CD4+ T cells [40]. The EBV homologues of two other herpesvirus glycoproteins, gM and gN, are also involved in viral entry and egress. These two proteins form a complex that is responsible for efficient envelopment and egress of the newly produced virions [41].

Following uncoating the EBV capsid is then transported to the nucleus where the viral DNA is released. This most likely occurs via transportation on microtubules using dynein and dinactin motors, similar to other herpesviruses. For instance, the EBV gene BFRF3 is a

homologue of HSV UL35, which is a small capsid protein responsible for interacting with dynein light chains [42]. Also, the EBV BVRF1 tegument protein is a homologue of HSV UL25 cork protein [43]. It is possible that BVRF1, with portal protein (BBRF1), may orient the capsid relative to the nuclear pore, aid in disassembly and permit import of viral DNA into the nucleus. However, the exact mechanism for EBV capsid trafficking and genome release in the nucleus is unknown.

Within 16 hours of infection newly circularized EBV episomes are detected and utilized for latent gene expression [44]. In addition to expression of latent genes, which aid in the survival and differentiation of B cells, EBV undergoes a switch to the lytic cycle for production of new infectious virus particles. The exact timing of the switch from latency to the lytic cycle is not known, but can vary from 3 days to more than 9 days after infection before secreted virus is detected [45]. The lytic cycle for all herpesviruses follows a similar cascade of gene expression: immediate early, early and late genes. Specifically, the lytic cycle for EBV begins with the expression of the immediate early gene BZLF1, which codes for the protein ZEBRA or Z [46]. ZEBRA is considered the dominant protein responsible for lytic cycle induction. Since ZEBRA is a homologue of the AP-1 family of transcription factors [45], it binds to AP-1 motifs known as Z-responsive elements (ZREs) [47]. ZEBRA activates both immediate early and early lytic promoters, such as the BMLF1 promoter, but requires the cooperative function of another immediate early gene BRLF1, or Rta [48]. The BMLF1 gene product transports intronless transcripts, which includes both early and late EBV gene transcripts, from the nucleus to the cytoplasm for translation, and is essential for virus replication and virion production [49,50]. The genes that code for the viral DNA polymerase and associated viral replication machinery proteins are also transcribed during the early lytic phase.

Both BZLF1 and BRLF1 bind directly to oriLyt, the origin of lytic EBV DNA replication, but only BZLF1 is required for replication from oriLyt [51,52]. Following DNA replication, the viral genome is clipped in the terminal repeats region to form linear double-stranded DNA genome for packaging into virions [16]. Late viral genes are expressed following the onset of viral DNA replication, and include structural proteins such as nucleocapsid proteins, viral glycoproteins, and cytokine (IL-10) and Bcl-2 homologues. The linear DNA genome is moved into the capsid through a dodecameric complex of Portal protein (BBRF1). Assembly of the DNA genome and capsid occurs in the nucleus, as with other herpesviruses.

Release of EBV particles occurs similarly to other herpesviruses, although the exact mechanism of egress is not known [53]. During egress, the viral capsid acquires its first envelope via budding through the inner nuclear membrane. Subsequent fusion with the outer leaflet of the nuclear membrane, or membrane of the endoplasmic reticulum (ER), results in the loss of this primary envelope and tegument proteins, as well as the translocation of the viral capsid/DNA into the cytoplasm. The final layer of tegument proteins is most likely acquired in the cytoplasm [54,55]. Unlike previous reports suggesting that the tegument layer in herpesviruses is amorphous and unstructured, more recent studies have suggested that herpesvirus tegument layers are increasingly complex and that tegumentation itself likely follows an intricate network of protein-protein interactions. The final envelopment occurs as tegumented capsids bud into the trans-Golgi network vesicles, which already contain the various viral glycoproteins in the vesicular membrane that will be incorporated into the virions [55]. Interaction of the tegument proteins with the cytoplasmic tails of the viral glycoproteins may drive the final budding process.

EBV also has a tropism for epithelial cells, and can complete its life cycle within these cells. Epithelial cells express neither CD21 (CR2), the surface receptor required for EBV

attachment in B cells, nor MHC Class II molecules, the surface receptor required for EBV fusion and entry into B cells. Therefore, EBV entry into epithelial cells is unique from entry into B cells, though the surface receptor responsible for EBV entry is not known. EBV infection of these cells requires the complex of gH and gL, which is also part of the complex required for viral entry in B cells. Unlike B cells, fusion of EBV with epithelial cell membranes does not require gp42, and, in fact, is inhibited by the presence of this glycoprotein. Studies have shown that EBV produced in MHC II negative epithelial cells is at least 2 logs more infectious for MHC II positive B cells compared to EBV produced in B cells. Similarly, EBV produced in MHC positive B cells is approximately 5 times more infectious for MHC II negative epithelial cells. Differences in infectivity may be the result of higher levels of gp42+ virus in epithelial cell supernatants compared to B cell derived supernatants, possibly due interactions of gp42+ virus with MHC II molecules that directly lead to their degradation. These results suggest that gp42 may function as a switch of virus tropism and may be relevant to the spread of EBV between tissues *in vivo* [56]. Previous research has suggested that the BMRF1 protein, a viral envelope protein, interacts with α5β1 integrins at the basolateral surface of polarized epithelial cells that results in high levels of infection [57].

1.2.3 Establishment of latency *in vivo*

During primary infection, incoming virus infects and establishes an initial site for lytic replication in the epithelial cells of the oropharynx. The newly produced virus then spreads throughout the lymphoid tissues, where it encounters and infects B cells. It remains unclear whether EBV preferentially infects naïve or memory B cells early after primary infection *in vivo*,

but infection *in vitro* does not appear selective for either B cell subset. Initial infection of B cells results in expression of all 9 latency genes, which includes 6 EBV nuclear antigens (EBNA1, EBNA2, EBNA-LP, EBNA3A, EBNA3B, EBNA3C) and 3 latent membrane proteins (LMP1, LMP2A and LMP2B), as well as EBV-encoded noncoding RNAs (EBERs) and BamHI A Rightward Transcripts (BARTs). This is known as Latency III, or the growth program, which is responsible for driving the activation and proliferation of the infected B cell population **(Table 1)**. Latency III is also expressed in B cell infection *in vitro*, and is responsible for the conversion of resting B cells into lymphoblastoid cell lines (LCLs). Although all latency genes are expressed in LCLs, only a subset of these genes are considered indispensible for *in vitro* immortalization, which includes EBNA2, LMP1, EBNA3A and EBNA3C. The majority of these EBV-infected B cells are targeted and removed by the emerging latent gene-specific CTL response [58,59].

A small number of infected B cells escape the CTL response by downregulating latent gene expression to Latency II, or default program **(Table 1)**. Latency II includes expression of EBNA1, LMP1, LMP2A, LMP2B, EBERs and BARTs. B cells expressing the Latency II program are characteristic of germinal center B cells, which undergo affinity maturation and differentiation into memory B cells much like their uninfected counterparts [60]. For uninfected B cells, interactions with cognate antigen, displayed by follicular dendritic cells (FDCs), and CD40L, provided by CD4+ T cells, lead to B cell proliferation and somatic hypermutation of BCR variable region, results in changes of antigen-binding affinities. Only B cells with the highest affinities for specific antigens are selected for survival and terminal differentiation into either memory B cells or plasma cells, which are responsible for secretion of large amount of antibody with the same antigen affinity as the BCR (surface immunoglobulin) [61]. Interestingly, EBV infection provides latent genes that mimic survival signals B cells would receive from

interactions with antigen and CD4+ T cells. These genes are known as LMP1 and LMP2A. Specifically, LMP1 provides a ligand-independent, constitutive signal that is similar to the signal provided by CD40 after engaging its ligand, CD40L, while LMP2A provides a ligand-independent, constitutive signal much like the signal provided by the BCR [62,63]. However, it is unclear whether these genes play an active role in selection of EBV-infected B cells for differentiation into long-term memory B cells.

Memory B cells are the main reservoir for EBV, and can express either Latency 0/I, also called latency program [23]. Latency I is characterized by expression of EBNA1 and LMP2A, as well as EBERs and BARTs **(Table 1)**. Latency 0, or true latency, includes expression of EBNA1, EBERs and possibly LMP2A without expression of any of the other latency genes. EBNA1 is usually only detected intermittently, particularly when the B cell undergoes cell divisions, due to its function as an episome/chromosome tethering protein during latency that allows for replication and subsequent distribution of EBV to daughter cells [64]. The steps involved in establishment of EBV latency in vivo are shown in **Figure 2**.

1.2.4 EBV-associated malignancies in the immunocompetent

The majority of EBV-infected individuals exhibit a benign and asymptomatic infection for the duration of their lives. However, a small percentage of individuals will develop malignancies that are associated with the presence EBV, though additional risk factors are usually correlated with disease development. Several studies have concluded that EBV-associated malignancies follow a specific pattern of latent gene expression (described in **Table 1**).

Table 1. EBV latency programs and associated malignancies

Latency Program	EBV Gene Expression	Phenotypes in healthy carriers	Associated Disease
Latency 0	EBERs, possibly LMP2A	Memory B cells	None
Latency I	EBERs, BARTs, EBNA1, LMP2A, BARF1 in epithelial cells	Memory B cells	Burkitt's lymphoma, Gastric carcinoma
Latency II	EBERs, BARTs, EBNA1, LMP1, LMP2A, LMP2B, BARF1 in epithelial cells	Germinal Center B cells	Hodgkin's lymphoma, NPC
Latency III	EBERs, BARTs, EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, LMP2A, LMP2B	Newly infected B cells	PTLD, lymphoproliferative diseases in immunosuppressed

EBV expresses two proteins that have been catalogued as oncoproteins, LMP1 and BARF1. LMP1 mimics the signal provided by CD40, and is essential for proliferation and immortalization of B cells. The other EBV oncogene, BARF1, is a colony stimulating factor 1 (CSF-1) mimic that is expressed during the viral lytic cycle in B cells, and has also been detected in epithelial cell tumors [65]. Therefore, the presence of putative EBV-encoded oncogenes points to the strong possibility that EBV infection is associated with malignant disease. Several malignancies develop in individuals with intact immune systems, and include Burkitt's lymphoma, Hodgkin's lymphoma, T/NK cell lymphomas, as well as carcinomas that include nasopharyngeal carcinoma and gastric carcinoma. With the exception of T/NK cell lymphomas, all of these malignancies are of B cell or epithelial cell origin [58,66]. These diseases will be described in detail below.

1.2.4.1 Burkitt's lymphoma

Burkitt's lymphoma (BL) is B cell-associated tumor with unusual presentation in sites such as the jaw, orbit of the eye, and ovaries. There are two classifications of BL, sporadic and endemic. The endemic form of BL was initially discovered by Denis Burkitt in 1958 in children residing in equatorial Africa, and is almost always associated with the presence of the latent EBV genome [67]. In contrast, sporadic BL is not as closely associated with childhood or the presence of EBV, only 30% of sporadic BL are EBV+. In endemic areas most children become infected with EBV within the first two years of life. Endemic BL in children has been characterized by elevated antibody titers against EBV lytic antigens, viral capsid antigen (VCA) and early antigen (EA), emphasizing the strong association of pediatric endemic BL with EBV infection [68]. Children that are most affected by endemic BL live in geographical regions that are also associated with endemic malaria. Studies have shown that the malaria parasite can contribute to development of BL by inducing mitogenic effects in B cells and suppression of the T cell response, creating the ideal milieu for outgrowth of infected B cells and somatic hypermutation/differentiation in the germinal center [66].

BL cells have characteristics associated with germinal center B cells. They carry somatic hypermutations in their BCR genes, albeit in the absence of Ig isotype switching, and express CD10 and CD77. These cells also express high levels of the transcriptional repressor protein Bcl-6, which inhibits B cell differentiation via targeting of Blimp1, a transcription factor associated with plasma cell differentiation. Interestingly, these cells do not express surface antigens that are associated with B cell activation, which includes CD23, CD30, CD39 and CD70, and are also low or absent for cell adhesion molecules CD54, CD58 and CD80. All BL cells contain a translocation of the c-myc proto-oncogene from its location in chromosome 8 to one of three

possible immunoglobulin loci located on chromosomes 2, 14 or 22, though translocations between chromosomes 8 and 14 are the most common. Translocation of the c-myc gene to the highly active Ig locus results in high expression of c-myc, which causes malignant transformation of the affected B cell [69].

The role of EBV in the development of BL is poorly understood. Endemic BL cells are characterized by the expression of the Latency I program, which includes expression of EBNA1, LMP2A, as well as EBERs and BARTs. There are hypotheses that suggest EBV+ BL cell lines have greater chromosomal instability than EBV- BL cells, which may contribute to the translocation of c-myc to the Ig locus [70]. EBV may also sustain the BL cells after tumorigenesis, which is evident by EBV positivity and the presence of clonal EBV genomes in BL tumors [71]. Also, EBNA1 and EBERs may contribute directly to the development of BL. EBNA1, in the absence of LMP1, inhibits loading of peptides on MHC Class I molecules, thereby inhibiting an effective T cell response against virus-positive cells. EBERs have been shown to upregulate the anti-apoptotic gene Bcl-2, which may contribute to survival of tumorigenic B cells in BL [72].

1.2.4.2 Hodgkin's lymphoma

Hodgkin's lymphomas (HL) are very treatable diseases that constitute approximately 10-15% of all malignant lymphomas worldwide. Approximately one third of HL are associated with EBV in North America and Europe, and a higher percentage, closer to 100%, are associated with EBV in Africa and Latin America. HL exhibits a bimodal age distribution, with most of the affected falling into either young adult or elderly groups. Although infection of EBV alone does not increase the risk of HL, development of IM following EBV infection is considered a risk factor

for HL. IM has not been associated with increased risk for development of non-Hodgkin's lymphoma [73].

There are two major subtypes of HL, including classical and lymphocyte predominant forms. Of these two subtypes, only classical HL is associated with EBV. There are histological subtypes of classical HL that include nodular sclerosis, mixed cellularity, lymphocyte-rich classical and lymphocyte depleted. EBV has been mostly associated with the mixed cellularity subtype of classical HL, and has been associated with lymphocyte-depleted subtype in the context of HL in HIV infection [66,69].

A hallmark of HL is the presence of a low percentage (1-3%) of malignant Hodgkin Reed-Sternberg (HRS) cells in a background of non-neoplastic cells that includes infiltrating CD4+ T cells, plasma cells, macrophages and eosinophilic granulocytes [74]. The infiltrating immune cells produce large amounts of various cytokines, creating a microenvironment around the tumor that promotes the malignant process. The presence of the virus in EBV+ HL most likely contributes additional cytokines to this microenvironment, which further prevents an effective immune response from entering and destroying the tumor [75].

Similar to BL cells, HRS are germinal center B cells that carry complete EBV genomes in the form of covalently closed episomes. In contrast to BL cells, HRS cells express a wider variety of EBV latent genes, known as the Latency II program **(Table 1)** [76]. Also, HRS cells are weakly positive for B cell surface markers, such as CD19, CD20 and CD79A, unlike BL cells that are highly positive for B cell pan markers CD19 and CD20. Although these malignant cells do not express B cell identification markers or surface Ig, they do retain expression of surface receptors necessary for T cell interaction, such as CD40 and CD80. Analysis of the EBV

genome revealed that HL tumor cells also had clonal EBV episomes, like BL tumors, that suggest the tumor originated from a single infected cell [75,77].

Somatic hypermutations in the Ig heavy chain genes demonstrate that HRS cells are GC-derived, though HRS cells have nonfunctionally rearranged Ig that cannot be expressed on the B cell surface. Although the lack of a functional surface Ig (BCR) would normally lead to induction of apoptosis, HRS cells are protected from apoptosis, which is most likely the result of survival signals provided by LMP2A expression that resemble those from an intact BCR. LMP1 may also contribute to development of HL via survival signals and activation of NF-κB [78].

1.2.4.3 T/NK cell lymphomas

Although EBV is mainly B cell tropic, it has been detected in certain types of NK/T cell lymphomas, which includes extranodal NK/T cell lymphoma of nasal type, aggressive NK cell leukemia, angioimmunoblastic T cell lymphoma and extranodal enteropathy-type T cell lymphoma. The nasal extranodal NK/T cell lymphoma is the most common of the NK/T cell lymphomas, though it is still generally a rare disease constituting merely 1.4% of all investigated lymphomas.

These lymphoma cells are characterized by frequent necrosis/apoptosis, expression of Latency II program **(Table 1)**, and the presence of clonal EBV episomes [79]. The malignant cells are either NK cells (CD3- CD56+, no TCR rearrangements) or T cells (CD3+ CD56-/+ with TCR rearrangements) and are most frequently found in the nasal region. EBV infection of T cells carries a high risk of lymphomagenesis, possibly due to the expression of LMP1 and LMP2A.

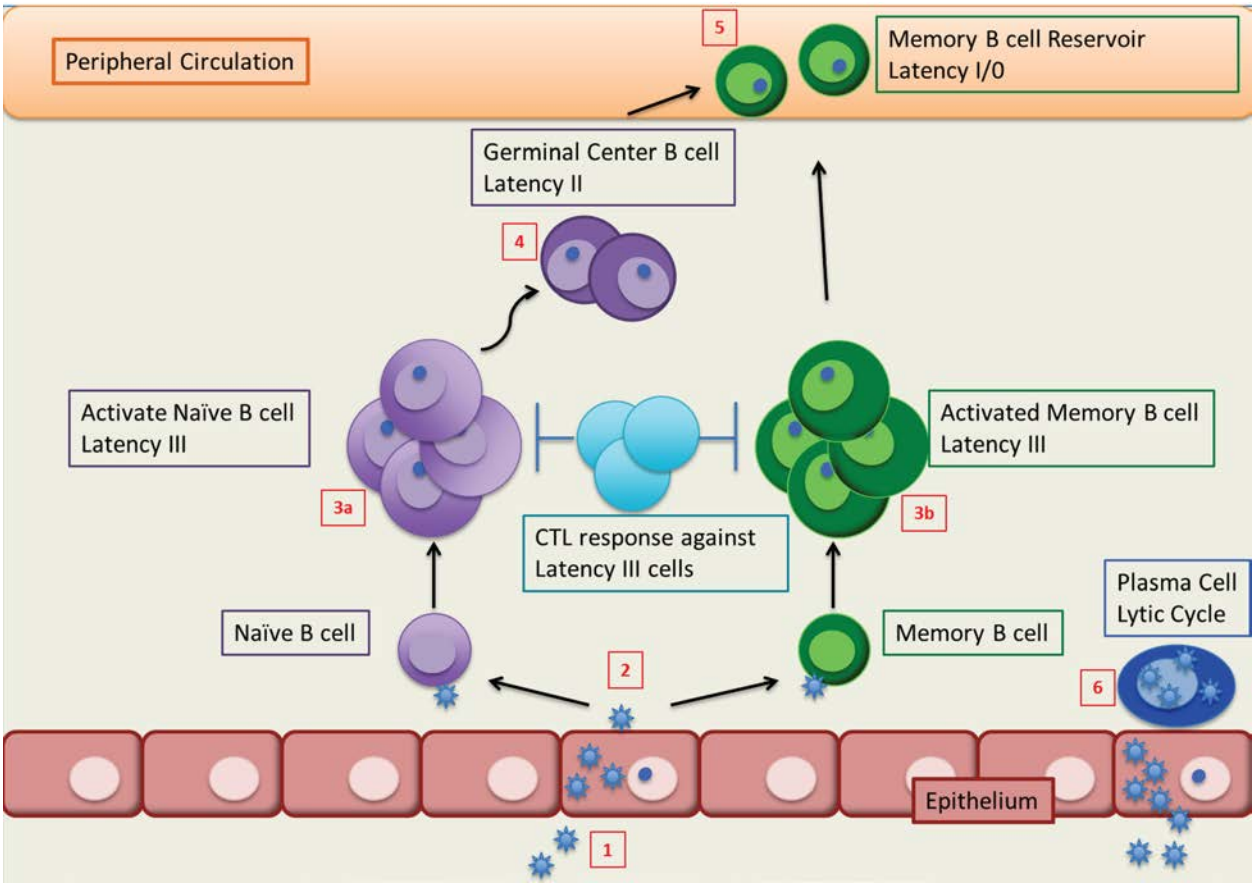

Figure 2. Primary infection and EBV persistence in memory B cells.

[1] EBV infects the epithelium of the oropharynx, where it is amplified. **[2]** EBV infects naïve and memory B cells, activating them to proliferate via expression of Latency III program **[3]**. CTL responses remove B cells expressing Latency III. **[4]** Naïve B cells restrict EBV gene expression to Latency II and differentiate into germinal center B cells that will eventually escape into the **[5]** peripheral blood as memory B cells expressing Latency I/0. **[6]** Terminal differentiation of memory B cells into plasma cells results in lytic reactivation. Progeny virus infect nearby epithelial cells where large amount of virus is produced and released into the saliva.

1.2.4.4 Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is a tumor derived from the epithelial lining of the nasopharynx that is most prevalent in Southeast Asia, responsible for 20% of all cancers in China and Taiwan. There are two major subtypes of NPC, keratinizing squamous cell carcinoma and

non-keratinizing carcinoma. The non-keratinizing subtype can be further divided into differentiated and nondifferentiated carcinomas.

Nearly 100% of undifferentiated NPC worldwide are EBV+, specifically containing clonal EBV episomes, which suggests that EBV is an essential cofactor for development of NPC. Similar to BL, NPC patients have elevated antibody titers against the lytic antigens, VCA and early antigen (EA). Additionally, NPC patients have elevated IgA antibodies against these antigens; it was discovered that elevation of IgA antibodies preceded disease onset in NPC patients by several years. These data suggest that EBV replication precedes NPC and may occur at mucosal surfaces [80,81].

Risk for development of NPC can be associated with geography, genetic predisposition, and environmental factors. High incidence rates for NPC are observed in certain ethnic populations in Southeast Asia, North Africa and the Inuit population in the Arctic Circle [82]. The disease is also more common in men, occurring 2-3 times more often than in women. There are also familial risks for developing NPC, and other published data suggest that HLA alleles A2, B14 and B46, as well as polymorphisms in nitrosamine metabolism genes are associated with increased risk of disease [83,84]. Additionally, environmental factors, such as diet, seem to play a role in NPC risk, with diets high in salted fish and other preserved foods the strongest indicator for NPC development.

Similar to HL, NPC tumors are accompanied by a tumor microenvironment that includes tumor-infiltrating lymphocytes (TIL) that are most commonly CD4+ Th-17 cells, as well as tumor-associated macrophages and fibroblasts. Within the tumor microenvironment expression of proinflammatory cytokines such as IL-1β, IL-6, IL-8 and IL-10, as well as the viral encoded homologue vIL-10 contributes to tumor growth and immune suppression [85]. The tumors

express Latency II, which is also found in HL **(Table 1)**. However, NPC cells also express BARF1, an oncogenic growth factor and CSF-1 receptor mimic that blocks binding of antiviral CSF-1 and subsequent macrophage differentiation and interferon production. Expression of this gene likely contributes to development of the tumors. LMP1 is detected in about 40-60% of the NPC tumors, and 100% of premalignant cells are positive for this gene. Additionally, expression of LMP2A and LMP2B are regularly detected in NPC. As with HL, the strong signaling capabilities of LMP1 and LMP2A suggest that these two proteins play a role in tumorigenesis. LMP2A and LMP2B can block differentiation and inhibit anchor dependence in epithelial cells, which could, in part, explain the metastatic activity of NPC cells [86].

1.2.4.5 Gastric carcinoma

Gastric carcinomas are tumors that develop in the epithelium of the stomach mucosa with glandular differentiation. Incidence of gastric cancers is highly variable, with higher rates in the Andean mountain regions of South America, Eastern Europe and Eastern Asia, and lower rates in North American whites, Northern Europe and most African regions [87].

EBV+ gastric carcinoma is relatively rare before the age of 30, with incidence rates increasing with age. Risk factors associated with disease development include familial occurrence, tobacco smoking, certain diets, alcohol, as well as infection with *Helicobacter pylori.* Also, men are at higher risk for developing EBV+ gastric carcinoma than their female counterparts. Although there is evidence linking these risk factors with increased incidence of the disease, the exact demographics related to gastric carcinoma development are not known.

The EBV genome has been detected in approximately 10% of gastric carcinomas. The malignant cells contain clonal EBV episomes, which indicates that the tumor began as a single infected cell. Serological studies have confirmed the presence of elevated antibody titers against

VCA and EA at the time of or preceding diagnosis of gastric carcinoma. These tumors are characterized by an inflammatory microenvironment similar to NPC, but produce higher levels of IL-1β compared to other growth factors [88]. Like NPC, gastric carcinomas express Latency II program and BARF1. LMP2A expression in the absence of LMP1 is common in gastric carcinoma, suggesting that LMP2A expression may represent an important risk factor for development of disease [89]. EBV-associated gastric carcinomas have few chromosomal abnormalities; the effects on cancer genes are often mediated by hypermethylation [90].

1.2.5 EBV-associated malignancies in the immunocompromised

Following primary infection with EBV, there is a temporary outgrowth of EBV-transformed B cells. An effective T cell response is required to end this temporary outgrowth, forcing EBV to restrict expression of highly immunogenic genes, such as the majority of EBNAs. Downregulation to Latency I/II directs the infected B cells to differentiate toward long-lasting memory cells.

Patients with suppressed immune systems, such as transplant recipients and patients with acquired immunodeficiency syndrome (AIDS), cannot eliminate proliferating EBV-transformed B cells, which can lead to development of immunoblastic lymphomas that may be fatal. Post-transplant lymphoproliferative disease and AIDS-related lymphomas will be discussed in more detail below.

1.2.5.1 Post-transplant lymphoproliferative disease

In most cases of EBV infection the CTL response removes immunoblastic EBV+ B cells expressing Latency III genes. However, transplant recipients experiencing immune suppression

induced by treatment with anti-rejection drugs are particularly sensitive to unchecked outgrowth of EBV+ Latency III cells. Post-transplant lymphoproliferative disease (PTLD) is an important cause of morbidity and mortality in solid organ and hematopoietic stem cell transplant recipients, and is a frequent result of drug-induced immune suppression. Approximately 90% are EBV+ within one year of transplantation [91]. In solid organ transplant recipients, PTLD is derived from the recipient and can easily be controlled by alleviating the suppression of the T cell response. However, in hematopoietic stem cell transplantation, PTLD is often donor-derived and directly related to the eradication of the host (recipient) immune system. Infusions of donor T cells or ex vivo-derived EBV-specific CTLs are the only methods available to control PTLD in these cases [92-94].

PTLD is characterized as histologically and molecularly heterogeneous, and can include several different pathologies, such as polyclonal B cell lymphoproliferations, polymorphic PTLD, as well as malignant monomorphic B cell lymphomas such as diffuse large B cell lymphoma, BL/BL-like lymphoma and HL. Although PTLD tumor cells frequently express displays Latency III, heterogeneity in viral gene expression between and within tumors suggest that this level of gene expression may only be required in the early stages of disease development [95]. Germinal center or post-germinal center B cells are frequently detected in PTLD malignancies, though the disease can involve B cells from various stages of development. Interestingly, approximately half of PTLD tumors do not express a functional BCR, much like HRS cells in HL. Therefore, it is possible that LMP1 and LMP2A also play important roles in the expansion of these B cells, indicating that the pathology of some PTLD cases could mirror that of HL.

In solid organ transplant patients, the risk of developing PTLD can increase based on the type of organ transplanted, presence of other viruses, age, and EBV seronegativity prior to transplant. For instance, pediatric transplant recipients are at higher risk for developing PTLD. Also, transplantation of intestinal tissue, multiple organs and tissues with high numbers of lymphocytes are associated with elevated PTLD risk. The specific removal of T cells using monoclonal antibodies is highly associated with increased risk of PTLD, most likely due to their role in controlling proliferation of Latency III B cells. In fact, depletion of both T and B cells is not a risk factor for PTLD development. Additionally, the risk of PTLD increases after treatment of transplant recipients with calcineurin inhibitors, such as cyclosporine and tacrolimus [96,97].

1.2.5.2 AIDS-associated lymphomas

HIV-infected individuals, especially those who have progressed to AIDS, have a defective cellular immune response. As mentioned previously, T cells play an important role in regulating EBV infection. In the absence of CTLs, EBV-infected B cells continuously proliferate, which can lead to development of malignancies. BL is a common malignancy observed early in AIDS progression, and resembles the sporadic subtype with exact characteristic chromosomal translocations [98]. Approximately 60% of malignancies observed in AIDS patients are EBV-associated, demonstrated by the presence of monoclonal episomes in tumor cells. EBV is primarily detected in HL and central nervous system (CNS) lymphomas in HIV-infected individuals [99]. The specific genetic or environmental factors involved in the development of these EBV-associated malignancies remains unclear. However, there is evidence that HIV infection could influence the cytokine expression, as IL-6 and IL-10 are notably elevated during HIV infection. Importantly, these two cytokines promote growth of EBV-infected B cells [100].

1.3 EBV-CELL INTERACTIONS

1.3.1 Latent Genes

1.3.1.1 EBNA1

EBNA1 is one of six EBV-encoded nuclear antigens **(Figure 3)**. EBNA1 homologues can be found in all known members of the lymphocryptovirus family. However, proteins with homologous sequences to EBNA1 are not present in the rhadinovirus family, though there is a protein with functional homology called LANA-1. EBNA1 is a DNA-binding protein that binds to specific sequences within the origin of plasmid replication (OriP), and is necessary and sufficient for replication and maintenance of the EBV episome. The EBNA1 dimer binds to 30bp repeat elements in the family of repeats (FR) and dyad symmetry (DS) domains within OriP, which results in the tethering of the EBV episome to the cellular chromosome and subsequent replication and segregation of viral genome into daughter cells duringing cell division. Attachment of EBNA1 to metaphase chromosomes occurs via its N-terminal domain, specifically in RGG-rich domains. Several cellular factors mediate chromosome tethering, including the chromatin-associated cellular protein EBP2 and telomeric factors that bind adjacent to EBNA1 in the DS region of OriP to promote plasmid maintenance [59,64].

Once bound to OriP, EBNA1 can function as a transcriptional activator via its N-terminal domain, specifically activating transcription from the Cp promoter and LMP1 promoter. It has been shown that EBNA1 can also transactivate cellular promoters through its FR domain when bound to OriP, which allows EBNA1 to influence cellular gene expression [101]. In addition to transcriptional activation, EBNA1 can act as a transcriptional repressor, regulating its own expression from the Qp promoter via binding to two specific sites in the promoter region [102].

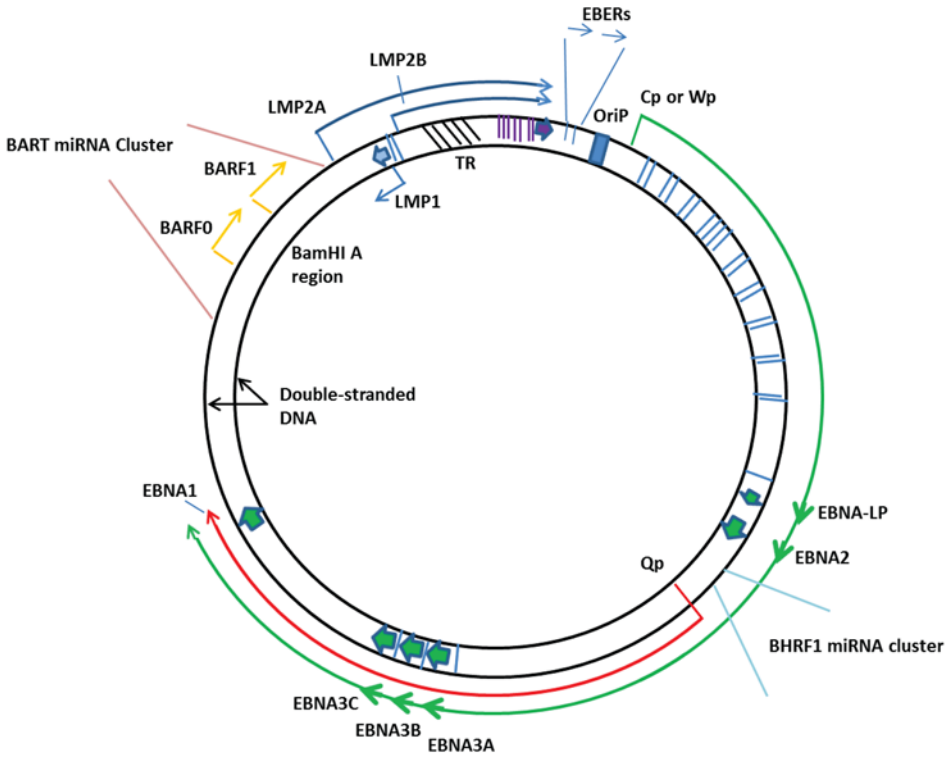

Figure 3. EBV Latent Genome

The double-stranded DNA form of the EBV genome is created by fusion of the terminal repeat (TR) region following uncoating in the nucleus. This allows for transcription of LMP2A and LMP2B, which cross the TR for complete transcription. The origin of plasmid replication (OriP) is shown in blue. The blue hash marks represent coding exons for the latency genes, and arrows represent the direction of transcription. Green arrows represent transcription of the 6 nuclear antigens (EBNA1, -2, -LP, -3A, -3B and -3C). The blue arrow represents transcription of the latent membrane protein LMP1, while the purple arrow represents transcription of LMP2A/2B. The BART and BHRF1 miRNA clusters are represented by pink and light blue lines, respectively. EBERs (EBER1 and EBER2) are represented by blue lines.

The size of EBNA1 can vary between 60-100 kDa, which is due to the presence of a glycine-alanine repetitive sequence. This region inhibits the processing of EBNA1 by the proteasome, a pre-requisite for peptide loading onto MHC class I molecules and subsequent presentation of EBNA1-specific epitopes to CD8+ T cells [103]. Lack of recognition by T cells allows for an extended half-life of EBNA1 proteins compared to other latency-associated proteins, and may contribute to the control of latency. EBNA1 is expressed in most EBV-

positive cell types, irrespective of cell phenotype, differentiation level or activation status, as in the case of lymphocytes.

EBNA1 is not considered essential for immortalization of infected B cells, even though it is essential for maintenance of the viral episome. However, previous studies have demonstrated anti-apoptotic activity in cells that express EBNA1, which could be explained by stabilization of p53 in the presence of EBNA1 [104,105].

1.3.1.2 EBNA2

Unlike EBNA1, EBNA2 is essential for the immortalization of B cells [106]. EBNA2 is a potent transcriptional transactivator of many cellular and viral genes, but, unlike EBNA1, does not directly bind to DNA. Although EBNA1 is expressed regardless of cell phenotype, EBNA2 expression is restricted to immunoblasts that express Latency III program, and is expressed from Wp or Cp promoters **(Figure 3)**. EBNA2 sequences between the two EBV subtypes, EBV-1 and EBV-2, are the most divergent of any other EBV gene, and may partially explain the differences in transforming capabilities between these two subtypes [58,64].

Expression of EBNA2 induces various activation markers such as CD23, CD21, c-fgr and c-myc, and is required for expression of the latent membrane proteins, LMP1 and LMP2 [107,108]. Transactivation of promoters by EBNA2 requires cellular factors, some of which include p300 and CREB-binding protein (CBP). CBP has been implicated in the activation of the c-myc promoter by EBNA2 [58].

EBNA2 also interacts with the cellular DNA-binding proteins CBF1/RBP-Jk and PU.1 to induce expression of various genes [109]. RBP-Jk is considered a downstream target of the Notch cell surface receptor. Under normal conditions RBP-Jk acts as a repressor via recruitment

of HDACs to targeted promoters. Interaction of cleaved Notch (Notch-IC) with DNA-bound RBP-Jk causes causes conversion of RBP-Jk to a transcriptional activator. Interaction of EBNA2 with RBP-Jk also converts this protein to a transcriptional activator, and, therefore, EBNA2 is regarded as a constitutively active homologue of Notch-IC [110,111]. In addition to activation of LMP promoters, EBNA2 transactivates the viral Cp promoter, thereby initiating the switch from Wp to Cp promoter that is characteristic of early B cell infection. The Cp, LMP1, LMP2 and CD23 promoter elements responsible for EBNA2 responsiveness were identified as RBP-Jk binding sites. EBNA2-mediated transactivation of LMP1/LMP2B promoter depends on additional interaction with the transcription factor PU.1 [59,64,112].

The essential role for EBNA2 in the immortalization of B cells stems from its transcriptional activation activities involving several viral and cellular promoters. LMP1 and LMP2A expression is regulated by EBNA2 in B cells expressing the Latency III program. LMP1 is essential for immortalization, as the CD40 signal mimic, while LMP2A may also play an important role in this process as the BCR signal mimic. Also, EBNA2 controls expression of various cellular oncogenes, such as c-myc, which most likely provides the link between EBNA2 and the cell cycle machinery [113].

1.3.1.3 EBNA-LP

EBNA-LP is an unusual viral protein that is created by splicing of the W1-W2 exons and the unique Y1 and Y2 exons **(Figure 4)**. The W1-W2 exons are found in the large internal repeat region (IR1) and encodes two domains of 22 and 44 amino acid repeats that contribute to the observed heterogeneity in the size and structure of the protein. The original mRNA transcript is bicistronic and also contains the EBNA2 gene at the 3' end. Although EBNA-LP localizes to the

nucleus, its distribution within the nucleus is variable and partially dependent on the cell phenotype. In BL cells, the distribution is diffuse throughout the nucleus, but localizes specifically to promyelocytic leukemia (PML) nuclear bodies in LCLs. Nonhuman primate lymphocryptoviruses express EBNA-LP homologues; the interaction of EBNA-LP and EBNA2 is conserved in these viruses [58,64].

In addition to EBNA2, EBNA-LP is one of the earliest latent genes expressed following B cell infection, and, like EBNA2, expression is restricted to B cells expressing Latency III. EBNA-LP cooperates with EBNA2 to enhance transcriptional activation of viral promoters, such as LMP1 and LMP2B and the Cp promoter [114]. Coactivation requires the nuclear location signal and conserved region 3 domains located in the W1 and W2 exons. There is no precisely defined role for EBNA-LP in B cell immortalization. However, immortalization is more efficient in the presence of this protein [115]. This could be due to enhancement of EBNA2-mediated transcription of essential proteins, such as LMP1, for the process of immortalization. Once LCLs are established there is no difference in growth kinetics between wild-type and EBNA-LP deficient LCLs.

EBNA-LP interacts with a large variety of cellular proteins, including sp100, pRb and p53. Early after EBV infection EBNA-LP interacts with sp100 causing its displacement from PML nuclear bodies. Both EBNA-LP and sp100 then co-activate EBNA2, which leads to transcription from EBNA2-responsive promoters. Later in infection, EBNA-LP and sp100 localize to PML nuclear bodies, leading to restricted EBNA2-associated gene expression. EBNA-LP also interacts with pRb, but the nature and function of this interaction is not clear. The interaction of EBNA-LP with p53 leads to proteasomal degradation of p53, blocking p53-mediated apoptosis [116-118]. Therefore, EBNA-LP plays important roles in the regulation of

EBNA2-mediated gene transcription, as well as a possible role aiding in the maintenancy of viral latency via blocking apoptosis.

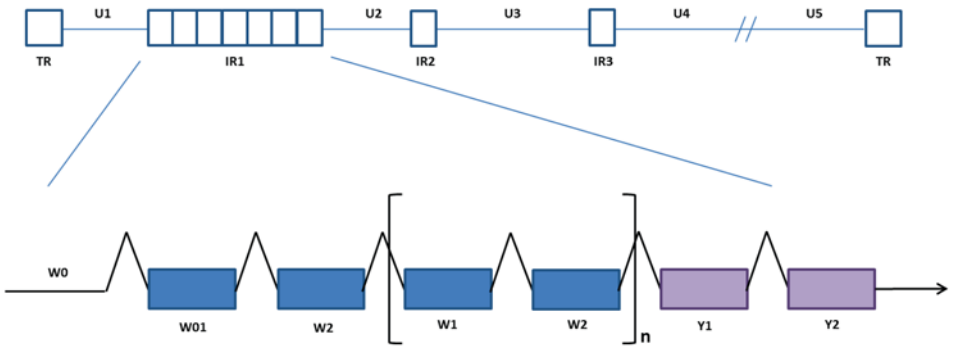

Figure 4. Splicing for EBNA-LP Transcript.

EBNA-LP is created by the splicing of the W1-W2 exons and Y1-Y2 exons. These exons are found in the internal repeat (IR) region 1 in the viral genome. The W1-W2 exons encode two domains of 22 and 44 amino acid repeats that contribute to heterogeneity in the size and structure of EBNA-LP.

1.3.1.4 EBNA3A, -3B, -3C

The EBNA3 proteins represent a family of large nuclear proteins that are located in tandem sequence in the EBV genome. Each EBNA3 transcript is generated by alternative splicing from a large transcript originating at Cp promoter **(Figure 3)** [119]. The EBNA3s have approximately 30% sequence homology, and are 70% homologous between EBV-1 and EBV-2 subtypes. Similar to EBNA2 and EBNA-LP, expression of this family of proteins is restricted to immunoblasts that express the Latency III program. Infected cells express only a few copies of each mRNA, indicating that expression of these genes is highly regulated. The EBNA3 proteins are very stable with long half-lives, likely due to interactions with C8/α7 subunit of the 20S proteasome complex [120]. These proteins are primary antigenic targets for CTL responses,

which demonstrate the importance for the regulation of their expression during viral infection [121]. The EBNA3 proteins, like EBNA2, cannot bind directly to DNA, but influence promoter activity via interaction with cellular DNA-binding factors, such as CBF-1/RBP-Jk.

Of the three EBNA3 family members, only EBNA3A and EBNA3C are essential for immortalization of infected B cells. EBNA3A and EBNA3C manipulate a variety of cellular processes for regulation of cellular and the viral gene expression. Specifically, these proteins can act as transcriptional repressors of viral Cp promoter via recruitment of HDAC1 to RBP-Jk. Since EBNA2 requires interaction with RBP-Jk to transactivate a variety of viral and cellular promoters, interaction of EBNA3A/3C with this DNA-binding factor inhibits EBNA2-mediated transcription [122,123]. Additionally, EBNA3C may tightly regulate cellular gene expression by directly competing with the binding of ProTα to p300, a cellular histone acetyltransferase [124,125]. EBNA3A/3C-mediated upregulation of the platelet and leukocyte C kinase substrate (Pleckstrin), which is involved in cytoskeletal rearrangements, could be an important factor for the maintenance of Latency III [126].

The role of EBNA3C in Latency III is highly diverse, which is exemplified by its regulation of genes involved in cell cycle regulation, apoptosis, ubiquitin pathway, proliferation, protein folding, and cytoskeletal rearrangement. For instance, EBNA3C forms a complex with cyclin A that enhances the kinase activity of this protein, resulting in hyperphosphorylation of the cell cycle regulator Rb. When Rb is hyperphosphorylated, the transcription factor E2F is released, allowing for subsequent gene expression and entry into S phase [127]. Interactions of EBNA3C with SCF^{Skp2} E3 ubiquitin ligase lead to proteasomal degradation of Rb and p27 and subsequent rescue of infected cells from early cell cycle arrest [128,129]. Additionally, both EBNA3A and EBNA3C interact with Chk2, another mechanism that allows LCLs to bypass cell

cycle arrest checkpoints [130]. EBNA3A and EBNA3C can induce oncogenesis directly by interaction with carboxy terminal binding protein (CtBP), which cooperates with Ha-Ras for transformation of primary rat fibroblasts [131,132]. Also, EBNA3C affects oncogenesis through stabilization of the c-myc protein and interaction with DP103, an ATP-dependent RNA helicase involved in cell proliferation and differentiation [133].

EBNA3A and EBNA3C prevent apoptosis during Latency III by downregulating expression of the Bcl2-interacting mediator of cell death (Bim) at the mRNA level, promoting an anti-apoptotic environment that is permissive for damaging events such as c-myc translocations [134]. The binding of EBNA3C with Spi-1/Spi-B aids EBNA2-mediated activation of transcription from the LMP2 promoter, a gene known to provide infected cells with anti-apoptotic signals [135]. Expression of EBNA3C has been shown to override the anti-metastatic function of the Nm23-H1 protein [136]. Interactions between EBNA3A and Hsp70 cellular chapereone protein suggest that viral proteins utilize native cellular chaperones to assist in the proper folding of viral proteins [137].

EBNA3A and EBNA3C have been shown to manipulate many cellular processes. EBNA3B is the least studied of the EBNA3 proteins. The treatment of a bone marrow transplant recipient with EBNA3B-directed CTLs resulted in the selection of EBNA3B deletion escape mutants, indicating that this protein is highly dispensable for cell transformation [138], which likely contributes to the lack of knowledge of the specific processes modulated by this protein [139]. Various studies have shown that functions of EBNA3B overlap those discovered for EBNA3A and EBNA3C. EBNA3B interacts with RBP-Jk similarly to EBNA3A and EBNA3C, and, therefore, also functions as a repressor of EBNA2-mediated transcription. Like EBNA3C, EBNA3B upregulates expression of vimentin, an intermediate filament protein, associated with

cell ruffling that may play a critical role in cell transformation [140]. Therefore, EBNA3A and EBNA3C have diverse mechanisms for controlling a multitude of cellular processes that contribute to EBV-associated B cell proliferation and tumorigenesis.

1.3.1.5 LMP1

Latent Membrane Protein (LMP) 1 is the only EBV latent gene considered an oncogene, and is, thereby, also essential for immortalization of infected B cells. The structure of LMP1 describes a 356-amino acid (aa) protein that contains a short intracellular N-terminus, 6 transmembrane (TM) domains, and 150-aa C-terminal cytoplasmic tail. LMP1 produces a ligand-independent constitutively active CD40-like signal, providing cells with proliferation and survival signals. Therefore, LMP1 is considered a functional homologue of the TNF receptor (TNFR) family of proteins, and can bind to several proteins of the TNFR signaling pathway via its cytoplasmic C-terminal tail [62,64,141].

The CD40-like signals are initiated by two regions within the C-terminal cytoplasmic tail called C-terminal activation regions (CTARs), which activate signaling pathways that culminate in NF-κB-dependent upregulation of gene expression [142]. The CTARs are required for LMP1 to signal via TNFR-associated factors (TRAF) molecules, such as TRAF3. Unlike similar domains in CD40, CTAR2 does not bind TRAF2, but instead binds to TNFR-associated death domain protein (TRADD). This interaction leads to initiation of MAP kinase signaling that results in activation of ERK, JNK and p38, and subsequent stimulation of the JAK/STAT signaling pathway [143-146]. Both CTAR1 and CTAR2 induce activation of the NF-kB signaling pathway and are essential for proliferation of B cells [147]. Additionally, LMP1 has

been shown to activate the phosphatidylinositol 3-kinase (PI3-K) pathway, which induces cell survival through Akt kinase, and also induces actin polymerization and cell motility [148].

Although known as the CD40 signal mimic, LMP1 and CD40 are not functionally interchangeable proteins. LMP1 forms a complex that is distinct from CD40, with preferential binding of TRAF3 and TRAF5, as opposed to TRAF1 and TRAF2 [149]. Induction of CD40 signaling requires trimerization and localization to lipid rafts, while the 6 TMs provide aggregation and complex formation needed for LMP1 signaling [150]. LMP1 can replace CD40 in transgenic mice and allow for functional B cell development, but these B cells are functionally different than B cells expressing natural CD40 [151]. Both proteins share the ability to downregulate Bcl-6 expression, which blocks the formation of germinal centers [152]. LMP1 is a highly expressed latent gene with a high turnover rate. Degradation of LMP1 is mediated by its short N-terminal tail, which contains a site for ubiquitylation [153].

1.3.1.6 LMP2A

Similar to LMP1, LMP2A induces a ligand-independent constitutively active signal, which, in this case, mimics the signal provided by the BCR. LMP2A is a protein isoform of the LMP2 gene that also includes LMP2B. Both LMP2A and LMP2B transcription require ligation of the terminal repeats **(Figure 1 and Figure 3)**. The LMP2A gene includes 119aa N-terminal cytoplasmic tail, encoded by its initiating exon, 12 TMs and a short C-terminal cytoplasmic tail. Localization patterns reveal that LMP2A is found in lipid rafts in the plasma membrane, where interactions with src-family kinases routinely occur [154,155]. LMP2A is consistently detected in many stages of latency in B cells (Table 1), as well as several B cell and epithelial cell

malignancies, including HL and NPC. Therefore, LMP2A is presumed to play an important role in viral replication, persistence and EBV-associated diseases.

The N-terminal cytoplasmic signaling domain (CSD) provides the BCR-like signal within infected cells **(Figure 5)**. LMP2A has been shown to produce signals similar to tonic BCR signaling, which provides infected B cells with strong survival signals [156-158]. The CSD includes an immunoreceptor tyrosine-based activation motif (ITAM) that interacts with the src-family kinases Lyn and Syk [158]. These interactions initiate signal transduction pathways, such as PI3-K/Akt pathway, that lead to expression of anti-apoptotic genes like Bcl-2 and Bcl-xL [159-161]. Also, activation of PI3-K/Akt pathway by LMP2A inhibits TGF-β-induced caspase activity, another way LMP2A protects infected cells from initiation of apoptosis [162]. Experiments using transgenic mice have revealed that LMP2A provides B cells with survival signals in the absence of a functional BCR, which allows for development of B cells that would normally be targeted for apoptosis [163].

In addition to anti-apoptotic signaling, LMP2A provides infected cells with proliferation signals, and is a major player in the maintenance of viral latency. For instance, in epithelial cells, activation of PI3-K/Akt pathway can result in inactivation of GSK-3β and subsequent accumulation of β-catenin, an inducer of cell proliferation [164,165]. The role of LMP2A in providing proliferation signals to infected B cells has not been clearly shown prior to this study. LMP2A aids in viral maintenance by physically excluding the BCR from lipid rafts, which successfully inhibits the ability of the BCR to produce pro-apoptotic signals, and calcium fluxes that would stimulate the viral lytic cycle [166]. Additionally, the polyproline (PPPPY) motif in the LMP2A CSD can interact with WW domains of Nedd4 family of E3 ubiquitin ligases [167]. This interaction allows LMP2A to negatively regulate expression of genes, such as Lyn and Syk

that are integral for initiation of signaling from the BCR, as well as the ability to regulate its own expression [155]. Signaling through the BCR has been shown to initiate the switch to the lytic cycle. Therefore, blocking BCR signaling creates an environment that does not allow for lytic induction, which exemplifies the function of LMP2A as a major protein in the maintenance of viral latency.

LMP2A signaling has been shown to strongly affect epithelial cells, a possible explanation for its oncogenicity in NPC. LMP2A has been shown to activate ERK/MAPK and JNK/MAPK pathways that result in expression of genes associated with cell proliferation [168]. Also, previous studies have determined that expression of LMP2A coincides with inhibition of epithelial cell differentiation and loss of anchor dependence, two hallmarks of oncogenesis and metastasis in epithelial cells [86]. LMP2A can repress the effects of LMP1 by inactivating NF-κB and STAT3.

The C-terminal cytoplasmic region of LMP2A has been reported to possess a clustering signal that the protein utilizes for self-aggregation, or aggregation with the other LMP2 isoform, LMP2B. Protein aggregation may also be associated with the TMs. However, the main role of the TMs appears to be protein localization, as removal of any one TM results in re-localization of the protein to other parts of the cell [169].

The role of LMP2A in immortalization of B cells *in vitro* remains controversial. The work presented in this dissertation will better elucidate the role of this protein in this process.

1.3.1.7 LMP2B

LMP2B is another protein isoform of the LMP2 gene. This gene has an initiating exon and promoter that is distinct from LMP2A; LMP2B shares a bidirectional promoter with LMP1.

Similar to LMP2A, LMP2B transcription requires circularization of the EBV genome, as the transcript crosses the TR region **(Figure 1 and Figure 3)**. Unlike LMP2A, the first exon of the LMP2B gene is noncoding. Therefore, LMP2B does not contain an N-terminal CSD, and translation initiates at Exon 2, which is the start of the first TM. LMP2A and LMP2B share their remaining genetic information, which includes 12 TMs and a short C-terminal cytoplasmic tail that the proteins use for self-aggregation and, possibly, aggregation with each other and other proteins. LMP2B localizes to perinuclear regions, which is determined by the 12 TMs. The removal of the TM domains results in localization of LMP2B at the plasma membrane [64,169].

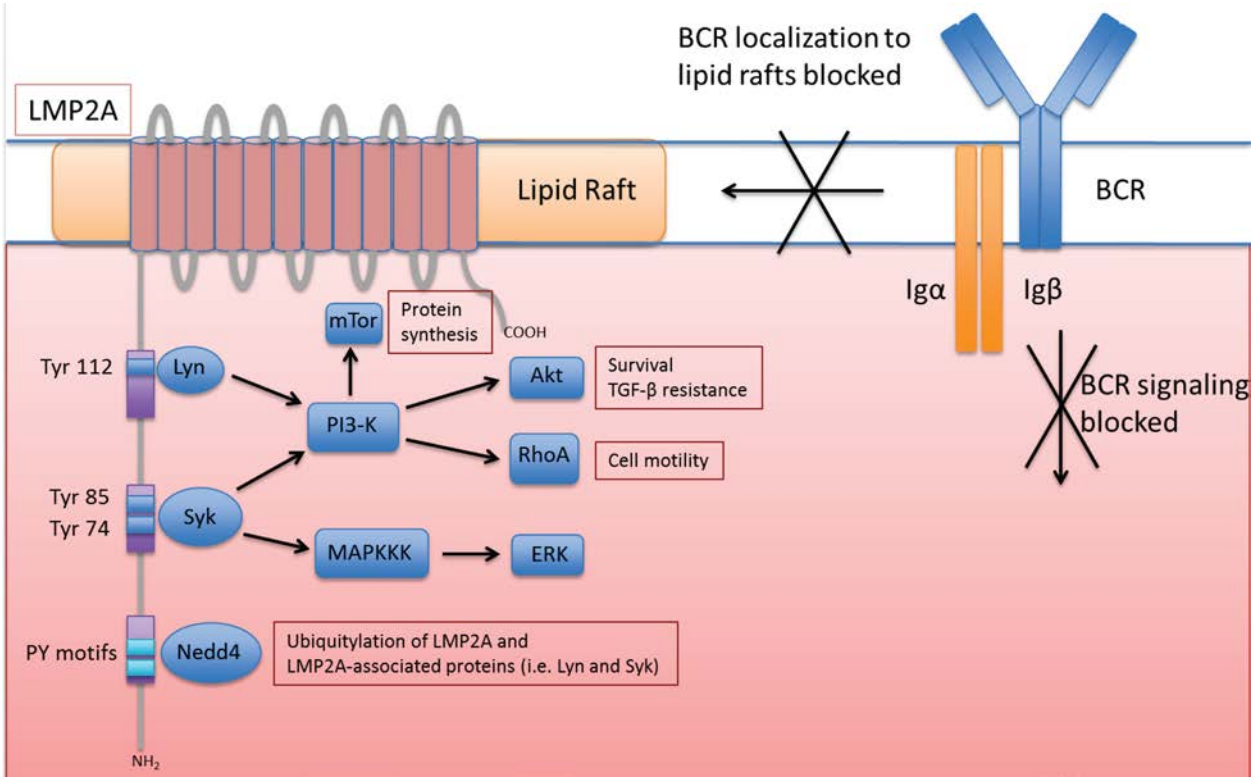

Figure 5. LMP2A produces BCR-like signals and blocks BCR signaling.

LMP2A is an integral membrane protein with 12 transmembrane domains that localizes to lipid rafts in the plasma membrane. The N-terminus contains tyrosine motifs used for interactions with src-family kinases and Nedd4 that result in modulation of cellular signaling pathways. The presence of LMP2A in lipid rafts results in exclusion of BCR from these locations, thereby inhibiting BCR signaling.

Since LMP2B does not contain a signaling domain, the function of this protein remains largely unknown. However, recent studies have concluded that LMP2B may have a role in the modulation of LMP2A activity [170-172]. The ability of the two proteins to aggregate suggests that LMP2B can sequester LMP2A away from lipid rafts and into perinuclear regions. In the absence of src-family kinases, LMP2A would be unable to produce its signal. Other studies have found that LMP2B inhibits phosphorylation of the CSD of LMP2A, which would also block the ability of LMP2A to signal. Interestingly, expression of LMP2B in the absence of other EBV latent genes results in global inhibition of phosphorylation following BCR cross-linking [173].

Overexpression studies discovered that LMP2B can override the LMP2A-induced block of the BCR, re-establishing native BCR signaling and calcium fluxes that result in lytic reactivation [170-172]. Therefore, LMP2B is considered an important protein for the regulation of lytic switch. It is possible that LMP2B elicits signaling effects via interactions with known signaling molecules. For example, previous studies have found that LMP2B, but not LMP2A, colocalizes with CD19, a signaling protein that is part of the BCR co-receptor complex [174].

Although LMP2B does not share a signaling domain with LMP2A, studies have found that these two isoforms have similar functions in epithelial cells. The presence of either LMP2A or LMP2B is important for transformation. Specifically, LMP2A and LMP2B have been shown to inhibit cell differentiation, while enhancing cell spreading and anchor independence, allowing for metastasis. The mechanism of these effects is not clear, but is independent of PKC, PI3-K, ERK and PLC-γ [86]. Also, both LMP2A and LMP2B promote the degradation of interferon receptors IFNAR1 and IFNGR1, which leads to inhibition of interferon signaling in epithelial cells [175]. These effects suggest that LMP2B, in the absence of direct signaling ability, can contribute to tumorigenesis in epithelial cells.

1.3.1.8 EBERs

The EBV-encoded small RNAs (EBERs) are 166bp and 172bp single stranded RNAs, called EBER1 and EBER2 **(Figure 3)**. Both EBER1 and EBER2 are transcribed by RNA Polymerase III and contain intragenic sequences typical of A and B boxes found in polIII transcripts. EBERs are noncoding RNAs that have uncapped 5' triphosphates and 3' strings of U nucleotides, and contain several stem-loop secondary structures. EBERs are highly abundant, transcribed at rates as much as ten times higher than the most highly expressed latency-associated mRNA [59,64].

EBER1 and EBER2 are expressed during all stages of latency, and are consistently detected in nearly all EBV-associated malignancies. The relative level of EBER1 and EBER2 can vary with isolates, but EBER1 is most often the predominant species expressed in latently infected cells. EBER1 and EBER2 are not essential for immortalization of B cells [176]. These two RNAs normally localize to the nucleus, but can also localize to the cytoplasm where they bind to the kinase PKR, an interaction that may be important for viral persistence via protection of infected cells from interferon-induced apoptosis [177]. The expression EBER-induced IL-10 could represent an important component of the pathogenesis of EBV+ BL [178]. EBERs assemble into stable ribonucleoprotein particles with the ribosomal protein L22 and the La protein, which may help EBERs regulate their own expression [179].

1.3.1.9 BARTs and microRNAs

Mature microRNA can form an active complex called RNA-induced silencing complex (RISC), which will bind to and repress translation of mRNA with only partial complementarity. EBV encodes 23 species of microRNAs; 3 are adjacent to the BHRF1 gene and the other 20 are located in introns of the BART transcripts. BARTs, or BamHI A Rightward Transcripts, were

first discovered in NPC, and have since been identified in other EBV-associated malignancies such as BL, HL, nasal T cell lymphoma, and in the peripheral blood of healthy carriers [76,180-182]. BARTs encode a number of potential open reading frames (ORFs), which include BARF0, RK-BARF0, A73 and RPMS1. Although the protein products of these ORFs have not been identified and remain controversial, they potentially function as negative regulators of EBNA2 and Notch activity (RPMS1), as well as modulators of kinase signaling (A73) [183,184]. The BART cluster of microRNAs are predominantly expressed in Latency I and Latency II **(Figure 3)** [185]. BART2 microRNA is antisense to the BALF5 ORF, and, therefore, may have a role in regulation of EBV-encoded DNA polymerase [186].

The BHRF1 microRNAs are expressed during Latency III and Cp/Wp promoter usage **(Figure 3)**. The lack of sequence similarity with viral genes suggests that BHRF1 microRNAs may play a role in repression of cellular gene expression [185].

1.3.2 Lytic Reactivation

Similar to all other herpesviruses, EBV exhibits a biphasic life cycle, in which the virus exists in a latent or lytic phase. The purpose of latency is to activate B cells and induce their proliferation, and, ultimately, allow EBV to persist within the memory B cell population for the lifetime of the host. In contrast, the purpose of the lytic cycle is to produce infectious viruses for transmission between hosts. The induction of lytic reactivation can be accomplished *in vitro* most often with histone deacetylase inhibitors (Sodium butyrate) and protein kinase C activators (phorbol esters, 12-O-tetradecanoylphorbol-13-acetate or TPA). Chemicals known to alter certain intracellular regulator pathways, such as calcium ionophores, BCR cross-linking reagents, demethylating

agents such as azacytidine, and reagents that activate the unfolded protein response can also induce lytic reactivation [187]. The calcium fluxing and protein kinase C activation that is downstream of BCR engagement has been associated with lytic reactivation of EBV *in vitro* [188].

Lytic reactivation *in vivo* does not clearly involve signaling through the BCR, as interaction of an EBV-infected B cell with its cognate antigen would be a rare event. Lytic reactivation *in vivo* is most likely due to help from bystander T cells, or differentiation of memory B cells into plasma cells, the cell type most often associated with the lytic cycle [60]. Upregulation of the transcription factors XBP-1 and Blimp1 with concomitant downregulation of Pax-5 leads to inhibition of B cell proliferation and subsequent terminal differentiation into plasma cells [189,190]. Previous studies have determined that XBP-1 binds to the ZEBRA promoter to induce its expression, further emphasizing the link between EBV lytic induction and terminal differentiation of B cells. However, lytic induction can occur *in vitro* in the absence of plasma cell differentiation; induction of the unfolded protein response pathway by reagents such as thapsagargin resulted in increased expression of Zebra without upregulation of plasma cell associated markers CD138 or plasma cell associated transcription factors such as Blimp1 [191].

Epithelial cells have been used for production of recombinant viruses. In this case, HDAC inhibitors and phorbol esters can be used to drive lytic induction, but it is more common to introduce ZEBRA-expression plasmids [51], which was performed for this study. Previous studies have shown that introduction of ZEBRA alone is sufficient to drive the lytic cycle [51]. However, it is important to note that ZEBRA requires the other immediate early gene and lytic promoter transactivator, Rta [48]. Together ZEBRA and Rta induce the expression of all early

gene promoters, which includes genes for the DNA replication machinery as well as viral cytokine homologues.

The majority of EBV-associated malignancies are characterized by the expression of latency genes. Lytic reactivation has been widely studied as a treatment for several EBV-associated diseases. A recent study demonstrated that treatment regimens that included reagents for induction of lytic reactivation (gemcitabine and valproic acid) coupled with reagents that limit the spread of infectious EBV (gancyclovir) were effective in controlling EBV-associated malignancies such as NPC and GC [192].

Several genes associated with the Latency III program inhibit the reactivation of lytic EBV. LMP2 plays a major role in the maintenance of viral latency via blocking of BCR signaling [156-158]. Studies have shown that loss of LMP2 expression correlates with reactivation of the lytic cycle following stimulation of BCR signaling [170-172]. LMP1 also contributes to maintenance of latency following BCR signaling. The Latency III program has been associated with suppressive effects on TGF-β signaling; TGF-β signaling induces expression from the Zebra promoter [162,193,194]. Also, expression of the Latency III and Latency II programs inhibits terminal differentiation of B cells and epithelial cells, thereby inhibiting reactivation of the lytic cycle. Latency III expressing B cells *in vitro* have displayed low levels of spontaneous lytic reactivation, but the actual number of cells within a population undergoing lytic reactivation remains unclear [45,195].

1.3.3 Formation of lymphoblastoid cell lines

The peripheral blood lymphocytes of chronic EBV carriers contain small numbers of EBV+ B cells that will regularly form lymphoblastoid cell lines (LCLs) in culture settings if T cell activity

is inhibited by addition of CyA [196-198], but these cell lines can also be established by direct infection of resting B cells with EBV in culture. LCLs have been an invaluable tool for many studies, including research on EBV latency as well as isolation and perpetuation of the genotype for individuals in genetic studies. Multiple copies of the viral episome can be found in LCLs [199,200], with EBV gene expression restricted to the nine latent genes: EBNA1, EBNA2, EBNA-LP, EBNA3A, EBNA3B, EBNA3C, LMP1, LMP2A and LMP2B [64]. In addition to latent genes, the BART microRNAs and EBERs are expressed. EBERs are expressed in all forms of viral latency, though the exact roles of BARTs and EBERs in LCLs are not clear.

LCLs have characteristics of immunoblasts with high levels of activation (CD23, CD30, CD39, and CD70) and adhesion marker (LFA-1, LFA-3 and ICAM-1) expression [201]. These markers are absent on resting B cells, but are upregulated when B cells are activated by antigen or mitogens and induced to proliferate, indicating that EBV activates and induces B cell proliferation through constitutive activation of cellular pathways that drive normal B cell activation and proliferation [3].

Specific latent genes have been shown to induce B cell proliferation when expressed alone in human B cell lines. EBNA2 is a transactivator of many cellular genes as well as the EBV genes LMP1, LMP2A and LMP2B. A functional homologue of Notch-IC, EBNA2, with RBP-Jk, activates expression of the oncogene c-myc, inducing B cell growth and LCL formation [113,202]. EBNA3A and EBNA3C are each important for initiation and maintenance of LCL formation through interactions with many cellular genes. For example, EBNA3A and EBNA3C interact with Chk2, a cell cycle protein, that allows for EBV-infected B cells to bypass the G2/M growth arrest checkpoint. EBNA2 and EBNA3A/3C upregulate expression of LMP1 [203], a protein that is essential for formation of LCLs. LMP1 produces a ligand-independent constitutive

CD40-like signal in B cells that hijacks cellular pathways stimulated following CD40-CD40L ligation during B-T cell engagement [62,141]. LMP2A is another viral protein that produces a ligand-independent constitutive signal, much like the signal produced by the BCR [166,204]. The role of this protein in LCL formation *in vitro* has been disputed [205-208]. Our study described LMP2A expression as advantageous for efficient activation, proliferation and later LCL formation. Additionally, LMP2B was described as having a possible role in the maintenance of viral latency, but the protein was dispensable for LCL formation *in vitro*.

A small number of cells within the LCL population appear to undergo spontaneous lytic reactivation, though actual release of lytic virions into the supernatant is negligible. In direct infection of resting B cells, transient expression of ZEBRA is detected. One previous study found that lytic induction aids in LCL formation due to constant reinfection of uninfected B cells [209]. However, more recent studies have disputed this claim, showing that lytic reactivation does not have any effect on LCL outgrowth [45,195].

2.0 STATEMENT OF THE PROBLEM

In this study we address the roles of the EBV terminal proteins, LMP2A and LMP2B, in early B cell infection and LCL formation *in vitro*. The function of LMP2A has been extensively studied. An N-terminal signaling domain encoded by its first exon contains an ITAM that initiates cellular signaling pathways that resemble those provided by an intact BCR. Therefore, LMP2A provides infected B cells with survival signals via the Ras/PI3-K/Akt pathway that result in induction of the anti-apoptotic genes Bcl-2 and Bcl-xL. A recent study has also shown that LMP2A may also provide B cells with BCR-like activation signals that result in Ca^{+2} fluxes and protein kinase C activation. However, the role of LMP2A in B cell proliferation has been intensely disputed; some studies have shown that LMP2A is dispensable for B cell proliferation and LCL formation, while others have discovered that inclusion of LMP2A expression provides EBV with a growth advantage, concluding that LMP2A plays an essential role in efficient LCL formation. The role of LMP2B in B cell proliferation has not been as extensively studied. Some studies have also determined that this protein is dispensable for B cell proliferation and LCL formation. Other studies have discovered a role for LMP2B in the regulation of the switch to lytic reactivation through negative regulation of LMP2A activity.

In order to study the roles of LMP2A and LMP2B in early infection and LCL formation, we generated complete recombinant EBV viruses with deletions in the LMP2 region. Specifically, the initial exons associated with LMP2A and LMP2B transcription were deleted via

recombination between flanking LoxP sequences that resulted in loss of transcription. Previous studies that demonstrated the dispensability of the proteins for proliferation of B cells, and subsequent establishment of LCLs used a technique, unlike our approach, that resulted in supernatants with a heterogeneous population of wild-type and mutant EBV, which created a difficult system for clearly assessing the true effect of LMP2 on B cell proliferation and immortalization. Another study determined that LMP2 was necessary for efficient immortalization of B cells via the use of mini-EBV, which is essentially an incomplete EBV genome that expresses all latent genes necessary for immortalization. Therefore, we believe that our approach utilizing the complete EBV genome with minimal deletions in the initiating exons of both LMP2A and LMP2B was more appropriate to investigate the roles of LMP2 in the efficient early activation, proliferation, and survival of infected B cells, as well as subsequent immortalization.

We hypothesized that LMP2 is important for early EBV infection of primary B cells *in vitro* and later LCL formation. Specifically, we reasoned that LMP2 elicits its effects on infected B cells through the processes of B cell activation, proliferation, and survival enhancing the probability that LCLs will be established. Also, we studied the possible role for LMP2 in the regulation of viral latency, which could also, if disrupted, adversely affect B cell proliferation and the establishment and maintenance of LCLs. To address this, the following specific aims were proposed:

SPECIFIC AIM ONE: Analyze the kinetics of early EBV infection in B cells in the context of LMP2A and LMP2B expression. Infection of B cells by EBV leads to expression of activation markers (CD23, CD30, CD39, and CD70) and adhesion molecules (LFA-1, LFA-3

and ICAM-1). Expression of these markers can be induced in resting B cells by antigenic/mitogenic stimulation, which suggests that EBV-induced effects can be elicited by constitutive activation of these same cellular pathways that drive normal B cell activation and proliferation. LMP1 and LMP2A produce ligand-independent constitutive signals, but only LMP1 has been proven to play a role in these processes. Therefore, we generated recombinant EBV with deletions in LMP2A and/or LMP2B to determine if these proteins play a role in early B cell infection via the cellular processes of activation, proliferation and apoptosis during the first two weeks post-infection.

SPECIFIC AIM TWO: Determine the roles for LMP2A and LMP2B in the formation of lymphoblastoid cell lines *in vitro*. The role of LMP2 in the immortalization of B cells has been intensely studied and disputed within the field of EBV research. Some studies have determined that LMP2 is dispensable for B cell immortalization, while others claimed it is important for efficient immortalization. Most research has focused on the role of LMP2A in this process. With our more appropriate experimental approach, infected cells were followed for 12-14 weeks after infection to determine the efficiency of LCL formation in wild-type and LMP2 knockout (KO) virus infections.

SPECiFIC AIM THREE: Establish the roles for LMP2A and LMP2B in regulation of viral gene expression and maintenance of viral latency in early infection. Previous research has shown that LMP2A blocks signaling by the native B cell receptor, inhibiting downstream calcium fluxes that can result in protein kinase C activation and subsequent lytic reactivation. The consequence of this function for LMP2A is the stabilization of viral latency. In

contrast, LMP2B has previously been shown to regulate the switch to lytic reactivation via negative regulation of LMP2A activity. Therefore, I investigated the effect of LMP2A and LMP2B deletions on the stability of viral latency in early B cell infection via addition of lytic inducing agents (BCR signal stimulators) to the infected cells.

Data included in a manuscript recently accepted for publication with the journal PLOS ONE, include:

From Aim 1 – Figures 1, 2, 3, 5-12 and Tables 2, 3 and 5

From Aim 2 – Table 10

From Aim 3 – Figures 19-23

From Discussion – Figure 24

3.0 MATERIALS AND METHODS

3.1.1 Generation of recombinant virus constructs

Recombinant wild-type (wt) EBV (p2089) [210] and LMP2A knockout (KO) BACs (p2525) [211] were a generous gift from Wolfgang Hammschmidt and Markus Altmann (Department of Gene Vectors, Helmholtz Center, Munich, Germany). EGFP and hygromycin resistance genes were inserted into the recombinant wt and LMP2 knockout EBV BACs for tracking infectivity of virus stocks and selection, respectively **(Figure 6)** [210]. Flag tags were inserted into p2089 (wt) and p2525 (Δ2A) for these studies using a technique from W. Hammerschmidt [described in [212]]. Specifically, 10kb fragment of EBV sequence that corresponded to the LMP2 region was cloned into the shuttle vector p2768.5 (produced by M. Altmann and W. Hammerschmidt). This vector was used for insertion of a 3X flag tag into exon 7 and a zeocin resistance gene between exons 8 and 9 of the LMP2 gene. Homologous recombination between EBV sequence in p2768.5 and p2089 or p2525 created flag-tagged constructs p2089.1 (wt) and p2525.1 (Δ2A). For removal of LMP2B, the 10kb EBV DNA fragment in p2768.5 was constructed with LoxP sequences flanking exon 1′ of LMP2B. Exon 1′ was removed via recombination between flanking LoxP sequences after introduction of Cre recombinase; the new constructs were named p2089.3 (Δ2B) and p2525.3 (Δ2A/Δ2B).

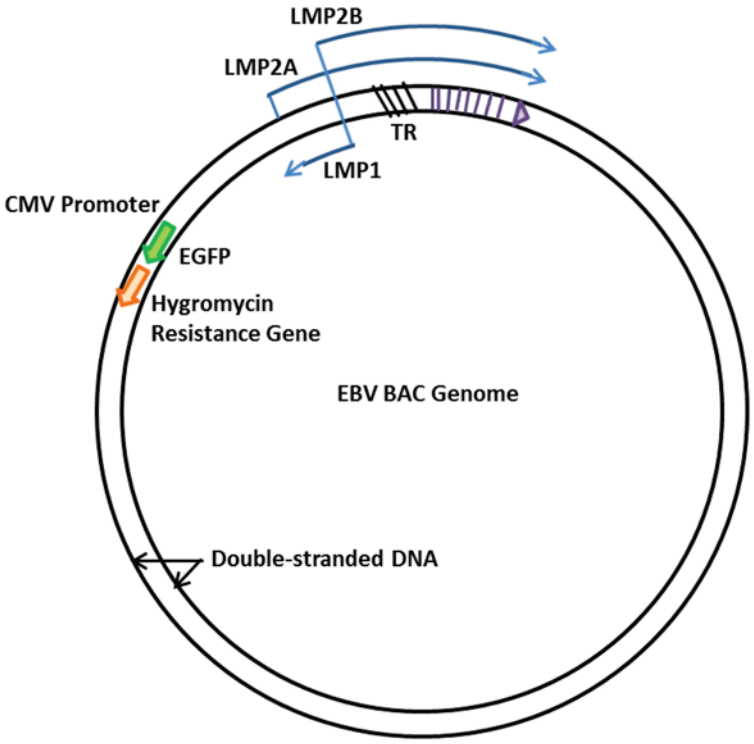

Figure 6. EGFP and hygromycin resistance gene placement in EBV BACs.

EGFP and hygromycin resistance genes were inserted in the BamHI A region of the EBV BAC genome. Expression of EGFP was under the control of the CMV promoter.

3.1.2 Validation of deletions by qcPCR

The integrity of the LMP2A and LMP2B deletion mutations were assessed using qcPCR. First, the wild-type and LMP2 KO BACs were dialyzed in dH_2O over a period of six hours to remove TE storage buffer. Fresh dH_2O was added after every hour throughout the six-hour period. Dialyzed DNA was then used for qcPCR using Novataq Master Mix (Novagen). Primers specific to the LMP2A and LMP2B exon 1 regions were used to determine the presence or absence of the respective first exons **(Table 2)**. Standard PCR conditions (94°C for 8 minutes, 35 cycles of

94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, ending with 72°C for 5 minutes) and ABI GeneAmp PCR System 9700 instrument were used.

Table 2. Primer sequences for validation of LMP2 deletions

Primer Name	Forward Primer (5′ – 3′)	Reverse Primer (5′ – 3′)
LMP2A Exon 1 Deletion	ATC CCT CTC GCC TTG TTT CTC	GAT GGT GTG GAT AAC ATC TCC
LMP2B Exon 1′ Deletion	GCG GTG TGT GTG TGC ATG TAA GCG T	ACC TCA TTC TGA AAT TCC CAT ATC C

3.1.3 Cell Lines and Cell Culture

All cells were incubated at 37°C with 5% CO_2. Raji B cells are a B cell line derived from Burkitt's lymphoma that was used for titering infectious EBV particles in supernatants from producer cell lines. These cells were maintained in R10 (RPMI, 10% FBS, 2 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin).

HEK 293 cells are an epithelial cell line derived from human embryonic kidney cells. The HEK 293 cell line (generous gift from Robert White and Martin Allday, Imperial College London, UK, described in [213]) was used for production of recombinant wt and LMP2 KO EBV BAC viruses as described previously [214]. HEK 293 cells were maintained in D10 (DMEM, 10% FBS, 2 mM L-Glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin). After transfection of recombinant EBV plasmids, the HEK 293/EBV cells were maintained in D10 + 75 µg/ml hygromycin to maintain selective pressure on cells harboring EBV. HEK 293 and HEK 293/EBV cell lines were split when confluency reached 90-100%. The addition of 2 ml

of trypsin allowed the cells to lift from flask and detach from each other for seeding of monolayer in new flask for cell line maintenance or 100mm plate for virus production.

Primary B cells were grown in R10. Established lymphoblastoid cell lines (LCLs) were maintained in R10. No hygromycin is needed to maintain selective pressure at this point because transformation of the B cells requires the presence of the viral genome. LCLs were split every 3-4 days, or when the cell density reached approximately $1x10^6$ B cells/ml.

3.1.4 Preparation of PBMC and primary B cells

Buffy coats from random, healthy donors were obtained from Pittsburgh blood blank. Ficoll-Hypaque overlay method was used to separate peripheral blood mononuclear cells (PBMC) from plasma and red blood cells. PBMCs were washed three times with 1X phosphate-buffered saline (PBS).

PBMCs were used for purification of primary B cells. The majority of experiments were performed using purified, primary B cells in order to simplify culture conditions and eliminate the possibility that EBV may infect and/or activate other cell types, including T cells and NK cells and thus skew the observations [215]. B cells were purified using negative selection (B cell Isolation Kit II, Miltenyi Biotec) to avoid the possibility of inducing unwanted activation during the separation process. Positive selection utilizes an antibody specific to CD19 for purification of B cells, which can cause background activation because this receptor is part of the BCR co-receptor complex and is capable of producing stimulating signals.

3.1.5 Transfection of HEK 293

Human embryonic kidney (HEK) 293 cells are used as producers of the recombinant viruses. First, long-term cell lines harboring the recombinant EBV plasmid must be acquired. Therefore, HEK 293 cells were seeded into 2 100mm plates so that the culture was 40-50% confluent within 24-48 hours. Upon reaching confluency, 6µg of each recombinant EBV plasmid was transfected per 2 100mm plates of HEK 293 cells using Genejuice (Novagen) via modified protocol from manufacturer's instructions. The EBV DNA was diluted in 600µl RPMI (no serum added), and the genejuice reagent (used at 3:1 dilution factor) was diluted in 600µl RPMI. The diluted genejuice was mixed gently and added to the diluted EBV DNA. The EBV DNA/genejuice was mixed gently by inversion and incubated at room temperature for 15 minutes. Following incubation, the DNA/genejuice mixture was added to the cells dropwise, 600µl per plate. The plates were incubated at 37°C for 24-48 hours.

3.1.6 Selection of virus production cell lines

After 24-48 hours incubation following transfection of the recombinant EBV DNA plasmids, the cells were observed for EGFP expression. Once EGFP expression was observed, usually within 48 hours of transfection, the cells were placed under selective pressure using 40-50 µg/ml hygromycin. Addition of hygromycin results in a high percentage of cell death due to the low transfection efficiencies using EBV DNA (~1-5% transfection efficiency). Therefore, the establishment of HEK 293/EBV cell lines can take 1-2 months. During this time, the cells were continually monitored for EGFP expression. After the first two weeks in culture, the

concentration of hygromycin was increased to 75 μg/ml. The use of 40-50 μg/ml hygromycin in the initial two weeks after transfection allows the majority of EBV+ cells to survive initial selection, but was not strong enough to prevent loss of the EBV genome over time. Addition of 75 μg/ml hygromycin immediately following transfection results in the death of the majority of cells. This can lengthen the amount time until producer cell lines are established to 2-3 months. Once EGFP+ colonies were the predominant cells in the plates, these cells were moved to 24-well plates to prepare for virus production.

3.1.7 Virus production and titering

Once EGFP+ HEK 293 cells were moved to 24-well plates and become 90-100% confluent, these "clones" were ready for virus production testing. Each well of the 24-well plates was replica plated for stimulation of the lytic cycle. The replica wells must reach 40-50% confluency for transfection of the p509 (BZLF1, also known as Zebra) [51] and p2670 (BALF4, also known as gB) [216]. Expression of Zebra was sufficient to initiate the lytic cycle. The inclusion of a viral glycoprotein, gB, in the process of viral production stemmed from evidence that addition of gB increases the infectivity of the progeny virus for B cells. The two plasmids were transfected into the HEK 293/EBV cells using Genejuice (Novagen). Following transfection, the cells were incubated for 5-6 days at 37°C for virus production. The supernatant was harvested, cell debris pelleted, and filtered using 0.45μm pore filters, and stored at either 4°C for short-term storage or -80°C for long-term storage.

Raji B cells were used to determine the number of infectious virions in supernatants from HEK 293/EBV cells [214,216]. The supernatants were serially diluted ten-fold, starting at

undiluted supernatant until reached 10^{-5} because the majority of viruses do not have titers that would require further dilution. After dilution of supernatants, $1x10^5$ Raji B cells were added per well. The Raji B cells and EBV were incubated for 48 hours at 37°C. After 48 hours 5mM sodium butyrate (histone deacetylase inhibitor) and 20nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA, protein kinase C activator) were added to each well to induce the lytic cycle and enhance EGFP expression for counting. The lytic inducers were incubated for 18-24 hours at 37°C. Infectious virus titers were determined based on the number of EGFP+ Raji B cells, called GIU or Green Inducing Units. This measurement was used to normalize virus supernatants for subsequent experiments.

Large-scale virus production utilized 100mm plates. HEK 293 cells were seeded in 4-6 100mm plates per cell line. When 40-50% confluency was reached, BZLF1 and BALF4 plasmids were transfected for virus production.

3.1.8 Determination of genome copy number in virus supernatants

For EBV genome copy number, virions were lysed with 10mM tris (pH 7.6) containing 50mM KCl, 2.5mM $MgCl_2$, 1% Tween 20 and 0.1mg/ml proteinase K. The lysed supernatants were used to detect a sequence in the BLLF1 gene that encodes the major viral glycoprotein gp350. The primers used for Real Time PCR were as follows: forward primer 5′-GTATCCACCGCGGATGTCA-3′; reverse primer 5′-GGCCTTACTTTCTGTGCCGTT-3′; and probe 5′-FAM-TGGACTTGGTGTCACCGGTGATGC-TAMRA-3′ [217]. Standards were used as mentioned previously [217]. Standards used for gp350 transcript were purified PCR products containing a known number of copies the target sequence. These standards were diluted in 10

mM Tris pH 7.6 and 0.1% Tween 20, with a background of 25 cell equivalents/µl DNA from the DG75 cell line. Real time PCR was performed with ABI 7500 instrument. All supernatants used in these studies were characterized and are presented in **Table 5**.

3.1.9 Proliferation experiments

For proliferation experiments, B cells were labeled with either Vybrant DiI (Invitrogen) or Violet Cell Trace (Invitrogen). Initial experiments utilized Vybrant DiI, which is a lipophilic membrane stain that is weakly fluorescent until it is incorporated into membranes. Vybrant DiI fluoresces orange-red, which is spectrally similar to tetramethylrhodamine (TAMRA) and R-Phycoerythrin (R-PE). To label cells with Vybrant DiI, $10x10^6$ B cells were resuspended at a density of $1x10^6$/ml in RPMI (no serum added). Then 5µl of Vybrant DiI was added to the cells and mixed well via pipetting. The cells were incubated at 37°C for 20 minutes, mixing the cells by pipetting every 5 minutes for uniform staining. After 20 minutes the cells were centrifuged at 1500rpm for 5 minutes at 25°C. The cells were resuspended in R10 (RPMI, 10% FBS, 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin) warmed to 37°C. This wash step was repeated two more times. $1x10^6$ B cells were fixed with 1% paraformaldehyde (PFA) for Day 0 time point analysis using FACSAria. The remaining B cells were used for EBV infection. $1x10^6$ B cells were infected with each recombinant virus, either wild-type or LMP2 KO viruses, at MOI 1. Infected B cells were harvested at 4, 8 and 16 days post-infection, fixed with 1% PFA and analyzed using FACSAria.

Violet Cell Trace was used in later experiments due to its capacity to trace multiple generations of cell division. To stain with Violet Cell Trace, up to $10\text{-}15x10^6$ B cells were

resuspended in 2ml of RPMI (no serum added). 2.5μM Violet Cell Trace dye was added to the cells, and mixed gently by pipetting. The cells were incubated at 37°C for 8 minutes. Addition of 10ml warm R10 quenched the labeling reaction, and the cells were centrifuged at 1200rpm for 3 minutes. The cells were washed with an additional 10ml of warm R10. $1x10^6$ B cells were infected with each recombinant virus, either wild-type or LMP2 KO viruses, at MOI 1. Infected/labeled B cells were analyzed using FACSLSRII.

Absolute counts of proliferating B cells were determined using Countbright Absolute Counting Beads (Invitrogen). The beads are set to a specific density, and were used in Violet Cell Trace experiments. 10μl of beads, which was equivalent to 9,900 beads, was added to each sample during fixation with PFA. During acquisition, the event gate was set to 5000 beads, which normalized the acquisition volume between samples and allowed for accurate, absolute counts of proliferating B cells.

3.1.10 Surface marker staining for flow cytometry

For surface staining, infected B cells were pelleted at 1200rpm for 5 minutes at 4°C, and washed in cold 1X PBS. The cells were then transferred to a 96-well plate for the remainder of the protocol, and pelleted at 1800rpm for 3 minutes at 4°C. Cells were resuspended in FACS monoclonal wash buffer (Hanks Balanced Salt Solution, 2.0% BSA, 5mM EDTA, and 0.1% sodium azide) for blocking. Anti-CD23 AlexaFluor 647 and anti-CD71 APC antibodies were added to the appropriate wells at known concentrations (10-20μl) in 100μl FACS monoclonal wash buffer. The cells/antibodies were incubated at 4°C for 60 minutes, then washed twice with 200μl FACS monoclonal wash buffer. The stained cells were then pelleted at 1800rpm for 3 minutes at 4°C. Fixation of the cells was performed using 1% PFA and incubated for at least 30

minutes at 4°C. The cells were then pelleted and resuspended in 0.1% PFA to prevent bleaching of fluorophores. Cells were analyzed using FACSAria.

3.1.11 Apoptosis staining for flow cytometry

EBV-infected B cells were stained with Annexin V antibody to determine the number of cells undergoing apoptosis. First, annexin-binding buffer was prepared: 10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4. The infected B cells were harvested and stained with live/dead cell indicator, Aqua. The Aqua stain was diluted to 1µl in 1ml RPMI (no serum added); 200µl was added per well of 96-well plate. The cells were then incubated at 37°C for 30 minutes. After the incubation the cells were then washed with R10 to quench the labeling reaction. Following Aqua staining the B cells were washed with annexin-binding buffer to prepare for Annexin-V staining. The B cells were resuspended in 100µl annexin-binding buffer, and 5µl Annexin V-APC was added to each well. The cells were incubated at room temperature for 15 minutes. After the incubation, the cells were pelleted and resuspended in 200µl annexin-binding buffer, and were analyzed within one hour of staining using FACSLSRII.

3.1.12 RNA extraction and Real Time PCR

RNA was extracted from HEK 293/EBV producer cell lines for Real time PCR in order to determine if all other latency genes could be expressed from LMP2 KO BACs. 5 EBNAs and 3 LMP mRNAs were PCR targets as mentioned below. RNA was also extracted from EBV-

infected B cells for analysis of early infection. Extraction was performed using a Qiagen RNeasy kit and resuspended in RNase free water. Conversion of RNA to cDNA was accomplished via a High-Capacity cDNA Archive Kit (ABI), which allowed for production of a greater number of cell equivalents per volume.

Standards were used for each viral transcript, which were purified PCR products containing a known number of copies of each target sequence. These standards were diluted in 10 mM Tris pH 7.6 and 0.1% Tween 20, with a background of 25 cell equivalents/µl DNA from the DG75 cell line. Viral gene expression was normalized to β_2-microglobulin cDNA (predesigned primer kit from ABI) and expressed as mRNA copies/cell. The standards used for the β_2-microglobulin measurement were cDNA dilutions from the IB4 cell line with known number of copies of the target sequence.

Real time PCR was performed for cDNA from HEK 293/EBV cells using an ABI 7500. Each PCR reaction volume was 25µl and contained 2µl of cDNA template. The PCR reaction included 1X PCR Buffer II, 0.5% Amplitaq Gold (ABI), 0.8% ROX reference dye (Invitrogen), 2.5 mM $MgCl_2$, 200 µM dNTPs (dATP, dGTP, dCTP, dTTP), 400 nM forward and reverse primers (IDT), and 200 nM probe (ABI custom FAM-TAMRA probes). The cycling program was set to 2 minutes at 50°C, 10 minutes at 95°C, 15 seconds at 95°C and 2 minutes at 50°C for 40 cycles, and 5 minutes at 60°C. Real time PCR for cDNA from infected B cells was performed using an ABI ViiA 7. Each PCR reaction volume was 10µl and contained 2µl of cDNA template. PerfeCTa® FastMix® II, Low ROX™ kit was used for PCR. The cycling program was set to 30 seconds at 95°C, and 40 cycles of 5 seconds at 95°C and 30 seconds at 60°C.

3.1.13 Viral PCR targets

The EBV mRNA targets for these experiments included 5 EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C) and 3 latent membrane proteins (LMP1, LMP2A and LMP2B. The lytic gene BZLF1 or Zebra was also a target for mRNA studies using infected B cells. The primer and probe sequences are in **Table 3**.

3.1.14 Microscopy

Nikon TS100 microscope was used to observe progress of EBV infection in B cells and PBMCs. The microscope has a SPOT Insight 2 camera that can be used with the SPOT Advanced software 4.0.5 (Diagnostic Instruments, Inc) to capture EGFP+ and proliferating B cells. The recombinant wild-type and LMP2 KO viruses contain an EGFP gene that can be used to track infections, as well as titer infectious particle numbers. The infected cells were monitored every 3-4 days within a specified period, usually either 6 weeks or 12-14 weeks, for EGFP expression, clumping and proliferation.

Table 3. Primers and Probes for Real Time PCR analysis of EBV gene expression

EBV Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Probe (5′-3′)
EBNA1	GAT TCT GCA GCC CAG AGA GTA GTC	TCG TCA GAC ATG ATT CAC ACT TAA AG	TCG TCG CAT CAT AGA CCG CCA GTA GAC
EBNA2	TAA CCA CCC AGC GCC AAT C	GTA GGC ATG ATG GCG GCA G	CAC CAC GTC ACA CGC CAG TGC TGG GT
EBNA3A	GAT TCT GCA GCC CAG AGA GTA GTC	CTT CTT CCA TGT TGT CAT CCA GG	CCC GGC CTG TCC TTG TCC ATT TTG
EBNA3B	GAT TCT GCA GCC CAG AGA GTA GTC	CCA CGC TTT CTT CAT TAT TCA GGT	TAG ACC GCC AGT AGA CCT GGG AGC AGA
EBNA3C	GAT TCT GCA GCC CAG AGA GTA GTC	CCA GGG TCC TGA TCA TGC TC	AAG ACC CAC CAT GGA ATC ATT TGA AGG A
LMP1	TCA TCG CTC TCT GGA ATT TG	TCC AGA TAC CTA AGA CAA GTA AGC AC	AGC ACA ATT CCA AGG AAC AAT GCC TGT C
LMP2A	CTA CTC TCC ACG GGA TGA CTC AT	GGC GGT CAC AAC GGT ACT AAC T	TGT TGC GCC CTA CCT CTT TTG GCT GGC G
LMP2B	CGG GAG GCC GTG CTT TAG	GGC GGT CAC AAC GGT ACT AAC T	TGT TGC GCC CTA CCT CTT TTG GCT GGC G
BZLF1 (Zebra)	TTC CAC AGC CTG CAC CAG T	AGC AGC CAC CTC ACG GTA GT	CAA CAG CCA GAA TCG CTG GAG GAA TGC G

3.1.15 Stimulation of BCR signaling

One aim of the study was to determine the effect of LMP2A and LMP2B on the maintenance of viral latency or lytic switch. The switch to the lytic cycle can be initiated via signaling through the BCR. Therefore, BCR signaling was stimulated via addition of soluble immunoglobulin with

specificity for IgM/IgA/IgG (Jackson Immunoresearch). Previous studies have determined that addition of this reagent to B cells will stimulate signaling through the BCR [218,219]. B cells were infected with recombinant EBV at MOI 1 and incubated at 37°C. After 10-12 hours incubation, 10 µg/ml soluble immunoglobulin was added to the infected B cells to stimulate lytic reactivation. The B cells were harvested at 12, 24, 48, 72, 96, 120 and 168 hours for RNA extraction to analyze latent and lytic gene expression in wild-type and LMP2 KO virus-infected B cells.

3.1.16 Long-term outgrowth assay

To determine the effect of LMP2 on the long-term outgrowth of infected B cells, 5 x 10^4 negatively enriched B cells were infected with wild-type and LMP2 KO viruses at MOI of 1. Long-term outgrowth was also determined for PBMCs. Infection of 5 x 10^4 PBMCs was performed with wild-type and LMP2 KO viruses at MOI 30. Infected cells were observed regularly over the course of 12-14 weeks to determine if efficient proliferation and LCL formation occurred. Standard proliferation assays are followed for approximately 6-8 weeks, but slow proliferation rates of LMP2A KO virus-infected B cells required longer observation. At the end of 12-14 weeks, the number of wells with proliferating cells were determined, counted and transferred to 25 cm^2 flasks.

4.0 SPECIFIC AIM ONE: ANALYZE THE KINETICS OF EARLY EBV INFECTION IN B CELLS IN THE CONTEXT OF LMP2A AND LMP2B EXPRESSION

4.1 ABSTRACT

Infection of primary B cells with wild-type EBV leads to activation and proliferation soon after infection. Specifically, the expression of activation markers (CD23, CD30, CD39, and CD70) and adhesion molecules (LFA-1, LFA-3 and ICAM-1) are induced by EBV infection. The viral proteins EBNA2 and LMP1 cooperate in the activation of B cells, specifically inducing upregulation of CD23 expression. Infected B cells are pushed towards transformation and establishment of LCLs by the ability of EBV to hijack signaling pathways associated with normal B cell activation and proliferation. Like LMP1, LMP2A is a latent protein that modulates cell-signaling pathways, especially those associated with the BCR. These signals promote the survival of infected B cells via activation of the Ras/PI3-K/Akt pathway, which inhibits apoptosis through induction of anti-apoptotic genes Bcl-2 and Bcl-xL. LMP2B does not directly produce signaling, due to a lack of a signaling domain, but appears to interact with other cellular signaling proteins, such as CD19 and LMP2A. It is possible that these interactions allow LMP2B to elicit unique effects in infected B cells.

In this study, I investigated possible roles for LMP2 in early B cell infection *in vitro* using recombinant LMP2 KO viruses. These viruses were used to infect purified, primary B cells

in culture. Following infection, the efficiency of specific cellular processes associated with EBV infection was observed. Data obtained from these experiments suggests that LMP2A is critical for activation following EBV infection, possibly indicating a cooperative function for activation alongside EBNA2 and LMP1. In contrast LMP2B did not significantly affect B cell activation, which indicated that the interactions between LMP2B and other cellular proteins (i.e., CD19) are seemingly not involved in this process. LMP2A appeared to be essential for proliferation of B cells immediately following EBV infection, while deletion of LMP2B resulted in delayed proliferation kinetics compared to wild-type EBV. Deletion of either LMP2A or LMP2B alone resulted in higher percentages of apoptotic cells. However, loss of both isoforms exhibited the most extreme loss of early proliferation and increase in apoptotic B cells.

4.2 RESULTS

4.2.1 EBV constructs used in this study

EBV BAC plasmids were used to generate recombinant viruses for these studies. These BACs include EGFP gene and hygromycin resistance cassette insertions. Expression of EGFP under control of the CMV promoter allowed for demonstration of infectious virus numbers in each virus stock, as well as tracking of infectivity during experiments. The hygromycin resistance cassette was used for selection of HEK 293 producer cell lines harboring the EBV plasmid. The parental wild-type EBV BAC used for these experiments was designated p2089 (a generous gift from Dr. Wolfgang Hammerschmidt) [210]. Another BAC with deletion of LMP2A Exon 1, designated p2525, was provided by Dr. Hammerschmidt for our studies [211]. Flag tags were inserted into Loop 11 of the LMP2 gene, which is located in Exon 7 **(Table 4)**. Previous work using these BACs established that insertion of the Flag tag in loop 11 did not affect the function of LMP2A or LMP2B [169].

The p2089 and p2525 BACs were used to generate the LMP2B Exon 1′ deletion BACs. Mutations were created using a shuttle vector, named p2768.5 (a gift from Markus Altmann and Wolfgang Hammerschmidt). The DNA in this shuttle vector that contained Exon 1′ with flanking LoxP sequences was inserted into the BACs via homologous recombination between shared EBV DNA sequences. Selection of BACs containing the recombinant DNA was performed using the antibiotic Zeocin. Later, introduction of Cre recombinase induced recombination between LoxP sequences resulted in removal of Exon 1′, creating the BACs designated 2089.3 (Δ2B) and

2525.3 (Δ2A/Δ2B). LMP2A and LMP2B transcripts cross the terminal repeat region of the viral genome, requiring circularization of the linear genome for transcription. Both proteins are isoforms of the LMP2 gene. Each transcript is spliced multiple times and includes 9 total exons. They only differ in their first exons, designated Exon 1 for LMP2A and Exon 1′ for LMP2B, and share the remainder of their genetic information (Exons 2-9). Exon 1 of LMP2A encodes the N-terminal CSD that elicits a signal similar to the BCR. Exon 1′ of LMP2B is noncoding indicating that this protein does not include a domain designated for signaling. Exons 2-9 encode the 12 transmembrane domains necessary for protein localization and a short C-terminal tail that is important for protein aggregation **(Figure 7)**.

Table 4. Recombinant EBV BACs used for this study

BAC Number Designation	Important Features	Virus Phenotype
2089.0	EGFP and hygromycin resistance genes	Wild-type, Wt
2089.1	EGFP and hygromycin resistance genes; flag tag insertion in loop 11	Wild-type, Wt
2089.3	EGFP and hygromycin resistance genes; flag tag insertion in loop 11; deleted Exon 1′	No LMP2B expressed, Δ2B
2525.1	EGFP and hygromycin resistance genes; flag tag insertion in loop 11; deleted Exon 1	No LMP2A expressed, Δ2A
2525.3	EGFP and hygromycin resistance genes; flag tag insertion in loop 11; deleted Exon 1 and Exon 1′	No LMP2A or LMP2B expressed, Δ2A/Δ2B

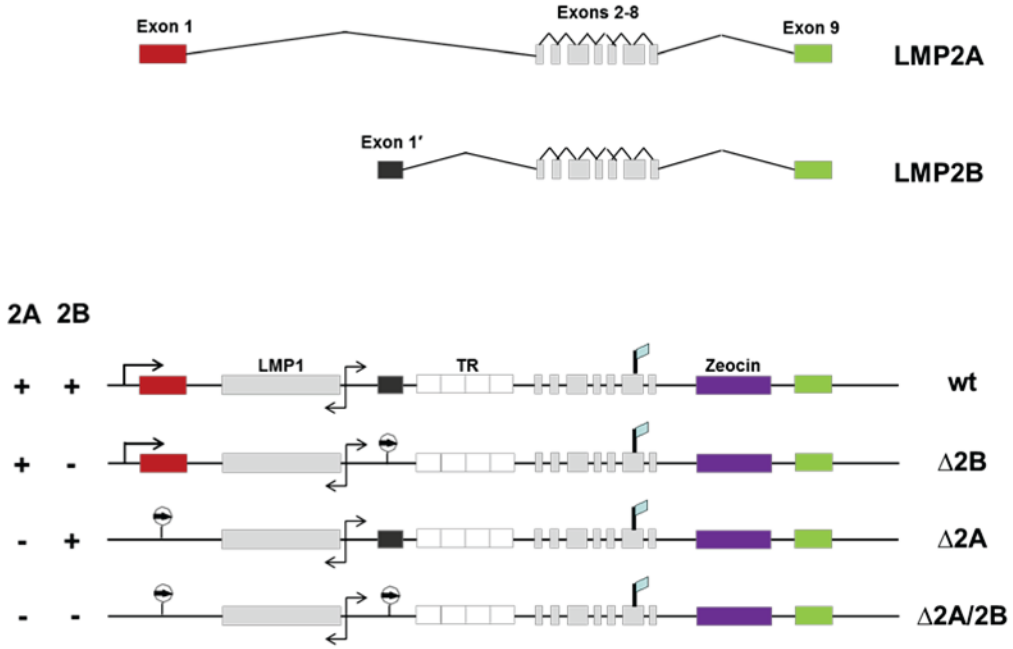

Figure 7. Virus constructs with deletions in LMP2.

This figure demonstrates the LMP2 region in virus constructs used for this study. The wild-type (wt) construct has no deletions within the LMP2 region. The EGFP and hygromycin genes were inserted approximately 40kb away from LMP2 region (near the BamHI A region) of the EBV genome that is not depicted here. Splicing of exons 1-9 results in full-length LMP2A transcript, while splicing of exons 1′-9 results in the shorter LMP2B transcript. The Δ2B construct has deleted Exon 1′ via LoxP sequence recombination, resulting in loss of LMP2B transcription. Deletion of Exon 1 in the Δ2A construct using the same method of recombination resulted in complete loss of LMP2A transcription. The Δ2A/Δ2B construct represents EBV BAC that has completely lost the ability to produce both LMP2A and LMP2B transcripts. Removal of these exons resulted in loss of LMP2A and LMP2B protein expression.

4.2.2 PCR reveals presence of exon 1 and exon 1′ deletions

Prior to virus production, we examined our wild-type and LMP2 KO BACs to validate the deletions of the initial exons for each isoform. Primers were designed with homology to either internal or external sequences associated with the deletion regions **(Table 2)**.

The results demonstrated the presence or absence of LMP2A Exon 1 and LMP2B Exon 1′ deletions in the appropriate BAC plasmids **(Figure 8)**. The LMP2A Exon 1 deletion primer set included a forward primer with sequence homology within the LoxP/deletion region that contains the LMP2A promoter and a reverse primer with sequence homology outside the deletion region. Therefore, a 522bp PCR product was observed for wild-type and Δ2B BACs, whereas no PCR product was detected for LMP2A deletion BACs (Δ2A and Δ2A/Δ2B). The LMP2B Exon 1′ deletion primer set included both forward and reverse primers with homology to sequences outside the deletion region. PCR for wild-type and Δ2A BACs yielded a 374bp product, while a smaller 167bp product was observed for LMP2B deletion BACs (Δ2B and Δ2A/Δ2B). Therefore, PCR results indicated that the correct deletions were present in the appropriate LMP2 KO BACs. Specifically, the LMP2A promoter and Exon 1 were deleted in the Δ2A and Δ2A/Δ2B BACs, and LMP2B Exon 1′ was deleted in the Δ2B and Δ2A/Δ2B BACs.

4.2.3 Gene expression assay in recombinant virus producer lines demonstrates intact viral genomes

The previous PCR experiment validated the correct LMP2 deletions were present in the appropriate BACs. However, that experiment did not reveal whether other parts of the viral genome remained intact following recombination. Therefore, the wild-type and LMP2 KO BACs were transfected into HEK 293 cells, which were also used for virus production. Once these transfected cells were grown into stable cell lines via hygromycin selection, $1x10^6$ EGFP+ cells were used for detection of EBV latent gene expression. Real time PCR results showed that the wild-type and LMP2 deletion BACs maintained expression of all other latency-associated genes,

indicating that the genomes remained intact following manipulations to the LMP2 region **(Figure 9)**.

EBNA2 and LMP1 were the highest expressed genes in producer cell lines. LMP2A transcript was not detected in Δ2A and Δ2A/Δ2B producer lines, again validating the correct deletion. Also, LMP2B transcript was not detected in Δ2B and Δ2A/Δ2B producer lines, validating the presence of the Exon 1′ deletion. EBNA1, EBNA3A, EBNA3B and EBNA3C were consistently expressed between producer lines; the only exception was elevated LMP1 and EBNA3A and decreased EBNA3B in Δ2B producer lines. However, the variation in expression of these transcripts was not statistically significant in these cell lines. However, it could explain a negative regulatory mechanism for LMP2B in the expression of EBNA3A and, interestingly, LMP1, which shares a bidirectional promoter with LMP2B. Expression of EBNA3B decreased in the absence of LMP2B, which also suggests a possible regulatory mechanism for this protein in the expression of EBNA3B. Aside from those minor differences, latent gene expression was consistent between EBV BAC producer lines, suggesting that all four BAC plasmids remained intact following manipulations to the LMP2 region. Additionally, the recombinant wild-type and LMP2 KO BACs were sequenced for mutations possibly acquired during the recombination process. Sequencing results did not indicate the presence of any large deletions within the mutant BACs compared to wild-type BAC. Therefore, we moved into the virus production phase with these producer lines.

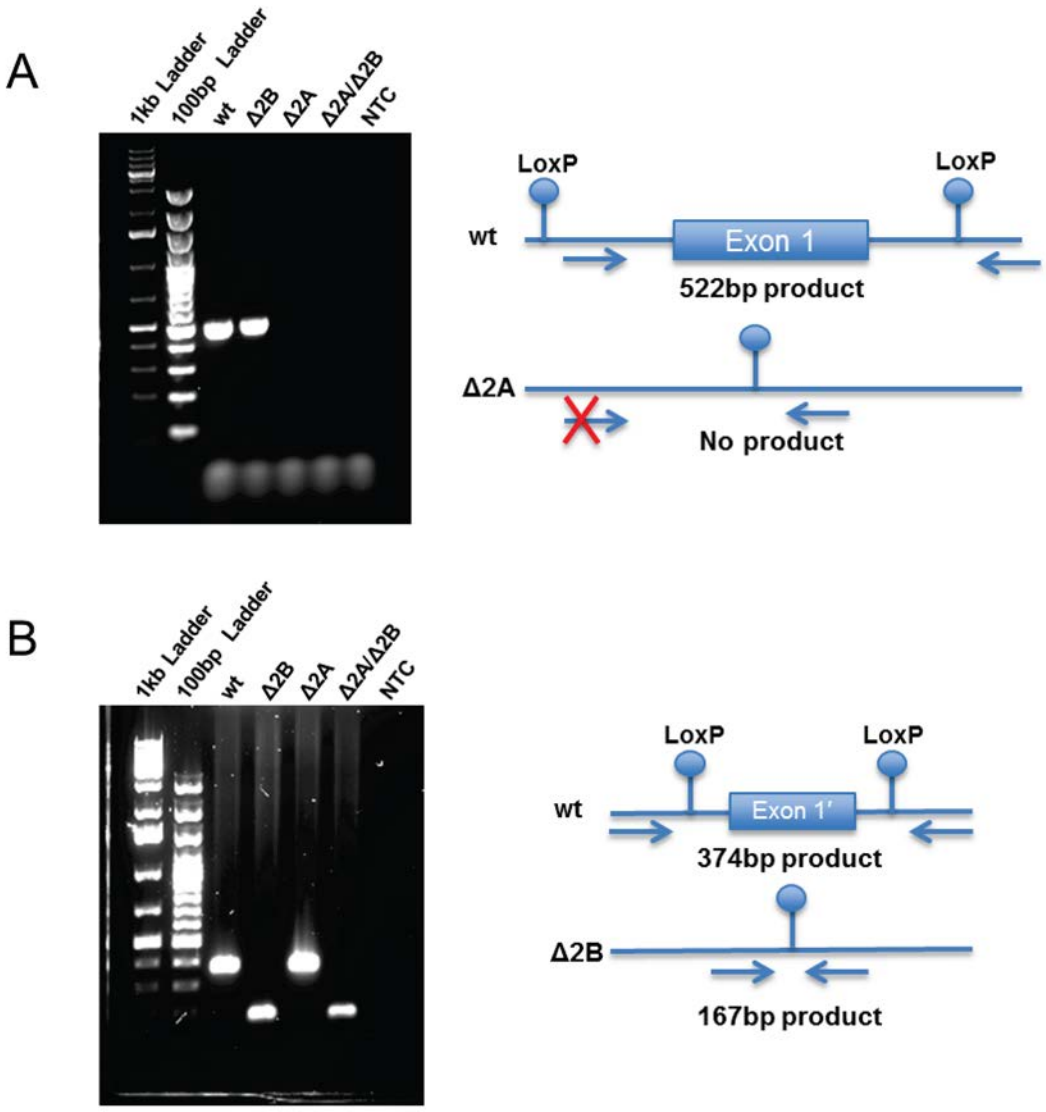

Figure 8. Confirmation of Exon 1 and Exon 1' deletion by qcPCR.

(A) Deletion of LMP2A Exon 1 was validated by lack of PCR product. The forward primer sequence was located inside the LoxP/deletion region, resulting in loss of the primer binding site following recombination. The loss of forward primer binding resulted in lack of PCR product. (B) Deletion of LMP2B Exon 1′ was validated by small 167bp PCR product. Both forward and reverse primer sequences were located outside the LoxP/deletion region, resulting in PCR product size differences between wild-type (wt) and Δ2B BACs.

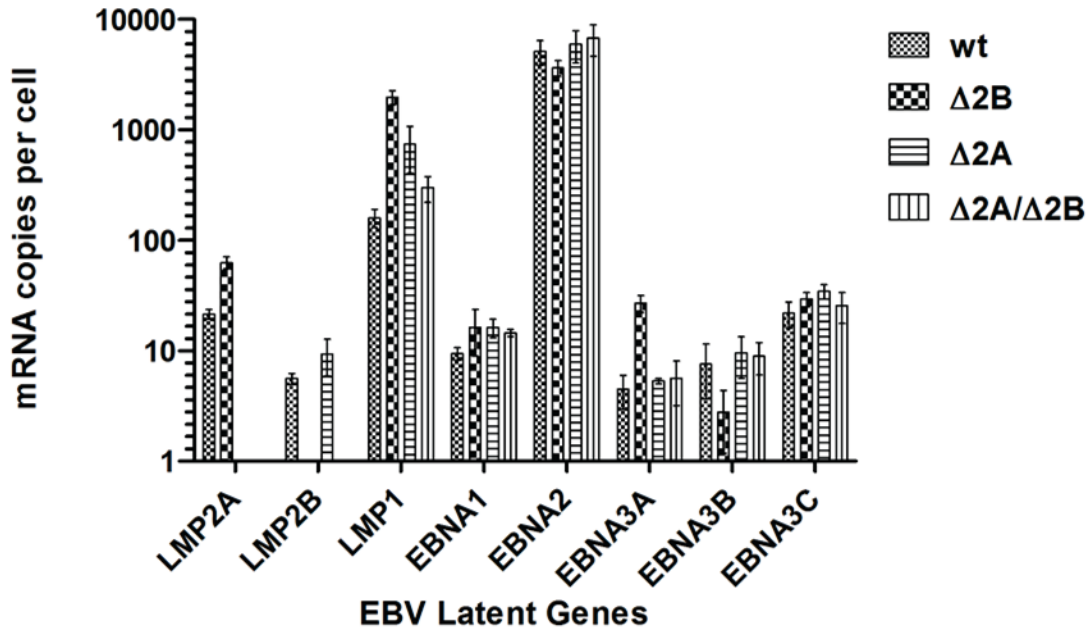

Figure 9. Latent gene expression is consistent between EBV BAC cell lines.

Gene expression analysis using Real Time PCR validated presence of Exon 1 and Exon 1′ deletions; LMP2A transcript was not detected in Δ2A or Δ2A/Δ2B producer cell lines, and LMP2B transcript was not detected in Δ2B or Δ2A/Δ2B producer cell lines. The other latent genes, which were not manipulated in these constructs, maintained consistent expression levels between wild-type (wt) and mutant BAC cell lines. There were no statistically significant differences based on Two-way ANOVA testing with Bonferroni Post-test comparison to wild-type.

4.2.4 Viruses produced from recombinant virus genomes have similar characteristics

Virus production from HEK 293/EBV cell lines is accomplished via introduction of Zebra and gB expression plasmids [51,216]. Zebra is one of two immediate early proteins that are responsible for initiation of the lytic cycle [48,220,221]. In conjunction with the other immediate early gene Rta, Zebra activates promoters for expression of early genes, such as those associated with the viral DNA replication machinery [48]. An Rta expression plasmid is not used for virus production because previous research has shown that expression of Zebra alone is sufficient for

lytic cycle entry; Zebra can activate the Rta promoter, inducing Rta expression [51,222,223]. The glycoprotein gB is necessary for viral entry, so gB expression plasmids were included to increase the infectivity of the progeny viruses for B cells [216].

Virus supernatants were tested for the numbers of infectious virions, as well as genome copy numbers **(Table 5)**. Infectious titers for the virus supernatants were determined by infection rates in Raji B cells. LMP2 KO viruses were able to efficiently infect Raji B cells, further confirming similarities to wild-type EBV outside of the LMP2 region of the genome **(Figure 10)**. Calculations of virus titer were determined by the number of EGFP+ Raji B cells per well, and were represented as green inducing units (GIU) per milliliter. Primers and probe specific for the major virus glycoprotein, gp350, were used to calculate genome copy number [217]. This allowed us to selectively use recombinant virus stocks with similar genome copy number to infectious unit (genome copy#/GIU) ratios for subsequent experiments, which reduced the potential for differences in early infection kinetics to be attributable to variation in particle doses.

4.2.5 Infection of B cells with LMP2 KO viruses exhibited delayed proliferation kinetics compared to wild-type virus

The recombinant virus stocks shown in **Table 5** were used to infect primary B cells for all subsequent experiments. Although previous research has conclusively shown that wild-type EBV induces activation and proliferation of human B cells during early infection and subsequent establishment of LCLs [224], the specific role of LMP2 in this process is not clear, and has, in fact, been intensely disputed within the field of EBV research [205-208]. Therefore, we first explored the effect of LMP2 KO viruses on proliferation kinetics in early B cell infection.

Primary B cells were purified from PBMCs by negative selection, and used for EBV infection. Previous studies have used PBMCs for kinetic experiments, but we preferred simplified culture conditions that eliminated the possibility of bystander effects, such as EBV infection of other cell types that could result in their activation/proliferation and skew our observations [215]. Live microscopy was used to follow EBV infection of B cells, allowing for observations of EGFP expression, morphological changes, such as increased cell size and clumping, and proliferation. The effects of LMP2 KO viruses on early B cell infection were assessed for up to 14 days post-infection. B cells were infected with wild-type and LMP2 KO EBV at an MOI of 1.

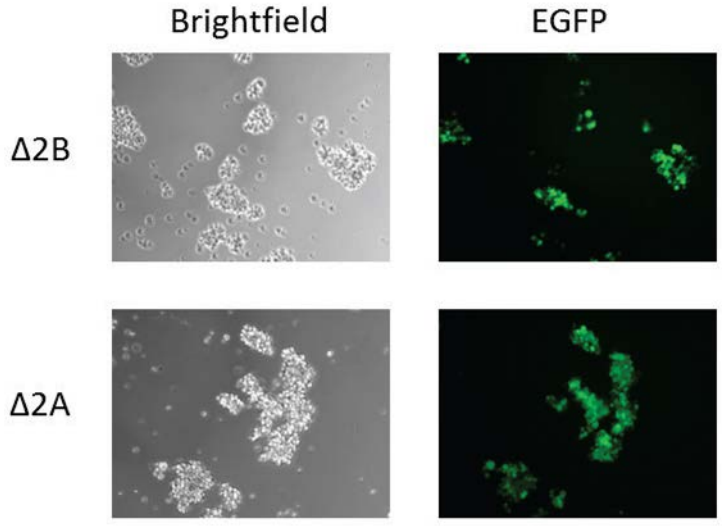

Figure 10. LMP2 KO viruses efficiently infect Raji B cells for titration.

LMP2 KO viruses infect Raji B cells to a high degree, indicating that infectivity of the viruses were not adversely affected by either of the LMP2 deletions. Also, the ease at which recombinant EBV infects this cell line shows that Raji B cells are appropriate for titration of virus stocks. Infection of Raji B cells with virus supernatants was performed numerous times (n>20), and similar results were observed for supernatants that contained high numbers of virus (GIU/ml $\geq 10^5$/ml)

Table 5. Characterization of recombinant viruses used for this study

EBV	Genome #/ml	*GIU/ml	Genome/GIU
Wild-type	4.3×10^7	1.9×10^6	23
Wild-type	1.01×10^8	4.1×10^6	24.6
Δ2B	6.1×10^7	4.8×10^6	13
Δ2B	10×10^6	5.45×10^5	18.4
Δ2B	15.1×10^6	5.6×10^5	27
Δ2B	7.8×10^7	2×10^6	39
Δ2A	6.2×10^6	1×10^6	6.2
Δ2A	5.1×10^7	9.5×10^5	53.7
Δ2A	1.14×10^8	2×10^6	56.9
Δ2A/Δ2B	1.3×10^6	8.8×10^5	1.5
Δ2A/Δ2B	2.3×10^8	2×10^6	115
Δ2A/Δ2B	1.13×10^6	7.9×10^5	1.4
Δ2A/Δ2B	12.1×10^6	6×10^5	20.2

*GIU is an acronym representing "green inducing units". Green inducing units are a unit of infectivity for the EGFP expressing recombinant viruses. The differences observed for the Genome to GIU ratios are most likely due to the differing abilities of virus producer lines to produce recombinant wild-type and LMP2 KO viruses after long period of time in culture conditions (>2-4 months). The observed differences in GIU/ml and Genome/ml and Genome/GIU numbers were not considered significant because a normalized multiplicity of infection (MOI = 1) was used for majority of experiments.

Microscopy experiments **(Figure 11)** revealed that EGFP expression could be observed at similar levels for all virus-infected B cells by 4 days post-infection. Interestingly, expression of EGFP was not observed for all proliferating B cells, which could be due to the presence of secreted factors by infected cells, such as cytokines (IL-10, IL-6, etc) that could influence the proliferation of neighboring uninfected B cells. By 4 days post-infection, the green cells coalesced into small, scattered clumps of approximately 2-10 cells, which was indicative of B cell activation. B cells will clump together in cell culture when activated due to upregulation of adhesion molecule expression, such as LFA1 and ICAM1 [225-227]. Fewer B cell clumps were observed in Δ2A and Δ2A/Δ2B wells compared to wild-type wells. Over time, the clumps increased in size in wells that contained B cells infected with wild-type and Δ2B viruses **(Figure 12 & 13)**, which appeared to be caused by the initiation of proliferation. However, the largest cell clumps, indicating the most rigorous proliferation, were only found in wild-type virus-infected B cell wells. There was a lack of significant proliferation in Δ2A and Δ2A/Δ2B virus-treated wells during the observation period, which was indicated by the small cell clump sizes in those wells **(Figure 12 & 13)**. Therefore, we conclude that expression of LMP2A is critical for efficient proliferation of B cells following EBV infection.

To further study the effect of LMP2 on B cell proliferation kinetics, we labeled B cells with a membrane dye for quantitation of proliferation defects observed in microscopic images. The orange-red emission spectra of the membrane dye, Vybrant DiI (Invitrogen) was advantageous for our experiment due to the presence of EGFP expression in B cells infected with recombinant EBV. DiI proliferation tracker experiments revealed that wild-type and Δ2B infected cells began to proliferate between 4 and 8 days post-infection **(Figure 14)**. High levels of proliferation were observed in wild-type and Δ2B virus-infected B cells by 8-16 days post-

infection **(Figure 14A)**. These experiments were repeated in a total of three B cell donors, and exhibited similar results between donors **(Figure 14B)**. The amount of proliferation detected in the cytometry experiments correlated with the appearance and size of clumps formed in undisturbed wells. However, Δ2B-infected wells seemed to lag slightly behind wild-type in clump size **(Figure 13)** compared to mean fluorescence intensity **(Figure 14A)**. Efficient proliferation was not observed in wells containing B cells infected with either LMP2A KO virus compared to wild-type virus (Δ2A p-value < 0.01, Δ2A/Δ2B p-value < 0.01) **(Figure 14B)**. Therefore, our results suggest that the inclusion of LMP2A in our recombinant viruses is essential for efficient proliferation of infected B cells in early infection.

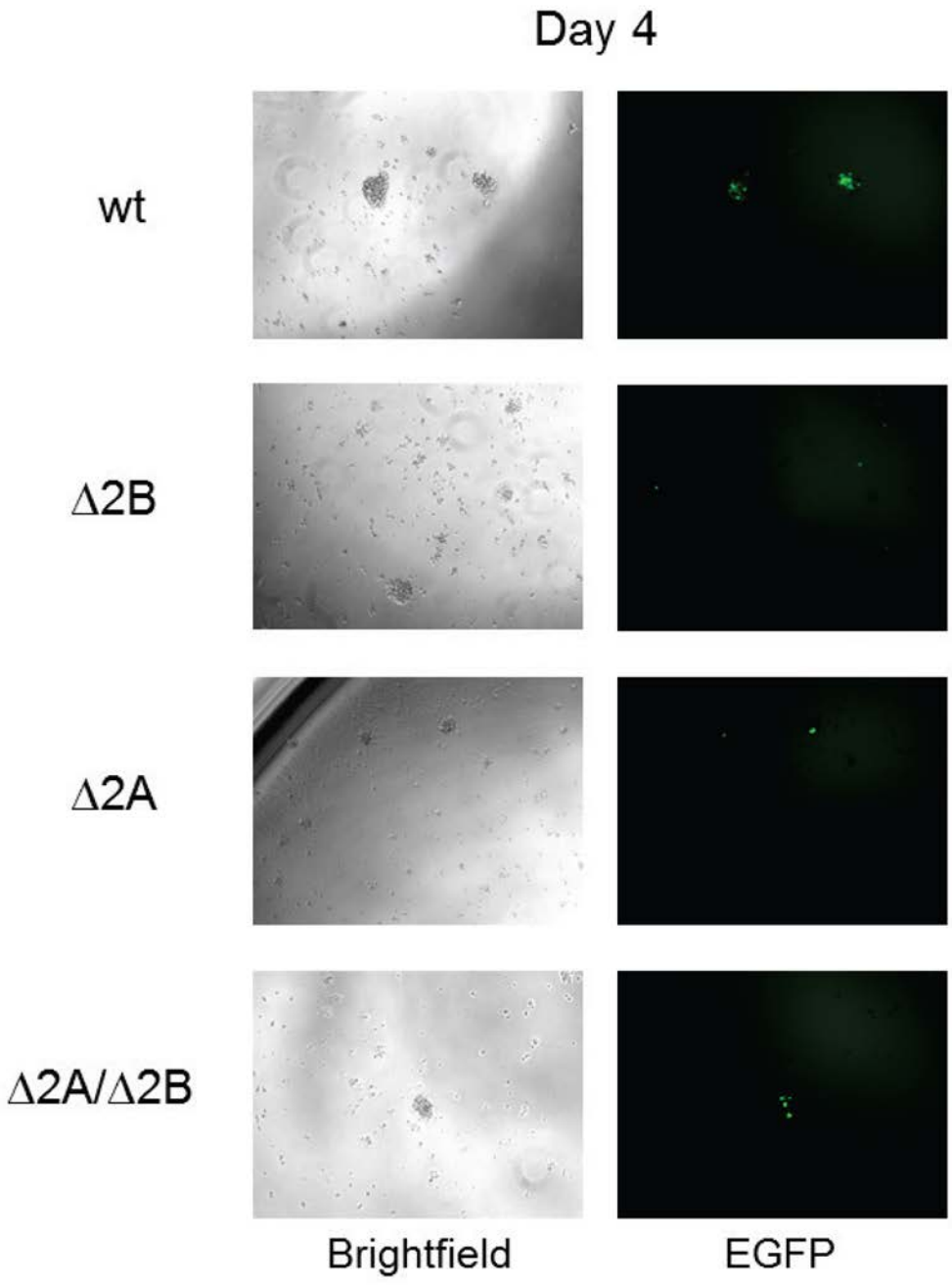

Figure 11. Proliferation of B cells infected with wild-type and LMP2 KO viruses at 4 days post-infection.

$5x10^4$ B cells infected with recombinant wild-type and LMP2 KO EBV at MOI 1. EGFP expression and small B cell clumps are evident in all wells. This data is representative for one of three donors used for this experiment.

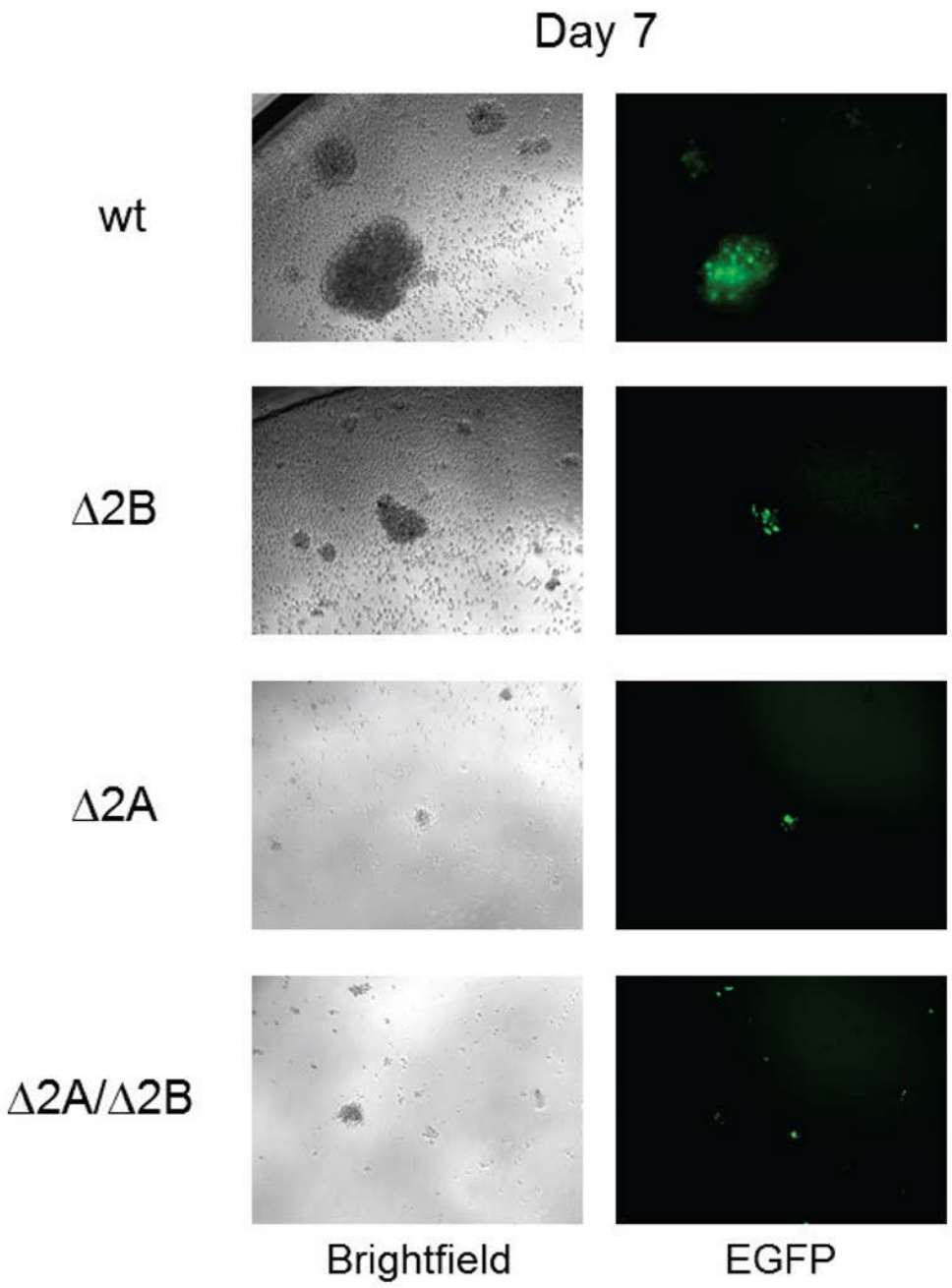

Figure 12. Proliferation of B cells infected with wild-type and LMP2 KO viruses at 7 days post-infection.

The size of cell clumps expanded in wells treated with wild-type and Δ2B viruses. Clump expansion is much slower in wells treated with Δ2A and Δ2A/Δ2B viruses. EGFP expression was still evident in all wells, indicating the presence of EBV. This data is representative for one of three donors used for this experiment.

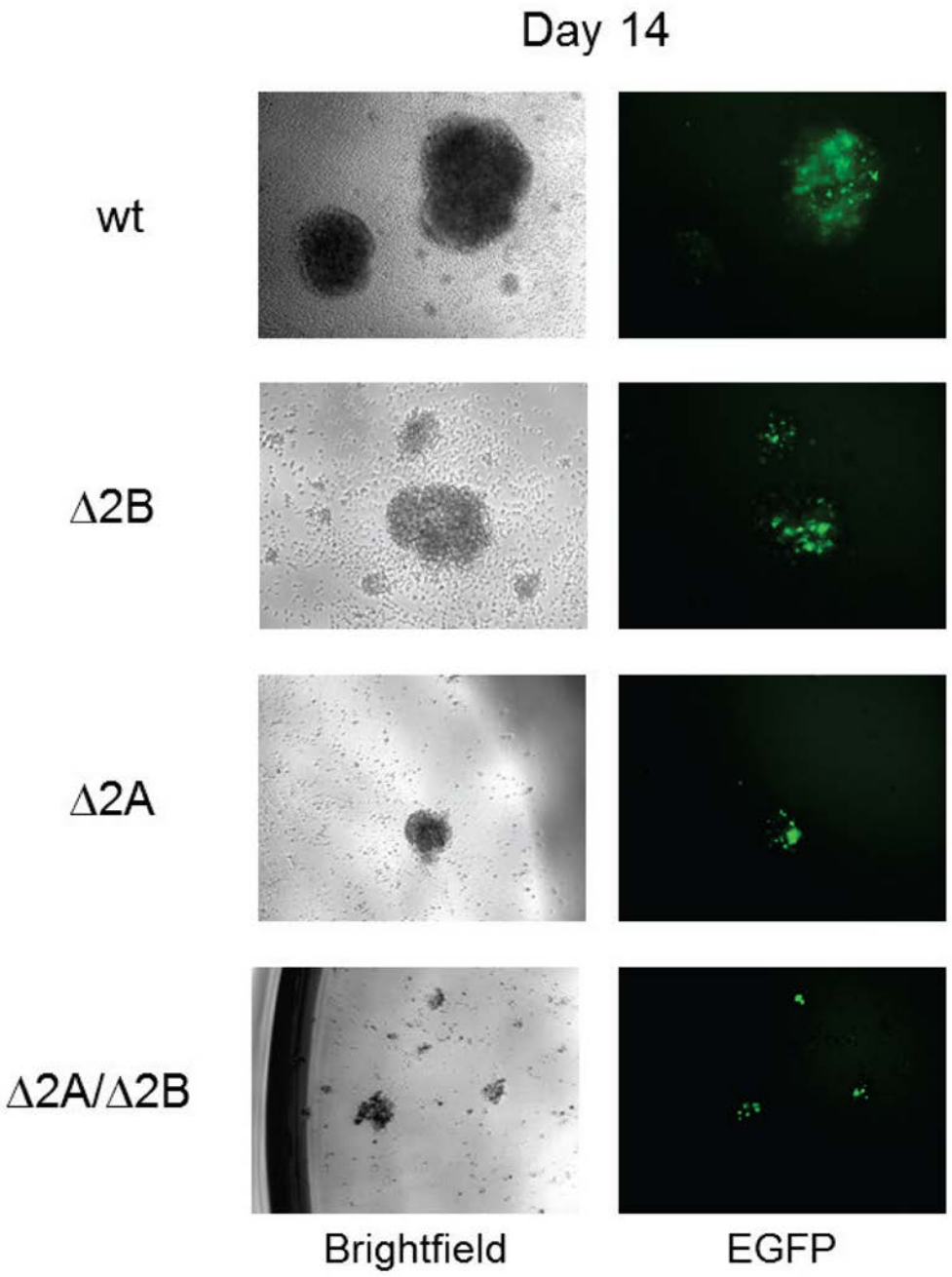

Figure 13. Proliferation of B cells infected with wild-type and LMP2 KO viruses at 14 days post-infection.

Expansion of cell clump sizes evident in all wells, particularly in wild-type virus treated wells. The most rigorous proliferation occurred in wild-type wells. Cells continued to grow efficiently in Δ2B-treated wells, but proliferation rates for Δ2A and Δ2A/Δ2B treated wells continued to lag behind. The presence of proliferating non-green cells could indicate the presence of secreted factors driving proliferation of uninfected B cells. This data is representative for one of three donors used for this experiment.

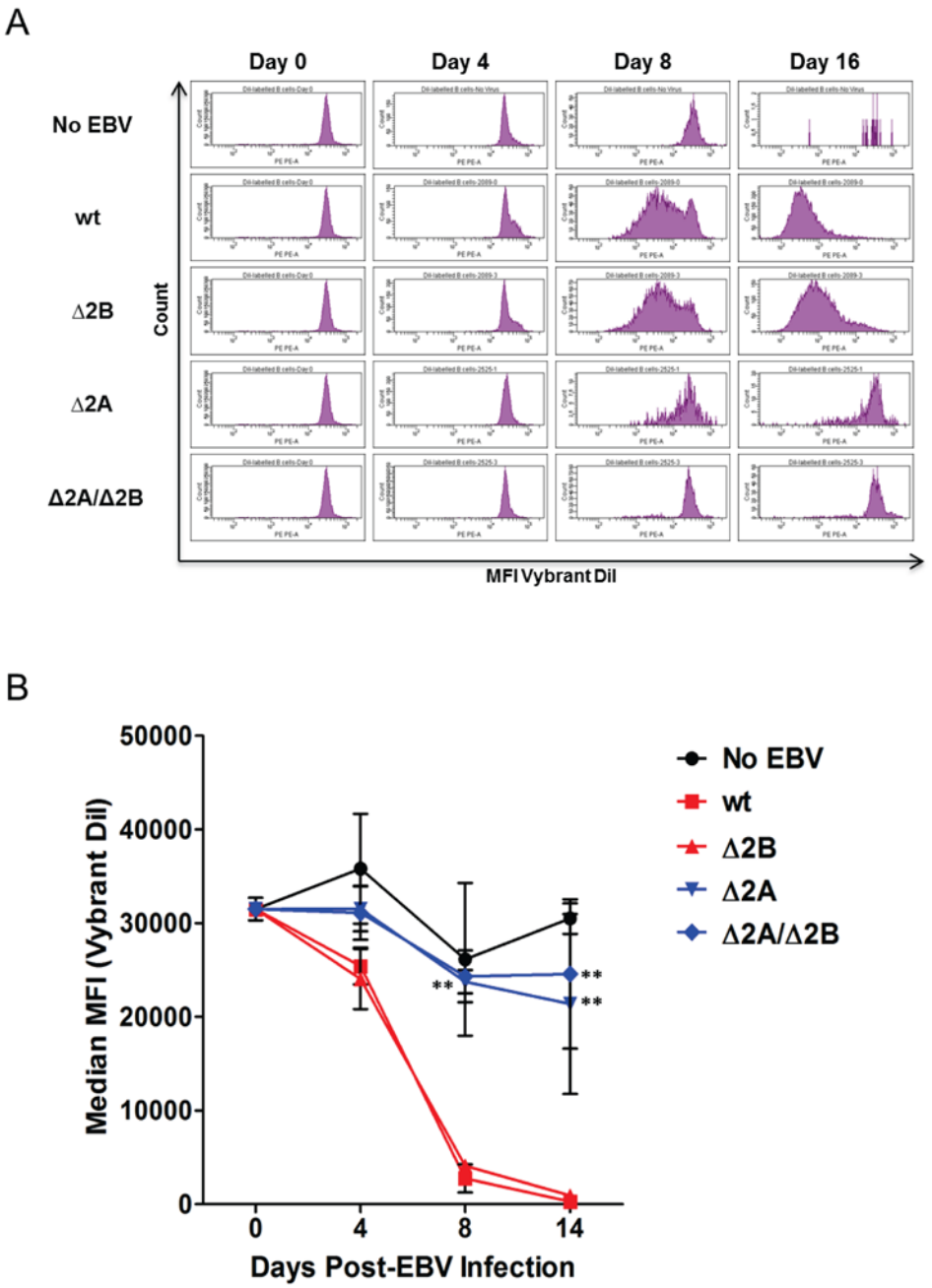

Figure 14. Quantitation of B cell proliferation following wild-type and LMP2 KO virus infection.

To quantify B cell proliferation, $1x10^6$ B cells labeled with proliferation dye (Vybrant DiI, Invitrogen) were infected with recombinant wild-type and LMP2 KO EBV at MOI 1. (A) Infected B cells were harvested and analyzed at 4, 8 and 16 days post-infection using flow cytometry. This data is representative for one of three donors used for this experiment. (B) Each data point is an average of 3 independent experiments ± SEM. Statistical significance was determined using Two-way ANOVA and Bonferroni Post-test. P-value (**) < 0.01 for both Δ2A and Δ2A/Δ2B viruses compared to wild-type at 8 and 16 days post-infection. There were no significant differences between proliferation of wild-type and Δ2B-infected B cells.

4.2.6 LMP2A KO viruses induced lower levels of activation marker expression

Since there were clear differences in the proliferation kinetics associated with Δ2A and Δ2A/Δ2B viruses in early B cell infection, and to a lesser extent the Δ2B virus, we reasoned that an event upstream of proliferation, such as B cell activation, could also be compromised by the LMP2 deletions. Additionally, previous studies have shown that LMP2A can produce a signal that mimics an activated BCR signal. Specifically, the expression of LMP2A was associated with activation of protein tyrosine kinases and Ca^{+2} initiation complexes that resulted in Ca^{+2} fluxes resembling those observed after BCR stimulation [228]. Activation of uninfected B cells can be triggered by BCR stimulation following antigen recognition and a second signal, such as CD40-CD40L signaling, which indicates that EBV latent proteins hijack cellular pathways that B cells normally use for activation and proliferation [3]. Therefore, it is reasonable to hypothesize that LMP2A, a protein that produces BCR-like signals, may play a role in B cell activation, and act in concert with LMP1, a viral protein that produces a CD40-like signal, to induce optimal B cell activation following *in vitro* infection.

For this experiment, infected B cells were probed for expression of two surface markers associated with B cell activation and proliferation, CD23 and CD71 [229]. The infected cells were analyzed at 0, 4, 8 and 16 days post-infection. By 4 days post-infection, higher percentages of B cells infected with wild-type and Δ2B viruses had upregulated expression of CD23 and CD71 **(Figure 15)**. The proportion of wild-type and Δ2B virus-infected B cells expressing CD23 and CD71 increased throughout the entire 16-day experiment, with levels reaching as high as 80-85% of B cells positive for the two surface markers **(Figure 15B and 15C)**. In contrast to wild-type and Δ2B viruses, a smaller percentage of Δ2A and Δ2A/Δ2B virus-infected B cells showed CD23 and CD71 expression. B cells infected with these viruses displayed delayed activation

marker expression kinetics that did not increase similarly to wild-type virus-infected B cells over the course of the experiment **(Figure 15A)**. At 16 days post-infection only 30-40% of B cells were positive for the activation markers, which significantly contrasted to activation marker expression in wild-type infected B cells (CD23 Δ2A 4-day p-value < 0.001, Δ2A 8-day and 16-day p-value < 0.0001) (CD23 Δ2A/Δ2B 4-day p-value < 0.01, Δ2A/Δ2B 8-day and 16-day p-value < 0.0001) (CD71 Δ2A 4, 8, 16-day p-value < 0.0001) (CD71 Δ2A/Δ2B 4-day p-value < 0.001, Δ2A/Δ2B 8-day and 16-day p-value < 0.0001) **(Figure 15B and 15C)**. Therefore, we conclude that LMP2A is involved in the process of B cell activation following EBV infection, which was consistent with our data demonstrating that LMP2A is also essential for efficient B cell proliferation.

4.2.7 LMP2 KO virus-infected B cells exhibited higher percentages of apoptotic B cells

Observations from our previous experiments suggested that there was a critical role for LMP2A in early activation and proliferation of primary, human B cells following EBV infection. In addition to the processes of activation and proliferation, several studies have established a role for LMP2A in B cell survival [63,211,230]. The BCR-like signal that LMP2A provides infected B cells has been shown to promote signaling through the Ras/PI3-K/Akt pathway, which contributes to B cell survival by inhibiting apoptosis in developing B cells through induction of anti-apoptotic genes such as Bcl-2 and Bcl-xL [230,231]. Therefore, we reasoned that the deficiencies in activation and proliferation of B cells by Δ2A and Δ2A/Δ2B viruses could be due to defective B cell survival signals leading to increased apoptosis.

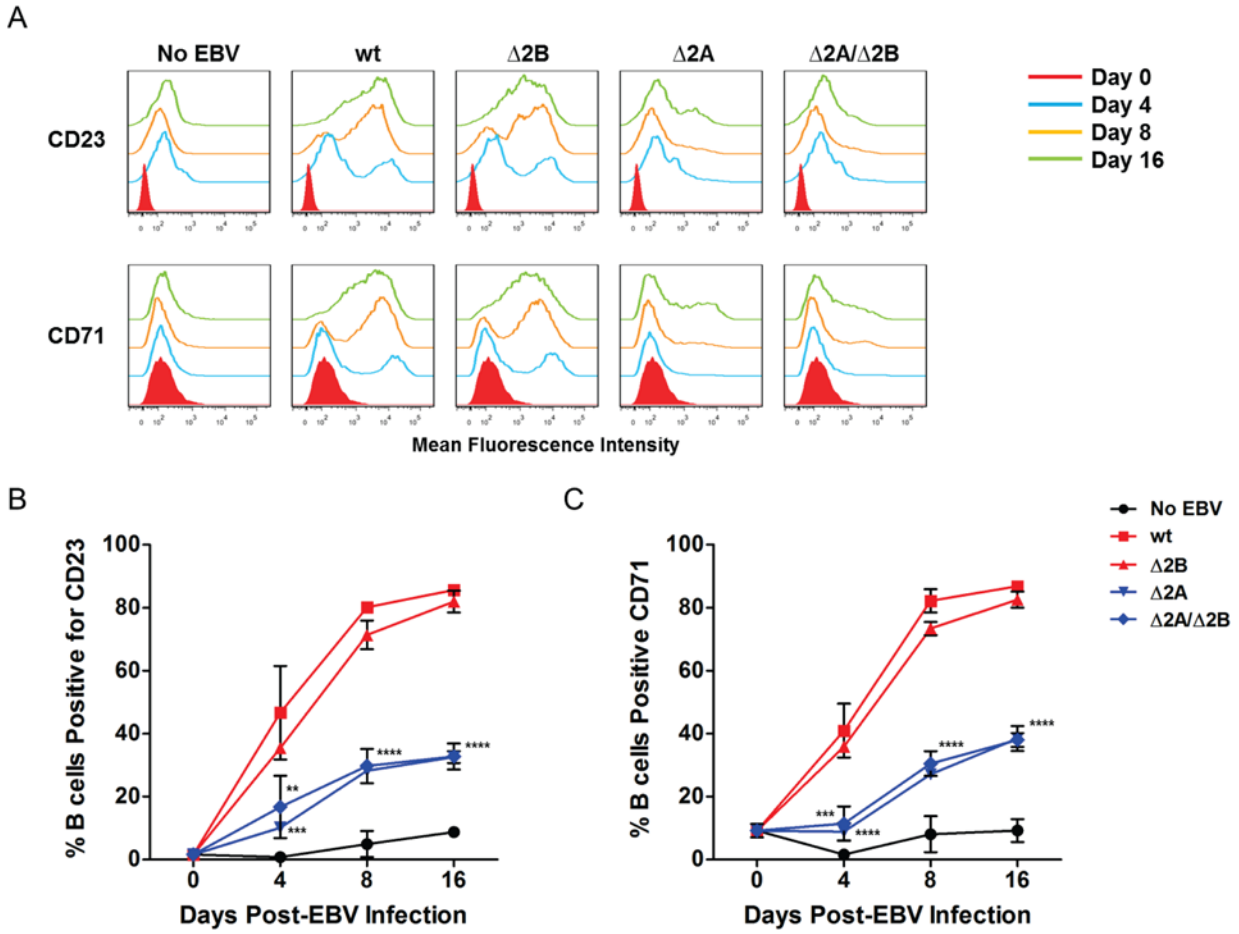

Figure 15. LMP2A is critical for sufficient activation of B cells.

$1x10^6$ purified B cells infected with recombinant wild-type and LMP2 KO EBV at MOI 1. B cells harvested at 4, 8 and 16 days post-infection for analysis of activation marker expression. (A) Expression of CD23 (marker of B cell activation) and CD71 (marker of lymphocyte activation and proliferation) assessed by flow cytometry and analyzed using FlowJo 7.6.1 software. (B) Expression of CD23 and (C) CD71 quantified and expressed as percent positive for surface markers. Each data point is an average of 2 independent experiments ± SEM. Statistical significance was determined using Two-way ANOVA and Bonferroni Post-test (compared to wild-type). P-value (**) < 0.01 for Δ2A CD23 expression at 4 days post-infection. P-value (***) < 0.001 for Δ2A/Δ2B CD23 expression at 4 days post-infection and Δ2A CD71 expression at 4 days post-infection. P-value (****) < 0.0001 for Δ2A CD71 expression at 4 days post-infection, and Δ2A and Δ2A/Δ2B viruses for both CD23 and CD71 expression at 8 and 16 days post-infection.

To examine this, purified B cells were labeled with Violet tracer proliferation dye and infected with recombinant wild-type and LMP2 KO viruses. Violet tracer enhances the ability to trace multiple generations of cell divisions. On the other hand, Vybrant DiI only showed whole population shifts, possibly missing cells undergoing one or two cell divisions, which could be due to its nature as a membrane dye, as opposed to a cytoplasmic dye like CFSE. For instance, analysis using mean fluorescence intensity (MFI) for Vybrant DiI experiments revealed that no proliferation occurs, even in wild-type virus-infected cells, by 4 days post-infection **(Figure 14)**. However, we reasoned that early proliferating cells were missed by this analysis, as was evident in the live microscopy images **(Figure 11)**. Also, the DiI data indicated that no proliferation kinetic differences existed between B cells infected with wild-type and Δ2B viruses **(Figure 14)**, which was not supported by data accumulated in live microscropy images that showed slightly slower proliferation of B cells infected with Δ2B virus **(Figure 12 & 13)**. Therefore, to understand more clearly the subtle differences between wild-type and Δ2B viruses, we labeled the cells with violet cell tracer to track proliferation and compare those rates to the rates of apoptosis in the same infected B cells.

Cells were collected at 4, 7 and 14 days post-infection for analysis, and stained with Aqua live/dead cell indicator and Annexin V for apoptosis. The cells were incubated with a specific number of Absolute Cell Counting Beads (Invitrogen) to standardize the acquisition volume of the sample on the cytometer (FACSLSRII), which allowed for accurate, absolute cell counts of proliferating B cells.

In order to better quantify these differences using flow cytometry, we collected proliferation and apoptosis levels simultaneously for wild-type and LMP2 KO viruses. For analysis, we used a series of gates to eliminate specific B cell populations **(Figure 16)**, such as

doublet, dead or dying cells as well as uninfected cells. With this gating strategy, the Violet Tracer and Annexin V gates were specific for the proliferating and apoptotic EBV-infected cells within the B cell population.

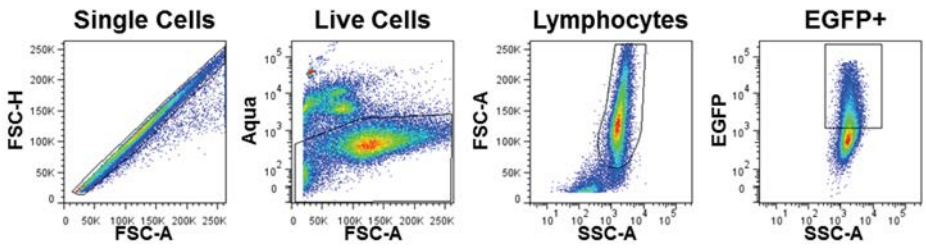

Figure 16. Gating strategy for proliferation and apoptosis analysis.

Cell doublets were first eliminated from total B cell population. Aqua live/dead cell indicator allowed for elimination of dead cells from our analysis to avoid overestimation of apoptotic cell number. Lymphocytes were selected for further gating, which eliminated any remaining cell debris or other cell types. The last selective gate before proliferation/apoptosis analysis was EGFP positivity, which allowed for elimination of uninfected cells from any further analysis. Uninfected cells would be more likely to undergo apoptosis and could possibly skew overall analyses**.**

Using these selection gates, results from three experiments (and three different donors) were combined. B cells infected with wild-type virus exhibited the highest levels of proliferation at all time points during the observation period **(Figure 17)**. Δ2B virus induced the second highest levels of proliferation. The proliferation kinetic rates were slower than wild-type infections, but were still considered efficient with the majority of infected B cells undergoing proliferation by 14 days post-infection. In contrast, B cells infected with either Δ2A or Δ2A/Δ2B viruses did not proliferate efficiently, especially in the first 4-7 days after infection. The numbers of proliferating B cells increased by 14 days post-infection, but were still significantly lower than the numbers of proliferating wild-type and Δ2B virus-infected B cells. Overall, the efficiency of B cell proliferation decreases dramatically when LMP2A expression is removed from the

infecting viruses. Interestingly, the number of proliferating B cells is lowest when both LMP2A and LMP2B are deleted.

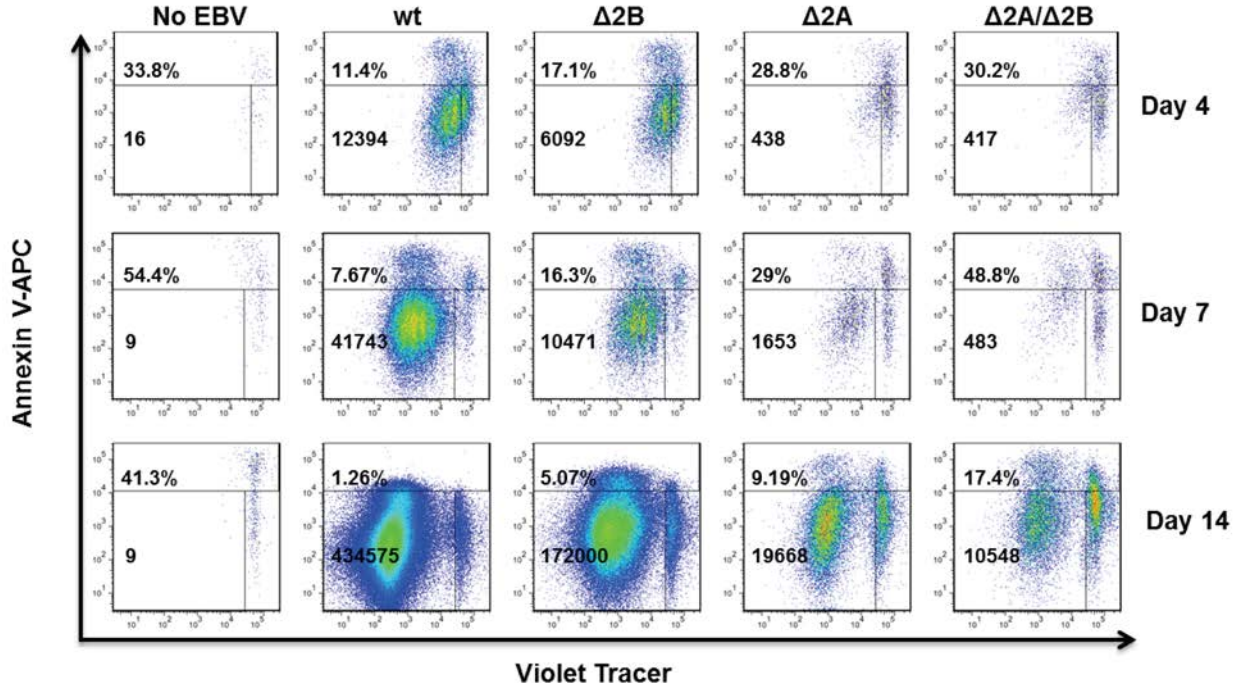

Figure 17. Efficient proliferation and survival of EBV-infected B cells requires LMP2.

Proliferation is most efficient when both LMP2A and LMP2B are present, with apoptosis rates at the lowest level. Loss of LMP2A expression corresponds with the most dramatic decrease in B cell proliferation efficiency and highest percentage of apoptotic cells. LMP2B may have a minimal role in proliferation and survival, which is reinforced by the additive effect observed in LMP2 double knockout virus infections; loss of both isoforms corresponds to the lowest proliferation rates and highest apoptosis rates. The rate of apoptosis decreased over time, with highest rates in the first week of infection.

The effects of LMP2 on apoptosis are inversely correlated with proliferation kinetics. Specifically, higher efficiency of proliferation corresponded to lower percentages of apoptotic cells **(Figure 17)**. Wild-type virus-infected B cells showed the lowest percentage of apoptotic B cells, while Δ2B virus infection exhibited the second lowest percentage of apoptotic B cells. The loss of LMP2A expression corresponded with the highest amount of apoptotic B cells compared

to wild-type and Δ2B virus-infected B cells. However, loss of both LMP2A and LMP2B demonstrated the highest amount of apoptosis, with the most dramatic increases associated with 4 and 7 days post-infection.

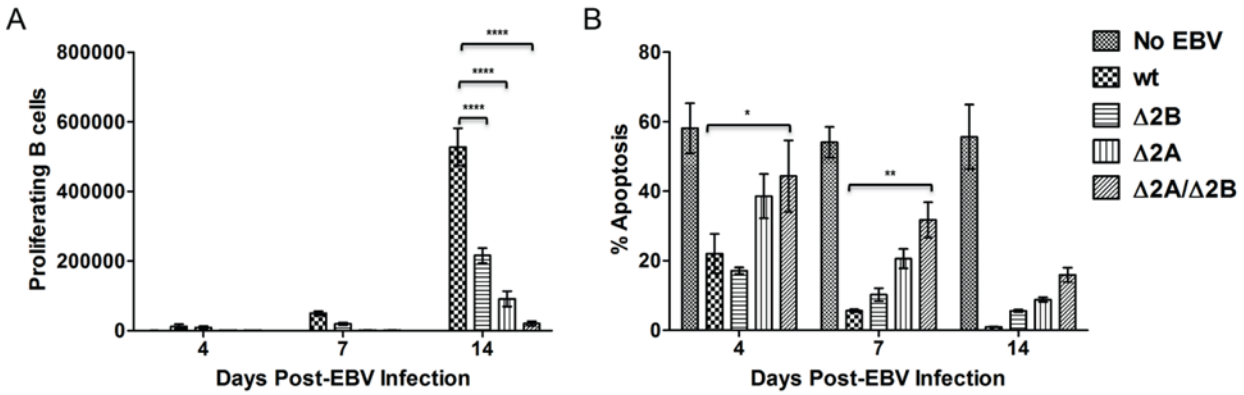

Figure 18. Analysis of 3 donors demonstrates effects of LMP2 on proliferation and apoptosis.

(A) Proliferation and (B) Apoptosis data points are an average of 3 independent experiments ± SEM. Proliferation and apoptosis rates are inversely correlated. Proliferation rates are highest for wild-type infection and lowest for LMP2 double knockout infection, while percentage of apoptotic cells highest for LMP2 double knockout and lowest for wild-type infection. Statistical significance was determined using a Two-Way ANOVA test and Bonferroni Post-test compared to wild-type. P-value (*) < 0.05, p-value (**) < 0.01 and p-value (****) < 0.0001.

The absolute number of proliferating wild-type-infected B cells was ≥2x the number of proliferating B cells in Δ2B wells (p-value < 0.0001), and ≥5x and ≥10x the number of proliferating B cells in Δ2A (p-value < 0.0001) and Δ2A/Δ2B (p-value < 0.0001) wells, respectively **(Figure 18A)**. Measuring absolute numbers of proliferating B cells demonstrated a significant difference in LMP2 KO virus-infected proliferating B cells compared to wild-type-infected cells. We next compared these proliferation results to the rates of apoptosis detected in the three separate trials. Wild-type-infected B cells exhibited the lowest percentage of apoptotic B cells **(Figure 18B)**, Δ2B virus-infected B cells had slightly higher levels of apoptosis

compared to wild-type virus-infected B cells **(Figure 18B)**, while Δ2A virus-infected B cells consistently had much higher levels of apoptosis compared to both wild-type and Δ2B viruses, though this difference was not statistically significant. Deletion of both LMP2 isoforms resulted in the highest levels of apoptosis at 4 (p-value < 0.05) and 7 (p-value < 0.01) days post-infection **(Figure 18B)** and lowest levels of proliferation **(Figure 18A)**. Together, the data indicate that there is an inverse relationship between the amount of apoptosis and proliferation occurring in the virus-infected populations, and we can conclude that LMP2A promotes the survival of EBV-infected B cells, particularly within the first week of infection. LMP2B alone did not significantly affect B cell survival, but removal of both protein isoforms appears to increase the percentage of apoptotic B cells. Therefore, it is possible that LMP2B can influence anti-apoptotic activities in infected cells, possibly through interactions with signaling proteins like LMP2A and CD19, which has been shown to promote B cell survival when in a complex with CD21 [232].

5.0 SPECIFIC AIM TWO: DETERMINE THE ROLES FOR LMP2A AND LMP2B IN THE FORMATION OF LYMPHOBLASTOID CELL LINES *IN VITRO*

5.1 ABSTRACT

EBV has been associated with the development of several human malignancies, such as Burkitt's lymphoma, Hodgkin's lymphoma and nasopharyngeal carcinoma. The transformative capabilities of the virus have been utilized *in vitro*, where infection of primary B cells leads to continuous proliferation and establishment of LCLs. The role of the LMP2 gene in this process is controversial. Several studies have claimed that the gene is dispensable for immortalization, while others have suggested that the gene plays an essential role in the process. These studies have utilized techniques that resulted in either wild-type virus contamination of mutant virus supernatants, or the use of incomplete genomes (mini-EBV), which only included expression of genes necessary for immortalization. Both of these experimental approaches created difficult models for studying the role of LMP2 in immortalization. Importantly, our study uses complete recombinant EBV with minimal deletions in the LMP2 region that can be produced in the absence of helper virus, thereby eliminating wild-type virus contamination.

In this study, we have used recombinant EBV with deletions in the LMP2 region to study the role of this gene in B cell immortalization. We used a classical immortalization assay, which includes infection of PBMCs with the viruses, as well as infection of purified B cells. For

classical immortalization assays, PBMCs were infected with recombinant EBV at an MOI of 3. The addition of 1 μg/ml CyA inhibited T cell responses that could occur in the presence of viral proteins. Infection of primary B cells was performed with recombinant viruses at an MOI of 1. CyA was not added in this experiment because T cells were removed during negative selection. Outgrowth assays were initiated in 96-well plates and observed by inverted fluorescent microscopy for cell clumping (activation) and proliferation. B cell proliferation was observed for 6-12 weeks post-infection. Establishment of LCLs was determined by the accumulation of continuously proliferating cells that required transfer to 25 cm^2 flasks, which usually occurs within 6 weeks of infection.

PBMC experiments resulted in frequent LCL formation for wild-type virus, whereas Δ2B virus infection rarely resulted in establishment of LCLs. Infection with Δ2A and Δ2A/Δ2B viruses did not establish LCLs for any of the six donors. In contrast, infection of purified B cells with wild-type and Δ2B viruses resulted in establishment of LCLs for all six donors. Also, Δ2A virus infection of purified B cells resulted in the production of LCLs for three of the six donors. However, two of those LCLs displayed extremely slow growth kinetics, requiring 12-14 weeks for establishment. The Δ2A/Δ2B virus was the least capable of the mutant viruses to produce LCLs. Only one LCL was produced from the six donors for the Δ2A/Δ2B virus, which also showed slow growth kinetics that required 12-14 weeks for establishment.

5.2 RESULTS

5.2.1 PBMC immortalization requires LMP2A and LMP2B

The classical immortalization assay involves infection of PBMCs with EBV to determine efficiency of outgrowth. Therefore, PBMCs were harvested from buffy coats of healthy donors. $5x10^4$ cells were seeded in wells of a 96-well plate, infected with wild-type and LMP2 KO viruses at an MOI of 3 and monitored for 6 weeks. CyA was added to the culture for the first 2 weeks of infection to inhibit T cell activity that could adversely affect EBV-infected B cells [233]. B cells infected *in vitro* express all nine latent genes, which include the EBNA3 proteins that are primary antigenic targets of CTLs [121]. EGFP expression was observed at ~3-4 days post-infection. Around the same time, small clumps of EGFP+ cells were observed for all virus wells. The presence of cell clumping is indicative of cell activation, which causes upregulation of adhesion molecule expression [224]. Overall there were fewer and smaller cell clumps in Δ2A and Δ2A/Δ2B virus wells, which most likely indicate inefficient activation.

After 9-days incubation, infected B cells were observed for signs of proliferation **(Figure 19)**. Small clumps of proliferating cells were evident in wild-type and Δ2B virus wells. In contrast, proliferation of infected cells was not evident in Δ2A and Δ2A/Δ2B wells at this time. At 17 days post-infection, similar proliferation rates were observed **(Figure 20)**. Wild-type virus-infected cells continued to proliferate at 17 days post-infection, but the rate of proliferation of Δ2B-infected cells appeared to decrease. This was evident by the continued presence of small clumps that did not appear to grow in size. Also, the number of clumps decreased in Δ2B wells

by this time post-infection. Proliferation of Δ2A and Δ2A/Δ2B virus-infected B cells was not observed, though EGFP expression was detected. The B cell clumps in these wells were small, comprised of only 2-3 cells in most cases, and did not appear to grow in size. By 30 days post-infection **(Figure 19)** (~4-5 weeks), it is clear that wild-type virus-infected B cells will establish LCLs, which is evident by observed high levels of proliferation. It is also apparent at this time point that PBMCs infected with LMP2 KO viruses would not establish LCLs. Day-by-day observations of the PBMC immortalization experiment are shown in **Table 6**. Also, **Table 7** shows a summary of the data obtained in microscopy experiments.

Table 6. Daily observations for PBMC infection with wild-type and LMP2 KO viruses

Day	Wt	Δ2B	Δ2A	Δ2A/Δ2B
3-4	Single, green cells	Single, green cells	Single, green cells	Single, green cells
6	Clumps of 20-40 cells	Clumps of 3-10 cells	Clumps of 2-5 cells	Clumps of 2-5 cells
9	Clumps of 50-100 cells	Clumps of 40-60 cells	Clumps of 20-40 cells	Clumps of 10-20 cells
12	Mostly clumps of >100 cells	Mostly clumps of 20-40 cells; a few 50-100 cells	Mostly clumps 5-10 cells; some 10-20 cells	Clumps of 5-10 cells
17	Mostly clumps of >100 cells	Mostly clumps of 20-40 cells	Mostly clumps 5-10 cells; some 10-20 cells	Mostly single cells; some clumps of 5-10 cells
20	Mostly clumps of >100 cells	Mostly clumps of 10-20 cells	Mostly clumps 5-10 cells; some 10-20 cells	Mostly single cells; some clumps of 5-10 cells
23	Mostly clumps of >100 cells, very large clumps	Mostly clumps 5-10 cells, a few 10-20 cells	Mostly clumps 5-10, a few 10-20 cells	Mostly single cells
30	Mostly clumps of >100 cells, very large clumps	Clumps of 5-10 cells, cells look sickly	Mostly single cells, some clumps of 5-10 cells	Mostly single cells

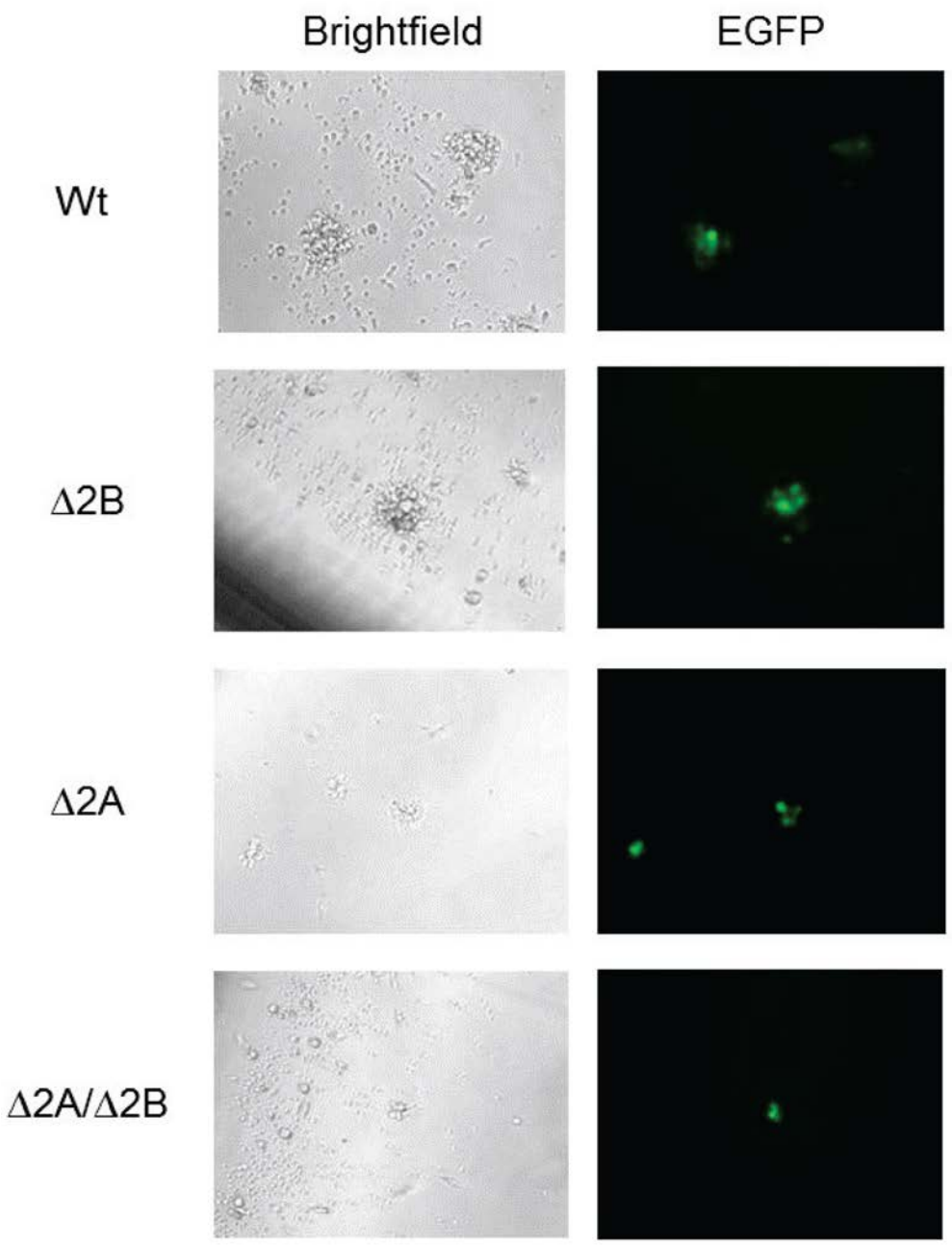

Figure 19. Immortalization of PBMC infected with wild-type and LMP2 KO viruses Day 9.

Within one week of infection both EGFP expression and cell clumping are evident in all virus-infected wells. Proliferation appeared to occur in wild-type and Δ2B virus wells, but did not occur in Δ2A and Δ2A/Δ2B wells.

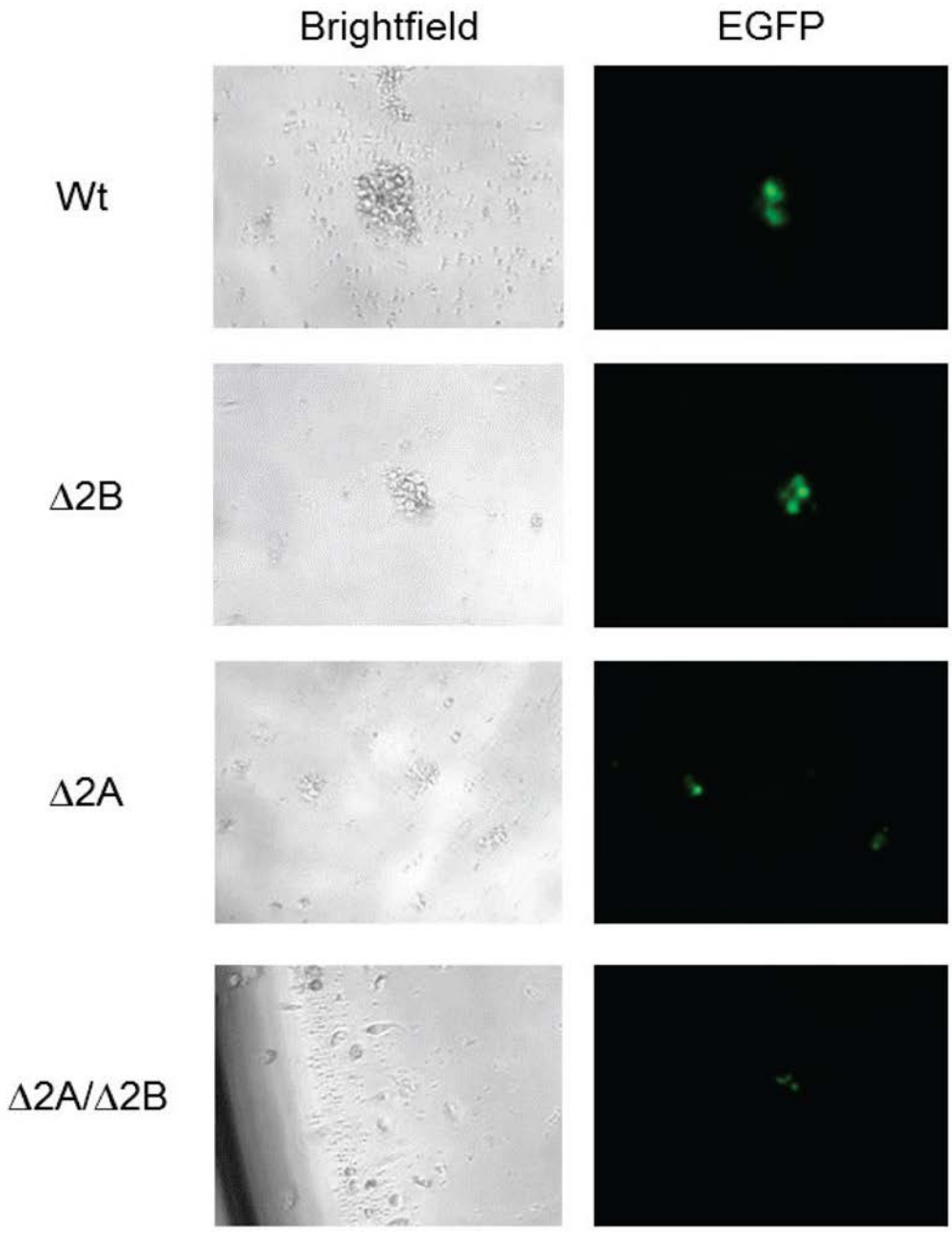

Figure 20. Immortalization of PBMC infected with wild-type and LMP2 KO viruses Day 17.

After two-weeks infected cells continued to proliferate in wild-type infected wells. Proliferation in Δ2B virus wells appeared to decrease, emphasized by observations of smaller clumps within the infected wells. Δ2A and Δ2A/Δ2B wells continued to show little, if any, proliferation.

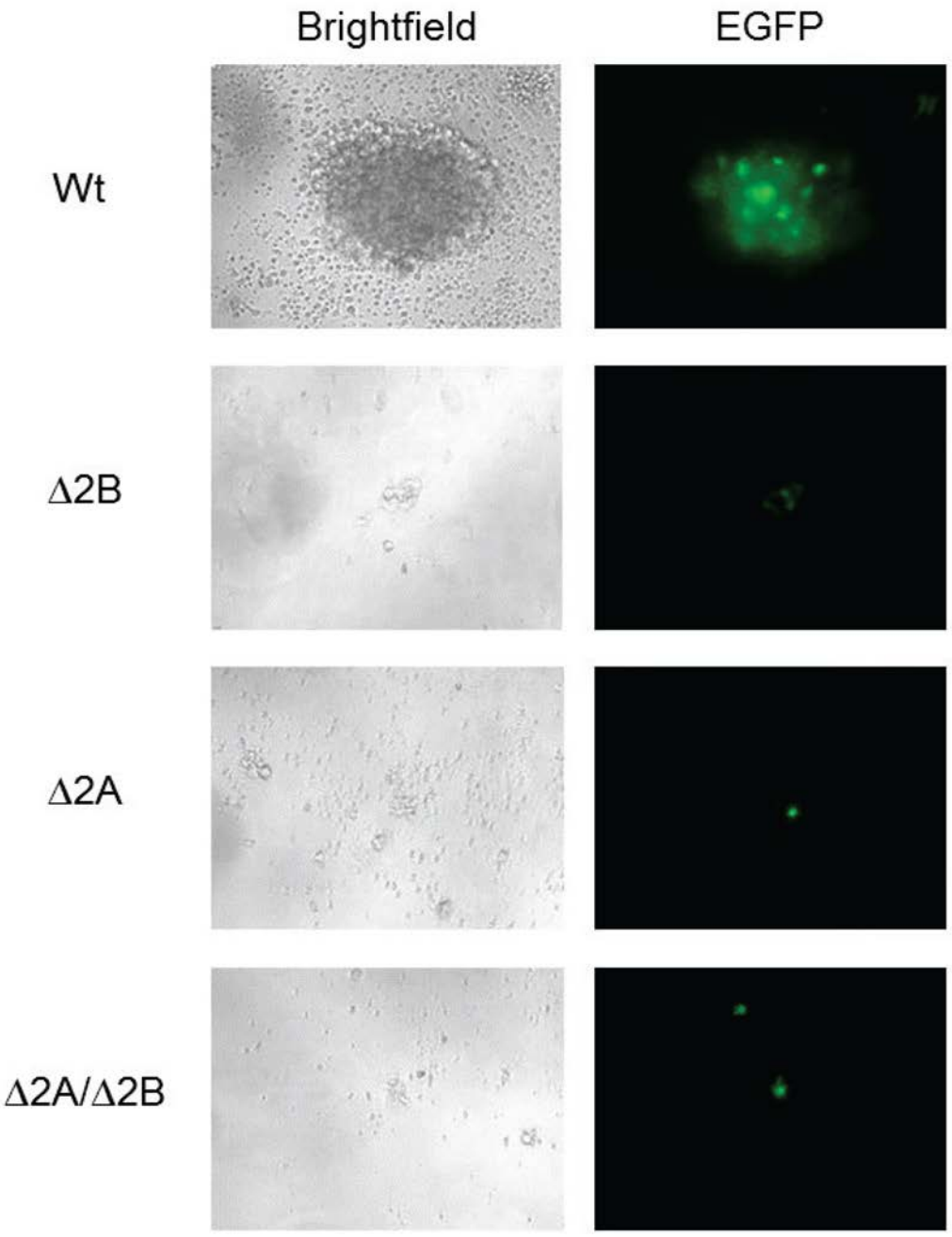

Figure 21. Immortalization of PBMC infected with wild-type and LMP2 KO viruses Day 30.

4-5 weeks after infection, wild-type infected cells continue to proliferate and will most likely establish LCLs. However, cells infected with Δ2B, Δ2A and Δ2A/Δ2B viruses do not appear to proliferate at this time. There is little chance of establishing LCLs from any of the LMP2 KO virus-infected wells.

Table 7. Summary of results from PBMC immortalization experiment

EBV	Infectious Titer/ml	Genome copy#/GIU	EGFP Expression	Activation (clumping)	Established cell lines
Wt	1.9×10^6	23	3-4 days	4 days	Yes
Δ2B	4.8×10^6	13	3-4 days	4-5 days	Not likely
Δ2A	1×10^6	6.2	3-4 days	5-6 days	No
Δ2A/Δ2B	8.8×10^5	1.5	3-4 days	6 days	No

The immortalization experiment was repeated using PBMCs from six healthy donors to determine the efficiency of B cell transformation by LMP2 KO viruses. As expected, infection of PBMCs with wild-type virus resulted in efficient establishment of cell lines for all six donors. In contrast, PBMCs infected with Δ2B virus only yielded LCLs for one donor. Δ2A and Δ2A/Δ2B viruses were incapable of producing LCLs from infected PBMCs, even when the cells were infected with a large amount of virus (MOI 3). This data is shown in **Table 8**. Therefore, our data suggests that both LMP2A and LMP2B are needed for establishment of LCLs from PBMCs.

Table 8. Establishment of LCLs from PBMC infection requires LMP2A and LMP2B

	Wild-type	Δ2B	Δ2A	Δ2A/Δ2B
Donor 1	+	-	-	-
Donor 2	+	-	-	-
Donor 3	+	-	-	-
Donor 4	+	+	-	-
Donor 5	+	-	-	-
Donor 6	+	-	-	-

(+) indicates LCLs were established for that donor with that specific virus within 6 weeks.
(-) indicates that LCLs were not established within the 6 weeks observation period

5.2.2 Efficient immortalization of purified B cells requires LMP2A

Since early infection kinetics studies were performed using purified B cells, we wanted to also determine the effect of the LMP2 deletions on immortalization of purified B cells. Additionally, early infection studies showed that wild-type and Δ2B viruses induced activation and proliferation similarly, though classical immortalization assays of PBMCs revealed an inability of Δ2B viruses to establish LCLs. Immortalization experiments using purified B cells would allow us to study the ability of these mutant viruses to induce immortalization without the inconvenience of T cell responses and possible adverse effects from addition of CyA. Therefore, B cells were purified with negative selection, which avoids engagement of CD19, a member of the BCR co-receptor complex, by antibody-conjugated beads and possible unwanted background activation. For this experiment, B cells were infected with wild-type and LMP2 KO viruses at an MOI of 1 and observed for 12-14 weeks for sustained proliferation. Initial infection was performed in 96-well plates with 50,000 B cells per well.

Observations at 5 days post-infection revealed the presence of EGFP+ cell clumps **(Figure 22)**. The presence of larger clumps in wild-type and Δ2B virus wells possibly indicates increased activation as well as initiation of proliferation at this time. B cells infected with Δ2A and Δ2A/Δ2B viruses expressed EGFP, but were generally associated with smaller clumps of approximately 2-5 B cells. This closely mirrored observations in PBMC immortalization experiments at early time points **(Figure 19)**. However, proliferation of purified, infected B cells occurred more rapidly compared to B cells infected in the presence of PBMCs.

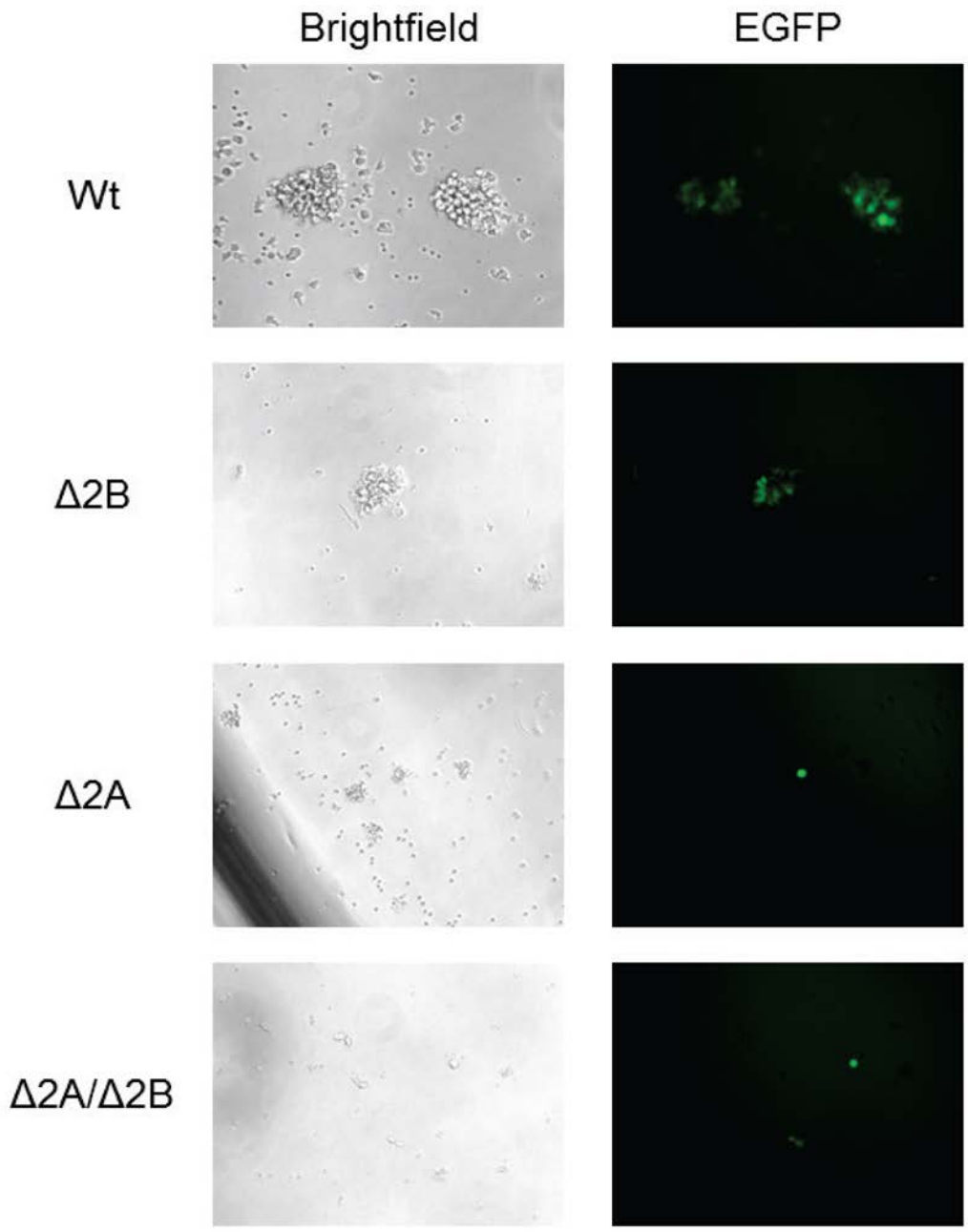

Figure 22. Immortalization of B cells infected with wild-type and LMP2 KO viruses Day 5.

Infected B cells expressed EGFP and appeared to form small clumps. The larger clumps were observed in wild-type virus wells, and most likely indicated initiation of proliferation. EGFP+ clumps were also observed in Δ2B wells. Δ2A and Δ2A/Δ2B viruses produced the smallest clumps of EGFP+ cells, which possibly indicated a lack of proliferation initiation at this time.

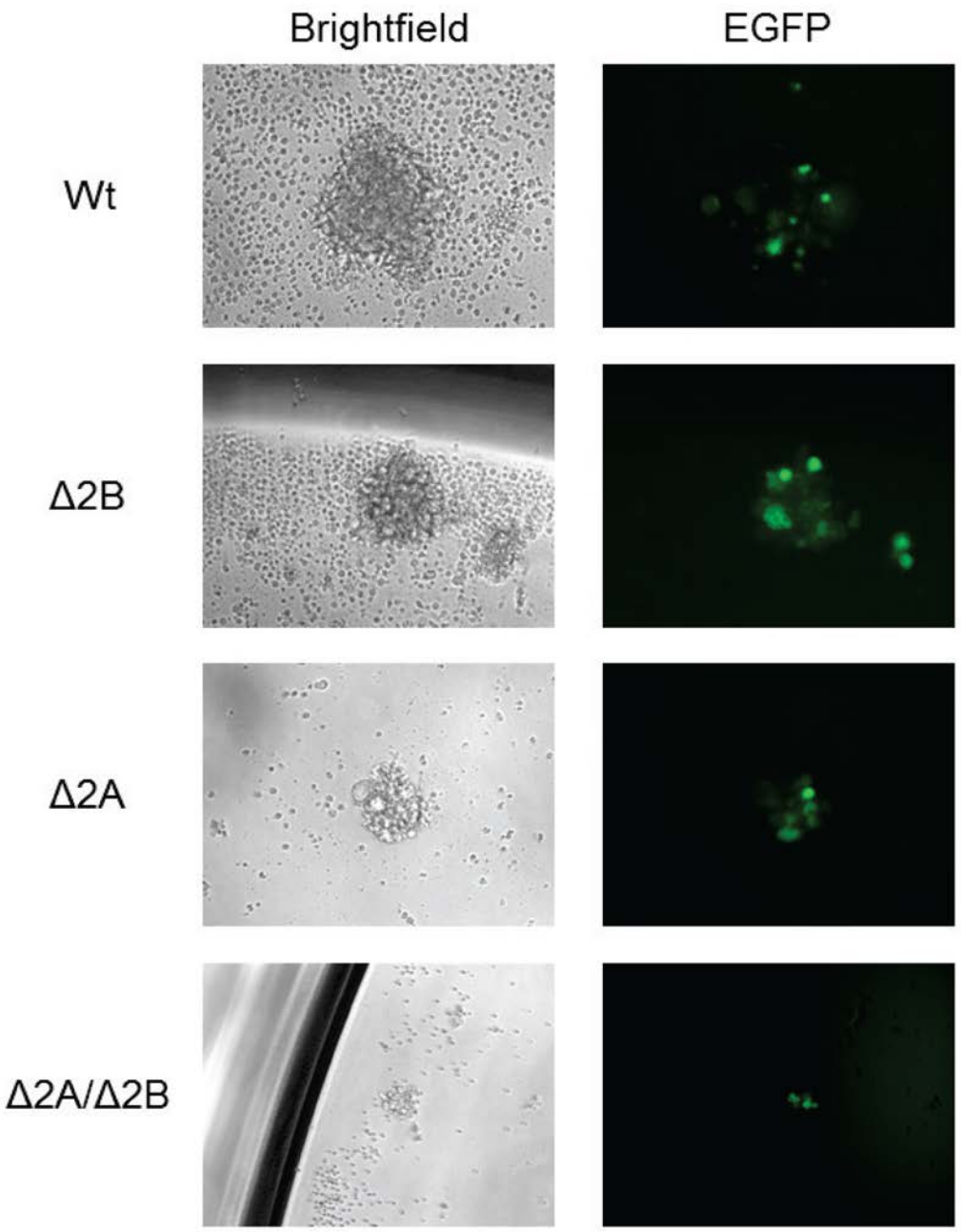

Figure 23. Immortalization of B cells infected with wild-type and LMP2 KO viruses Day 14.

After two-weeks infected B cells continued to proliferate in wild-type and Δ2B virus-infected wells. Infection with wild-type virus results in the highest rates of proliferation, evident from the high number of large clumps. Proliferation in Δ2A and Δ2A/Δ2B virus wells is slower, with fewer and smaller cell clumps in each well.

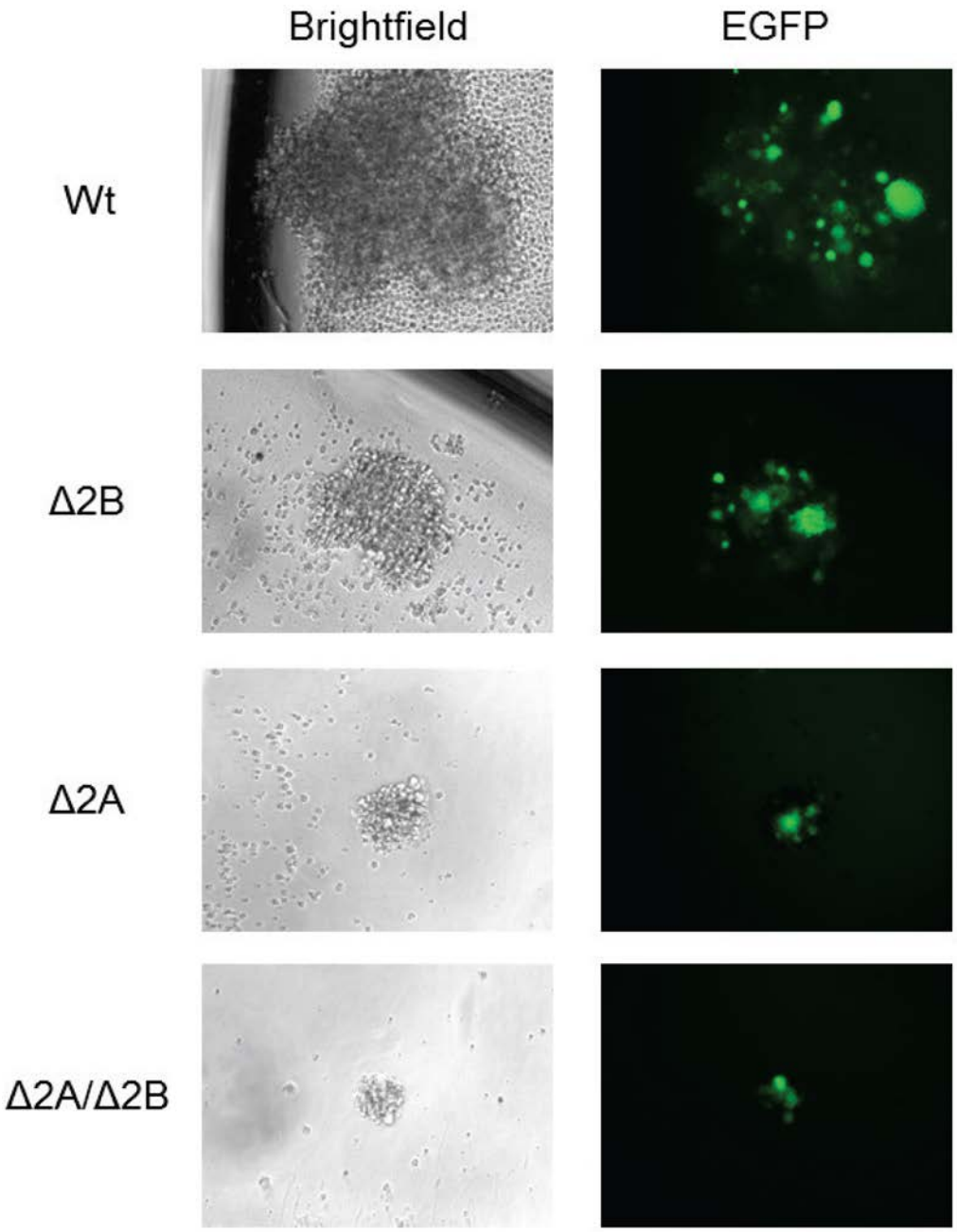

Figure 24. Immortalization of B cells infected with wild-type and LMP2 KO viruses Day 26.

After four-weeks infected B cells continued to proliferate in wild-type and Δ2B virus-infected wells. Infection with wild-type virus results in the highest rates of proliferation, evident from the high number of large clumps. Establishment of LCLs from wild-type and Δ2B wells is expected. Proliferation in Δ2A and Δ2A/Δ2B virus wells is slower, with fewer and smaller cell clumps in each well. Establishment of LCLs is possible, for Δ2A virus wells, but will most likely not occur for Δ2A/Δ2B virus wells**.** Again, the presence of EGFP+ and EGFP- cells in each clump indicates growth of uninfected B cells in these cultures, possibly due to secreted growth factors by infected B cells.

Table 9. Daily observations for purified B cells infected with wild-type and LMP2 KO viruses

Day	Wt	Δ2B	Δ2A	Δ2A/Δ2B
3-4	5-10 cells clumps, green cells	Small clumps, green cells	Single, green cells	Single, green cells
5	10-20 cell clumps	5-10 cell clumps	Small clumps	Mostly single cells
9	20-40 cell clumps	10-20 cell clumps	5-10 cell clumps	Small clumps
14	50-100 cell clumps	20-40 cell clumps	10-20 cell clumps	5-10 cell clumps
17	>100 cell clumps	50-100 cell clumps	10-20 cell clumps	10-20 cell clumps
20	>100 cell clumps	>100 cell clumps	10-20 cell clumps	10-20 cell clumps
23	>100 cell clumps, very large clumps	>100 cell clumps	20-40 cell clumps	10-20 cell clumps
26	>100 cell clumps, very large clumps	>100 cell clumps, a few very large clumps	20-40 cell clumps	Mostly 10-20 cell clumps, a few 20-40 cell clumps

By 14 days post-infection, purified B cells infected with wild-type and Δ2B viruses had grown into large clumps, though the most rigorous proliferation occurred in B cells infected by wild-type EBV **(Figure 23)**. At the same time, B cells infected with Δ2A and Δ2A/Δ2B viruses coalesced into small clumps of cells that were EGFP+. High proliferation rates for wild-type and Δ2B virus-infected B cells continued by 3-4 weeks post-infection **(Figure 24)**, and it is clear that these cells will continue to grow and establish LCLs. The proliferation of B cells infected with Δ2A and Δ2A/Δ2B viruses continued to lag behind rates observed for wild-type and Δ2B viruses by 3-4 weeks post-infection. At this point, continuous proliferation is observed at lower rates for Δ2A-virus infected B cells compared to wild-type virus-infected B cells, but it is not clear if establishment of LCLs will occur. The least amount of proliferation was observed for Δ2A/Δ2B

virus-infected B cells, with changes in B cell morphology consistent with loss of cell viability. **Table 9** describes observations at each time point during the experiment, and **Table 10** summarizes the data obtained from live images.

In order to determine the ability of LMP2 KO viruses to induce immortalization of purified B cells *in vitro*, the immortalization experiment (described above) was repeated using B cells from six healthy donors. The immortalization of infected B cells was determined by the accumulation of continuously proliferating B cells that require final transfer to 25 cm^2 flasks, indicating the establishment of cell lines. This experiment was monitored for a total of 12-14 weeks, a time period that was adjusted due to observations of delayed proliferation kinetics for B cells infected with Δ2A and Δ2A/Δ2B viruses. Incubation of only 5-6 weeks could eliminate any possible LCLs obtained by infection with these two viruses. All LCL data for the purified B cell experiment are represented in **Table 11**.

Table 10. Summary of results from purified B cell immortalization experiment

EBV	Infectious Titer/ml	Genome copy#/GIU	EGFP Expression	Activation (clumping)	Established cell lines
Wt	4.1 x 10^6	24.6	3-4 days	4 days	Yes
Δ2B	2 x 10^6	39	3-4 days	4 days	Yes
Δ2A	2 x 10^6	56.9	3-4 days	5 days	Not likely
Δ2A/Δ2B	2 x 10^6	115	3-4 days	6 days	Not likely

Wild-type infected B cells from all six donors established LCLs within 4-5 weeks after initial infection, which was similar to results observed for PBMC infection with this virus. The rate of LCL establishment was slightly faster for purified B cells at approximately 4-5 weeks post-infection, while this process averaged approximately 5-6 weeks for infected PBMCs. In

contrast to PBMC infections, purified B cells infected with Δ2B virus were able to establish LCLs. In fact, LCLs from Δ2B virus infections were established for B cells from all six donors within approximately 5 weeks. Infection with Δ2A virus resulted B cell proliferation, but continuous outgrowth and subsequent LCL formation were only observed for B cells from 3 of the 6 donors. However, it is important to note that two of these LCLs required a substantial amount of time for establishment (12-14 weeks), which is approximately 3 times longer than the amount of time required for outgrowth of B cells infected with wild-type and Δ2B viruses. Only one LCL was established from Δ2A/Δ2B virus infection, and this LCL, like two of those established for Δ2A virus, required 12-14 weeks for outgrowth and transfer to 25 cm^2 flasks.

Table 11. Efficient establishment of LCLs from purified B cells requires LMP2A

	Wt	Δ2B	Δ2A	Δ2A/Δ2B
Donor 1	+	+	+*	+*
Donor 2	+	+	+*	-
Donor 3	+	+	-	-
Donor 4	+	+	-	-
Donor 5	+	+	+	-
Donor 6	+	+	-	-

(+) indicates establishment of LCL within 5-6 weeks post-infection.
(-) indicates that LCLs were not established within 12-14 weeks post-infection
(+*) indicates that LCLs were established, but the length of time required was the total length of the experiment equivalent to 12-14 weeks.

Taken together, these results indicate that the inclusion of PBMCs in cell cultures leads to differing requirements for LMP2A, and especially LMP2B in long-term outgrowth of infected B cells. The presence of T cells, macrophages and other cells in PBMCs could inhibit outgrowth of LMP2 KO virus-infected B cells by the secretion of inhibitory cytokines or interactions with PD-1 on activated B cells. It is possible that LMP2B might negatively regulate expression of the PD-1 receptor on infected B cells, resulting in higher expression of PD-1 on the surface of Δ2B-infected B cells and subsequent increased susceptibility of those B cells to immune suppressive actions of T cells and macrophages in those cultures. This could explain the disparity in the ability of Δ2B viruses to promote long-term growth of B cells in the presence or absence of PBMCs.

6.0 SPECIFIC AIM THREE: ESTABLISH THE ROLES FOR LMP2A AND LMP2B IN REGULATION OF VIRAL GENE EXPRESSION AND LATENCY IN EARLY INFECTION

6.1 ABSTRACT

The maintenance and stability of viral latency has previously been described as a major function of the EBV-associated latency protein, LMP2A, which is accomplished via negative regulation of BCR signaling. LMP2A localizes to lipid rafts in the plasma membrane of infected cells, resulting in physical blockage of BCR entry to these locations. The BCR requires localization in lipid rafts for initiation of signaling pathways; crosslinking of the BCR by antigen binding and interactions with scr-family kinases occur within the regions of lipid rafts in the plasma membrane. Another mechanism of LMP2A-associated inactivation of BCR signaling involves Nedd4 ubiquitinase. LMP2A interacts with Nedd4 via poly-proline motif in the CSD and targets the src-family kinases, Lyn and Syk, for ubiquitylation and subsequent degradation by the proteasome. Inactivation of BCR signaling is paramount to the maintenance of viral latency and inhibition of the switch to the viral lytic cycle. Other studies have suggested an additional regulatory role for LMP2A in the control of latent gene.

In contrast, previous studies have shown that LMP2B is a regulator of the switch to the lytic cycle, which is accomplished by negative regulation of LMP2A. This is an intriguing

possibility due to the extensive sequence homology shared between the two proteins. Specifically, all 12 TMs and a short C-terminal tail are identical between LMP2A and LMP2B. The C-terminal tail has been hypothesized to act as an aggregation domain, which could represent a mechanism for LMP2B regulation of LMP2A activity. LMP2B has been shown to localize to intracellular perinuclear regions within infected cells. It is possible that LMP2B interacts with LMP2A through their C-terminal tails and sequesters the protein within these perinuclear regions, thereby inhibiting the ability of LMP2A to block BCR signaling, leading to lytic cycle induction.

In this study we have used our recombinant wild-type and LMP2 KO viruses (Figure 1, Table 4 & 5) to determine the effect of LMP2 deletions on the stability of latent gene expression and viral latency in early B cell infection. Our data suggests that neither LMP2A nor LMP2B play a role in the regulation of latent gene expression during early infection. Also, our Zebra expression data correlates with previous research that determined spontaneous lytic induction is rare in B cell infection *in vitro*. Interestingly, the loss of LMP2A did not correlate with elevated lytic switch during early infection, as we expected based on previous studies demonstrating a critical role for LMP2A in the maintenance of viral latency. Also, removal of LMP2B did not correlate with increased resistance to lytic cycle induction. Unexpectedly, the removal of both LMP2A and LMP2B demonstrated a significant drop in LMP1 expression and elevated Zebra expression, suggesting a possible compensatory role for LMP2A and LMP2B in the maintenance of viral latency in the absence of the other.

6.2 RESULTS

6.2.1 LMP2 does not affect latent gene expression

LMP2A has been described as an important protein for the maintenance of viral latency, possibly through direct regulation of cellular and EBV gene expression, such as EBNA2 [234]. During latency, EBV switches between two promoters, Cp and Wp, in order to tightly regulate latent gene expression [234]. In the initial stages of B cell infection, EBV utilizes the Wp promoter for the expression of EBNA2 and EBNA-LP [235], which also drives expression of the LMP1 and LMP2 proteins, EBNA proteins and cellular proteins associated with B cell activation and proliferation [236,237]. Transcription of EBNA2 and EBNA-LP from the Wp promoter is upregulated by the B cell specific transcription factor Pax-5. However, it has been shown that the presence of LMP2A expression correlates with downregulation of the activity of this transcription factor [238,239], and possibly EBNA2 expression. Consequently, it is possible to hypothesize that LMP2A regulates expression of other latent genes to maintain the ideal cellular environment for viral latency.

LMP2B could indirectly modulate gene expression via interactions with signaling molecules, such as CD19 and LMP2A [174]. For these reasons, we hypothesized that loss of LMP2 could cause changes in gene expression that could affect the ability of EBV to induce efficient activation and proliferation of infected B cells, as well as possibly lead to instability of viral latency and subsequent loss of proliferation. We utilized our recombinant wild-type and

LMP2 KO viruses to investigate possible roles for LMP2A and LMP2B in the regulation of latent gene expression and viral latency.

Infected B cells were probed for EBNA1, EBNA2, LMP2A, LMP2B and LMP1. As expected, Δ2A and Δ2A/Δ2B virus-infected B cells were negative for LMP2A transcript, and Δ2B and Δ2A/Δ2B virus-infected B cells were negative for LMP2B transcript **(Figure 25)**. Wild-type and Δ2B viruses displayed similar levels of LMP2A expression over the course of the experiment, with slightly higher levels for Δ2B-infected B cells at the latest time point (168 hours post-infection). Δ2A virus-infected B cells also exhibited similar levels of LMP2B expression at early time points compared to wild-type, but demonstrated significantly higher expression at the latest time point (168 hour p-value < 0.0001).

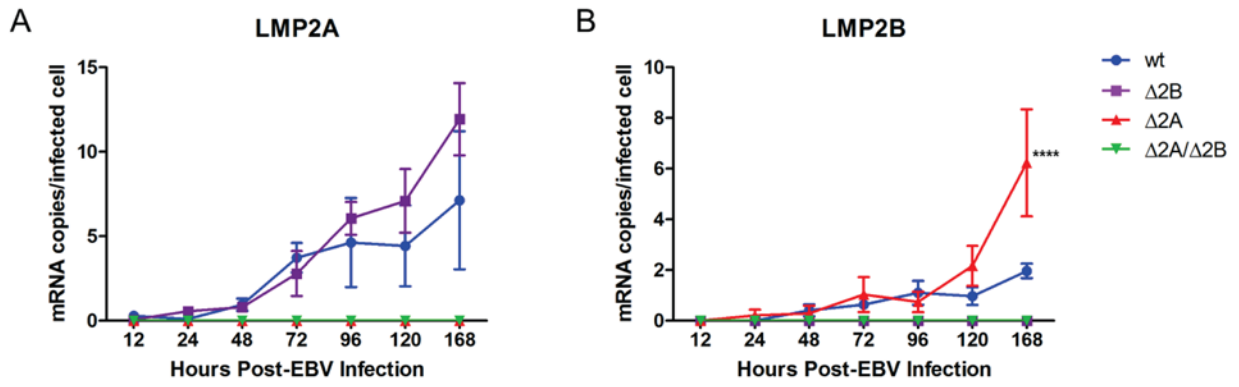

Figure 25. LMP2A and LMP2B transcription in wild-type and LMP2 KO virus infection.

(A) LMP2A and (B) LMP2B transcription not detected in the appropriate LMP2 KO virus-infected B cells. LMP2A transcript level is slightly elevated in Δ2B virus-infected B cells. LMP2B transcription is significantly elevated in Δ2A virus-infected B cells. mRNA per infected cell was determined by using the infection efficiency as a correction factor. Infection with wild-type and LMP2 KO viruses resulted in approximately 15-20% infection efficiency at 4 days post-infection. β_2Microglobulin mRNA was amplified for EBV gene normalization. Each data point is an average of three independent experiments ± SEM. Statistical significance determined using Two-way ANOVA and Bonferroni Post-test (each data point for LMP2 KO viruses was compared to wild-type to determine statistical significance). P-value (****) < 0.0001.

Wild-type and Δ2B virus-infected B cells exhibited similar expression levels of EBNA1, EBNA2 and LMP1 over the 1-week experimental period **(Figure 26)**. The trends in expression for these genes corresponded with previously published data for early wild-type EBV infection [209]. For instance, expression of EBNA1 was mostly stable with slight increases over the course of the week. EBNA2 expression was higher at early time points but trended toward a stable expression by 7 days post-infection. LMP1 levels were very low for the first 3 days of infection, but steadily increased over the last 4 days of the observation period. Importantly, LMP1 expression was comparable between wild-type and Δ2B virus-infected B cells, indicating that deletion of LMP2B Exon 1′ did not adversely affect the activity of the LMP1/LMP2B bidirectional promoter.

EBNA1 and EBNA2 expression in Δ2A and Δ2A/Δ2B virus-infected B cells were consistently lower, albeit not statistically significant, compared to wild-type infection at early time points, but became comparable to wild-type near the end of the observation period. Interestingly, EBNA2 expression was elevated for Δ2A virus-infected B cells compared to wild-type, but did not reach statistical significance. Expression of LMP1 was also lower in Δ2A and Δ2A/Δ2B virus-infected B cells throughout the 1-week period, but the difference in expression was not statistically significant. Importantly, LMP1 transcript levels increased throughout the experiment with kinetics similar to wild-type and Δ2B infections. There were no statistically significant differences in gene expression between wild-type and LMP2 KO virus infections **(Figure 26)**, except for increased LMP2B expression in Δ2A virus-infected B cells **(Figure 25)**. Therefore, these data suggest that the removal of LMP2A and LMP2B does not affect expression of other EBV latent genes, with the exception of increased LMP2B transcription in the absence of LMP2A.

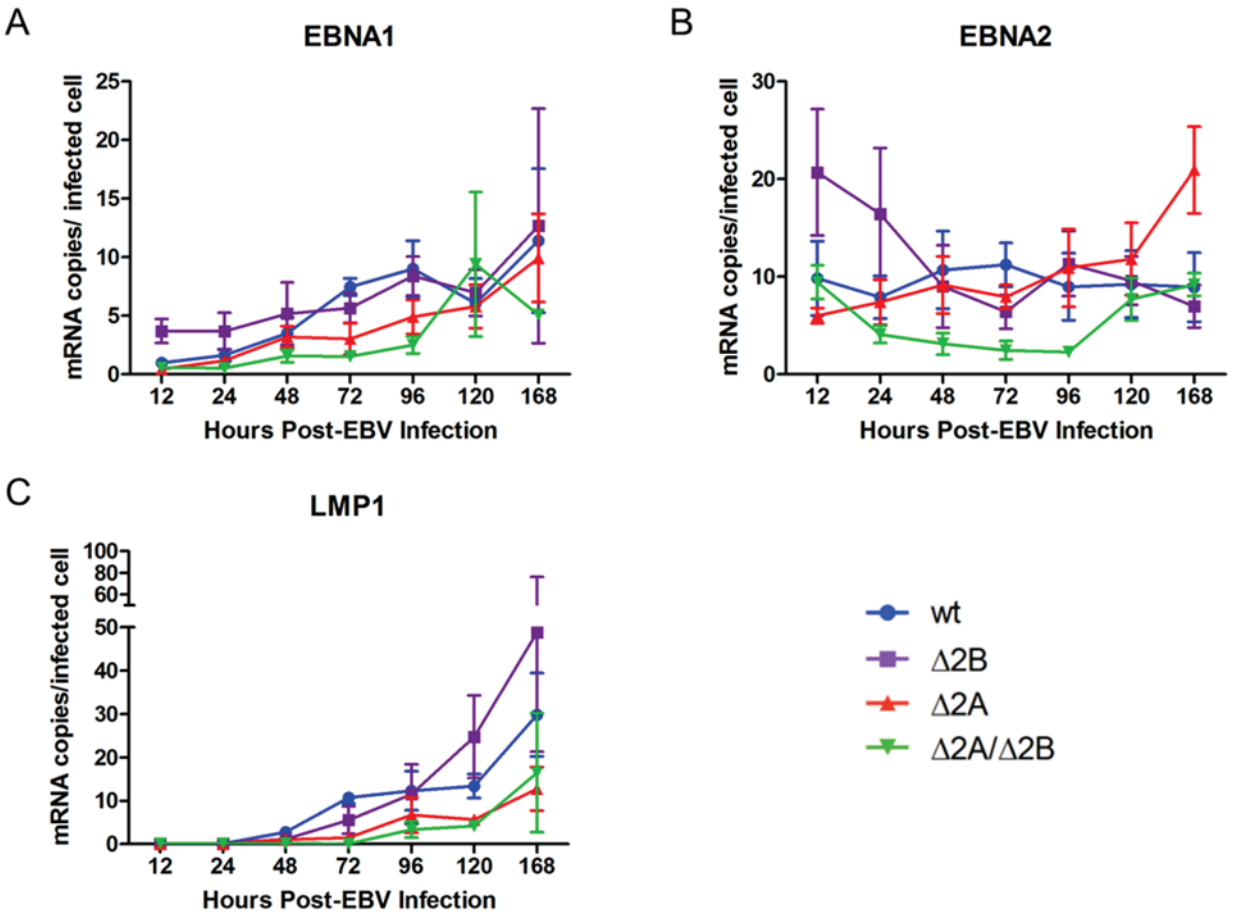

Figure 26. Latent gene transcription in wild-type and LMP2 KO virus-infected B cells.

(A) EBNA1, (B) EBNA2 and (C) LMP1 transcription levels were similar between wild-type and LMP2 KO virus-infected B cells. β_2Microglobulin mRNA was amplified for EBV gene normalization. Each data point is an average of three independent experiments ± SEM. Statistical significance determined using Two-way ANOVA and Bonferroni Post-test (each data point for LMP2 KO viruses was compared to wild-type infection to determine statistical significance).

6.2.2 LMP2 does not alter spontaneous lytic gene expression

In order to determine the necessity for LMP2A and LMP2B in viral latency during early infection, infected B cells were probed for changes in the expression levels of the immediate

early lytic transactivator BZLF1, or Zebra. As mentioned above, previous research has determined that LMP2A is a major player in the maintenance of viral latency via blocking of native signaling through the BCR [156-158]. Therefore, in the absence of LMP2A expression, we would expect to observe elevated Zebra expression, indicating possible initiation of a switch from latency to the lytic cycle. For LMP2B, previous research has indicated that this protein may also play a role in regulating viral latency, but the regulatory role involves control of the switch to the lytic cycle [170-172]. In this case, we reasoned that removal of LMP2B would increase resistance to lytic switch, and would, therefore, display lower levels of Zebra expression compared to wild-type infected B cells.

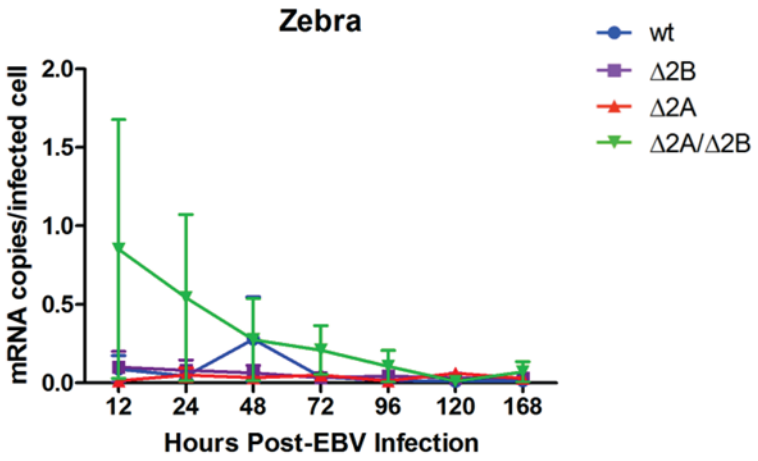

Figure 27. Lytic gene expression is very low during early infection.

Expression of the immediate early lytic transactivator gene, Zebra, was used to determine the amount of spontaneous lytic switch between wild-type and LMP2 KO viruses during early infection. The results indicate that only removal of both isoforms caused elevated Zebra expression, but these levels were not determined to be statistically significant by Two-way ANOVA**.**

In our study we found that neither removal of LMP2A nor LMP2B alone altered the expression of Zebra during early infection. Zebra expression in wild-type, Δ2B and Δ2A viruses were well below 1 copy/infected cell for all time points (~0.1 copies/cell), indicating lack of spontaneous lytic induction regardless of the presence or absence of LMP2 **(Figure 27)**. Interestingly, removal of both isoforms demonstrated slightly elevated Zebra expression during the earliest time points of the observation period (12-24 hours post-infection). However, this observed increase was not statistically significant (determined by Two-way ANOVA) due to significant variability in the measured mRNA values. The lack of spontaneous lytic induction in this setting corroborates previous studies that have shown a lack of appreciable lytic induction in directly infected B cells *in vitro* [157,158]. Additionally, we can conclude that LMP2A does not play a critical role in the maintenance of viral latency during early B cell infection. It is possible that established LCLs might be more sensitive to lytic switch in the absence of LMP2A, which could explain previous conclusions on this topic. We can also conclude, from our data, that LMP2B does not appear to regulate the switch to the lytic cycle during early infection.

6.2.3 BCR stimulation of wild-type and LMP2 KO virus-infected B cells

The low spontaneous Zebra expression levels suggested that very few virus-infected cells in any of the infected populations were undergoing lytic cycle induction, which is consistent with previous studies that showed lack of appreciable induction of the lytic cycle during early EBV infection *in vitro* [45,195]. However, it remained possible that sensitivity to a lytic inducing signal was altered in LMP2 KO viruses. Induction of the lytic cycle can be performed by a number of endogenous and exogenous stimuli. Many studies have used histone deacetylase

inhibitors, protein kinase C activators, azacytidine, unfolded protein response activators, as well as BCR crosslinking to induce the lytic cycle [187,188,240]. In this study, stimulation of BCR signaling was used to induce the lytic cycle in wild-type and LMP2 KO virus-infected B cells in order to assess the ability of LMP2 KO viruses to maintain viral latency. The latent status of the infected cells was determined via the processes of proliferation, apoptosis, as well as altered latent and lytic gene expression.

The stocks of wild-type and LMP2 KO viruses used in this study are described in **Table 5**. B cells purified by negative selection were infected with EBV at an MOI of 1. Stimulation of BCR signaling was accomplished via addition of 10μg/ml soluble immunoglobulin cocktail of IgM/IgG/IgA from Jackson Immunoresearch. The infected/stimulated cells were harvested at 4, 7 and 14 days post-infection for analysis. For proliferation and apoptosis studies, infected/BCR stimulated B cells were analyzed using the gating strategy described in **Figure 14**.

The effects of BCR stimulation on proliferation and apoptosis rates of wild-type and LMP2 KO virus-infected cells are shown in **Figure 28A and B**. BCR stimulation of wild-type infected B cells had a small but significant effect reducing the number of proliferating B cells (p-value < 0.05). The number of apoptotic B cells was unchanged by the addition of sIg, which suggests that the reduction in proliferation was not due to increased apoptosis caused by the stimulating reagent. It is possible that the observed decrease in proliferation after BCR stimulation could be due to increased induction of the lytic cycle. In contrast, addition of sIg to Δ2B-infected B cells significantly increased proliferation (p-value < 0.0001). Again, there were no significant changes in apoptosis throughout the experiment. BCR stimulation of LMP2A KO virus-infected B cells (Δ2A and Δ2A/Δ2B) did not significantly decrease the number of proliferating B cells or apoptotic B cells. It is possible that increased proliferation of Δ2B-

infected B cells following BCR stimulation could be due to resistance to lytic induction caused by the LMP2B deletion. Therefore, we believed that alterations in latent and lytic gene expression could reveal the underlying mechanism for this difference in proliferation in BCR stimulated Δ2B-infected B cells.

In order to determine changes in viral latency, we examined expression of LMP1 and Zebra transcripts during early infection **(Figure 27)**. LMP1 was used as an indicator of viral latency, as expression of this gene is essential for B cell proliferation in culture. Again, Zebra expression was used as an indicator of the lytic cycle. B cells were infected with wild-type and LMP2 KO viruses at an MOI of 1, and stimulated with 10µg/ml sIg.

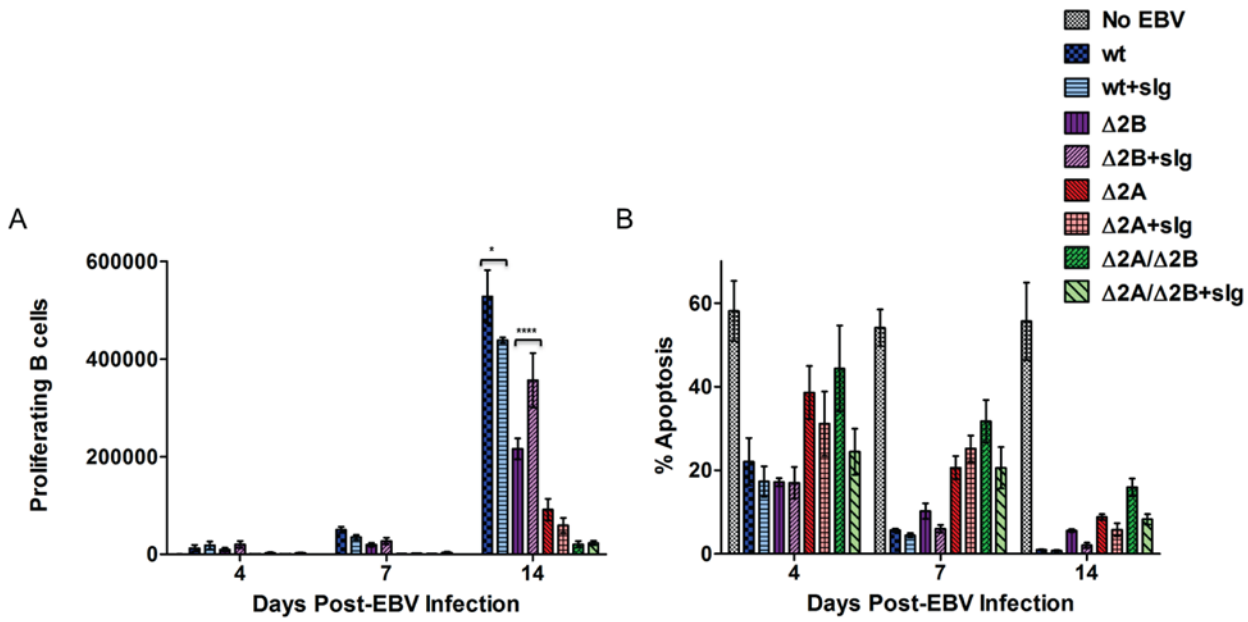

Figure 28. BCR stimulation effects on proliferation and apoptosis of wild-type and LMP2 KO virus-infected B cells.

Addition of sIg slightly decreased the number of proliferating B cells infected with wild-type virus. The proliferation of Δ2A and Δ2A/ΔB virus-infected B cells was affected by addition of sIg, while proliferation of Δ2B-infected B cells increased significantly. The numbers of apoptotic B cells was not affected by addition of the stimulating reagent. Statistical significance was determined by Two-way ANOVA, p-values obtained by Bonferroni Post-test comparison to virus alone. (p-value (*) < 0.05 and p-value (****) < 0.0001).

Stimulation of BCR signaling in wild-type-infected B cells did not decrease LMP1 expression **(Figure 29A)** or induce Zebra expression **(Figure 29B)**. This most likely indicates that latency was not disrupted in the wild-type infected B cells. Induction of the lytic cycle in wild-type EBV-infected B cells is difficult due to expression of the latency III program, in which all nine latency genes are expressed, including LMP2A. One explanation for the overall low Zebra expression levels is that only a small subset of infected B cells undergoes lytic cycle reactivation *in vitro*, which would skew the mRNA transcript/cell results. It is also possible that the strength of signals from latent genes, such as LMP1, EBNA2 and EBNA3C, during early infection is not easily overturned by lytic stimuli *in vitro*. LMP1 and Zebra expression levels were comparable between Δ2B and wild-type virus-infected B cells, which suggest that Δ2B-infected cells may not be more resistant to lytic induction.

LMP1 transcript levels were generally lower for Δ2A-infected cells compared to wild-type infection, but were only significantly lower at 168 hours post-infection (p-value < 0.05). However, Zebra expression remained low at less than 1 copy per cell, and did not exhibit significant differences when compared to wild-type virus. Therefore, we cannot conclude that B cells infected with Δ2A viruses are more sensitive to lytic induction than wild-type virus, which expresses LMP2A. Δ2A/Δ2B-infected B cells expressed significantly less LMP1 compared to wild-type infection (p-value 120 hours < 0.0001, p-value 168 hours < 0.001). Also, B cells infected with this virus expressed the highest level of Zebra transcript, though the difference is still not statistically significant when compared to wild-type virus. Therefore, removal of either LMP2A or LMP2B alone does not appear to significantly affect the ability of EBV to maintain latency following stimulation of BCR signaling. However, removal of both proteins significantly reduced expression of LMP1, possibly indicating instability in proliferative capacity.

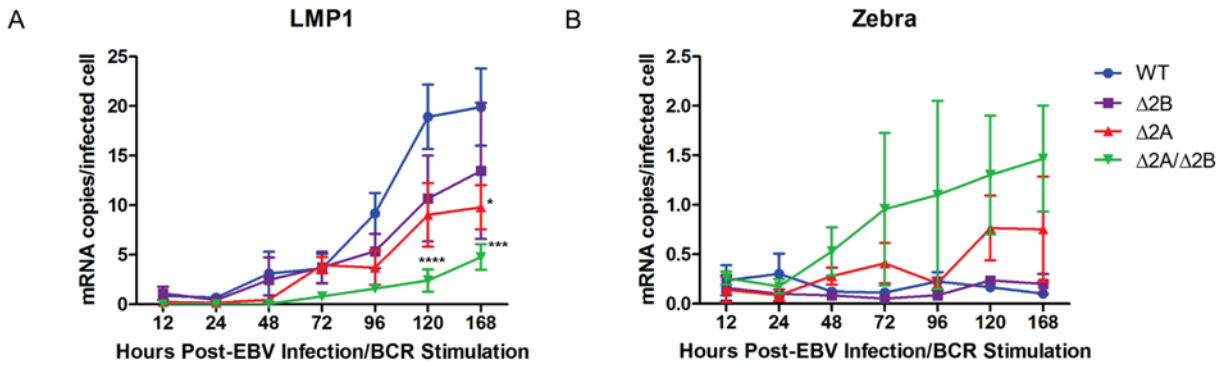

Figure 29. Latent and lytic gene expression following BCR stimulation.

B cells infected with wild-type and Δ2B viruses expressed similar levels of (A) LMP1 and (B) Zebra transcript. Δ2A virus infection did result in significantly lower LMP1 at 120 hours post-infection, but without appreciable changes in Zebra expression. The loss of both isoforms (Δ2A/Δ2B) resulted in significantly lower LMP1 expression with concomitant elevated Zebra. Data points are an average of 3 independent experiments ± SEM. Statistical significance determined using Two-way ANOVA, p-values determined using Bonferroni Post-test with comparison to wild-type.

7.0 DISCUSSION

7.1 COMPLETE RECOMBINANT EBV

In this study, we have demonstrated that expression of LMP2A augments early activation and proliferation of EBV-infected B cells. This appears to be critical for subsequent establishment of LCLs, since loss of LMP2A expression correlates with reduced LCL formation. Previous studies demonstrated the dispensability of LMP2A for establishment of LCLs [205-207], but did not include analyses of activation or proliferation in infected B cells during the early stages of infection. It is important to note that many of these previous studies utilized techniques that relied on recombination between mutated DNA and endogenous wild-type EBV. Production of virions within this system resulted in a highly heterogeneous population of both wild-type and LMP2 mutant EBV, which precluded clear assessments of the effects of LMP2 on B cell activation and proliferation from the time of initial infection.

Other studies have used mini-EBV plasmids, which are incomplete EBV genomes that express all latent genes known to be necessary for immortalization. With this system, the researchers were able to show that LMP2 was necessary for efficient immortalization of B cells [208]. However, it has been shown that mini-EBVs have an inherent high risk for introduction of small mutations in their sequences. This issue was exemplified by a report that showed spontaneous deletion of a cysteine residue in the EBNA3A ORF altered its function, which, in

turn, revealed an essential role for the gene in cell transformation [241]. Therefore, it was concluded that spontaneous mutation in other important genes for immortalization could have led to the observation that LMP2 is important for immortalization [207].

Our approach included the use of complete EBV genomes with deletions in the initiating exons of both LMP2A and LMP2B. Unlike previous studies, recombination between mutated DNA and wild-type EBV BACs with specific antibiotic resistance gene cassettes allowed us to procure EBV BACs with the preferred deletions without significant wild-type contamination. Also, sequencing analysis determined that no large deletions occurred outside the region of interest. Therefore our approach provides a more comprehensive system in which to investigate the roles of the LMP2 isoforms in the activation and proliferation events that occur shortly after B cell infection that, ultimately, lead to establishment of continuously proliferating lymphoblastoid cell lines.

7.2 EARLY INFECTION KINETICS WITH LMP2 KO VIRUSES

The underlying mechanisms of EBV persistence in humans and contribution to the development of cancer remain unclear. Among the viral genes expressed in LCLs, LMP2A and EBNA1 can be found in the majority of EBV-associated malignancies [2,64]. Additionally, LMP2B expression has been routinely found in NPC, and has been detected in other epithelial cell tumors [242-244]. The persistence of LMP2 expression, especially LMP2A, may indicate that this viral gene is an important risk factor for development of malignancies. Events associated with early EBV infection, including cell activation, anti-apoptotic signaling and early proliferation can be

used as an indicator of successful late infection events, such as cell transformation and LCL establishment *in vitro*. For instance, previous work has determined that efficient activation and early proliferation are paramount to successful immortalization of B cells by wild-type EBV [209]. Therefore, in this study, we have investigated possible roles for LMP2 in the kinetics of early events via cellular processes, such as activation, proliferation and apoptosis (survival), and have shown that LMP2A is critical for the successful initiation of these processes, as well as efficient cell transformation by the virus.

Previous studies have shown that LMP2A delivers signals suggested to mimic tonic BCR signaling, which is necessary for B cell survival [158,204,230,231,245]. LMP2A signaling initiates through activation of src-family protein tyrosine kinases (PTKs), such as Lyn and Syk, that normally form part of the BCR signaling complex [246]. Additionally, transgenic mouse studies have shown that LMP2A confers survival of BCR null B cells through constitutive activation of the Ras/PI3-K/Akt pathway [245], which results in inhibition of apoptosis in infected B cells via induction of anti-apoptotic genes Bcl-2 and Bcl-xL [159-161]. Our data support a role for LMP2A in B cell survival, which appeared to be important at the earliest times (4 to 7 days) after EBV infection when proliferation was initiating in the majority of infected cells. A role in survival seemed less critical by 14 days post-infection, which was suggested by the overall decrease in apoptosis percentages **(Figure 18B)**. In contrast, deletion of LMP2B did not result in significant changes in apoptosis. Unlike LMP2A, LMP2B does not encode a signaling domain, and therefore, has not been extensively studied for signaling capacity. Although loss of LM2B alone did not significantly contribute to changes in apoptosis, removal of both isoforms resulted in the highest percentages of apoptotic B cells, suggesting that LMP2B may play an indirect role in survival of infected B cells.

The BCR-like signal provided by LMP2A may also mimic an activation signal. Specifically, LMP2A has demonstrated the ability to stabilize β-catenin in epithelial cells through protein kinase C-mediated inhibition of glycogen synthase kinase-3 (GSK-3), a process usually performed by an activated BCR in B cells [164,247]. LMP2A may also mimic an activated BCR signal, inducing B cell activation and proliferation via Ca^{+2} fluxes and protein tyrosine kinase activation [228]. Both EBV-infected and BCR-activated cells express the surface activation markers CD23, CD40, CD44 and CD69 [107,224,248-250]. EBNA2 and LMP1 cooperatively upregulate expression of CD23, a surface receptor associated with B cell activation. Our data suggest that activation in the absence of LMP2A is inefficient, with lower levels of CD23 and CD71 expression compared to wild-type virus-infected B cells **(Figure 15)**. Therefore, it is possible that the signal provided by LMP2A cooperates with LMP1 and EBNA2 for the most efficient activation of B cells [107]. Again, in contrast to LMP2A, LMP2B did not affect activation of infected B cells.

Efficient activation of primary B cells should lead to optimal early proliferation (within 4-5 days), which would be consistent with our data (Δ2A vs wt infection) and represent a critical factor for long-term LCL growth establishment *in vitro*. However, an exceedingly higher level of early cell proliferation (hyperproliferation) has been shown to suppress growth of wild-type EBV-infected primary B cells by activation of ATM kinase through induction of the DNA Damage Response (DDR), and it appears that the attenuation of DDR in a small percentage of EBV-infected allows for subsequent outgrowth and establishment of LCLs [195]. Interestingly, the Δ2A-infected B cells showed only moderate proliferation at best, not hyperproliferation, early after infection when compared to wild-type and Δ2B viruses, and did not produce long-term LCLs as consistently. This suggests that high levels of early proliferation favor efficient

LCL outgrowth. Overall, it raises the possibility that LMP2A is able to both trigger early attenuation of DDR while supporting a signaling environment that enhances robust early proliferation of infected B cells. Although the major viral factor involved in DDR attenuation is EBNA3C via interactions with Chk2 [195], further investigation of a precise role for LMP2A in attenuation of DDR as well as enhancement of proliferation in early EBV-infected B cells is warranted.

The LMP2B isoform did not appear to be critical for B cell activation and proliferation since Δ2B-infected B cells exhibited a near wild-type level of activation and proliferation. This implies that most of the LMP2 gene effects on these processes are due to the signaling domain in LMP2A. There are possible mechanisms by which LMP2B may have effects on BCR or BCR-like signaling during infection. For instance, there are several lines of evidence showing that LMP2B can interact with signaling proteins such as LMP2A and CD19 [174], a member of the BCR co-receptor complex. In our experiments, LMP2B did not appear to play a direct role in events leading to activation, proliferation or protection from apoptosis of infected B cells. Therefore, the exact role of this protein in early B cell activation and proliferation remains unclear.

7.3 ESTABLISHMENT OF LCLS

The ability of EBV to immortalize infected B cells *in vitro* has been widely studied. LCLs established from these infections are an important model system for studying the malignant potential of the virus [3]. Specific latent genes have been discovered that are essential for the

process of immortalization, and include EBNA2, LMP1, EBNA3A and EBNA3C [141,212,214,251-254]. The role of LMP2 in this process has been highly debated, with some reports claiming that LMP2 is dispensable for immortalization [205-207], and others reporting that the gene is essential for the process [208]. As mentioned above, previous studies relied on experimental systems that either utilized mutant virus with high levels of wild-type virus contamination, or relied on incomplete virus genomes with a proclivity for acquiring mutations. Our experimental system incorporated the use of complete recombinant genomes without the burden of contaminating wild-type viruses.

In our studies, we infected PBMCs and negatively enriched B cells. Interestingly, we observed striking differences in the ability of the LMP2 KO viruses to establish LCLs in the different systems. Wild-type virus was able to produce LCLs regardless of the cell background. However, the kinetics of B cell growth in the PBMC setting was slower compared to the purified B cell system. Interestingly, Δ2B viruses established LCLs from purified B cells to the same extent as wild-type virus, but were almost completely incapable of producing LCLs from PBMCs. Δ2A viruses could establish LCLs in approximately 50% of experiments with purified B cells, but were never able to do so in PBMC wells. Only one LCL was ever established from Δ2A/Δ2B wells, produced from purified B cells, but required a lengthy amount of time in culture for establishment. The reason for this disparity is not clear, but could be the result of immune suppressive activities by other cell types within a PBMC culture (i.e. T cells, macrophages, etc).

The inability of LMP2A KO viruses (Δ2A and Δ2A/Δ2B) to establish LCLs from PBMCs was not surprising because these viruses could not efficiently produce LCLs from purified B cells either (Δ2A ~50% LCL establishment efficiency, Δ2A/Δ2Bv ~17% LCL establishment efficiency). However, the differences in LCL formation between PBMCs and purified B cells for

the Δ2B virus were much more striking. Also, proliferation rates for wild-type infected B cells were delayed in PBMCs compared to faster proliferation rates in purified B cells. There are a few possible reasons for this difference in LCL establishment and proliferation kinetics. For instance, infection of PBMCs with EBV requires the addition of CyA to inhibit CTL activity against the newly infected B cells. Specifically, CyA inhibits signaling through the T cell receptor (TCR) via formation of CyA/cyclophilin complexes that inhibit calcineurin and subsequent phosphorylation of NFAT. While CyA has not been shown to significantly affect B cell responses, it is possible that addition of CyA has some degree of inhibitory effect on BCR signaling, or LMP2A signaling in infected B cells, and thus reduces the proliferation rates of EBV-infected B cells.

Another possibility for decreased proliferation rates in EBV-infected B cells in PBMC experiments includes secretion or expression of inhibitory factors from bystander cells, such as T cells, macrophages, monocytes, or other cell types. Activated B cells express a surface receptor called programmed death 1 (PD-1) that contains an immunoreceptor tyrosine-based inhibitory domain (ITIM) [255,256], which becomes phosphorylated following interaction with ligand that allow for interaction with SHP-1 and SHP-2 phosphatases [257]. Therefore, PD-1 and its ligands are negative regulators of immune responses, most likely including B cell activation and proliferation. The ligands for PD-1, PD-L1 and PD-L2 are expressed on other cell types, such as macrophages and dendritic cells [258]. It is possible that slower proliferation rates or loss of LCL formation in EBV-infected PBMCs could be due to negative regulatory effects elicited by PD-1 or other similar receptors, but further investigation is required.

It is also possible that the differences in LCL establishment between B cells and PBMCs for Δ2B viruses are due to differences in cell number. B cells only comprise approximately 5-

10% of PBMCs. In purified B cell experiments, all 50,000 of the cells in each well are B cells, the primary target of EBV. However, in PBMC cultures, the number of B cells would fall to approximately 2500-5000 per well depending on the donor. Proliferation kinetics studies established a proliferation defect for LMP2B KO viruses, albeit not as extreme as the defect observed for LMP2A KO viruses. The combination of low B cell numbers and decreased proliferative capacity could contribute to the observed inability of Δ2B viruses to produce LCLs in PBMC cultures. However, the underlying cause of the observed discrepancy in LCL establishment for Δ2B viruses remains unclear, and requires further investigation.

7.4 LMP2 AND STABILITY OF VIRAL LATENCY

Previous research has demonstrated a role for LMP2A in the maintenance of viral latency [156-158]. Expression of LMP2A coincides with physical blockage of BCR entry into lipid rafts, and subsequent inhibition of interactions with Lyn and Syk needed to initiate BCR signaling. Lytic induction initiates when signaling through the BCR is restored. LMP2B has been shown to modulate LMP2A activity, allowing for BCR signaling to resume, which, in turn, triggers the switch to the lytic cycle [170-172]. It has been suggested that LMP2A may also maintain latency *in vivo* by regulating the expression of other latent genes [234], most likely as a way to avoid T cell recognition of targeted epitopes, such as those from EBNA2 and the EBNA3 family proteins.

Based on the aforementioned observations we reasoned that stimulation of BCR signaling in LMP2A KO virus-infected B cells would result in enhanced induction of the lytic cycle, which

could partially explain the loss of efficient activation and proliferation of infected B cells. However, in the context of our experimental system, stimulation of BCR signaling in Δ2A-infected B cells did not result in an enhanced lytic switch, suggesting that LMP2A did not play an important role in maintenance of viral latency in the early stages of outgrowth *in vitro*. It is possible that the need for LMP2A to elicit this particular function may be greatest after establishment of LCLs [157,158]. Since previous research demonstrated a role for LMP2B in the regulation of lytic switch [171,172], we had expected BCR stimulation of Δ2B-infected B cells to be more resistant to lytic reactivation. However, the observed levels of LMP1 and Zebra expression were comparable to wild-type, which suggests that Δ2B-infected B cells were not more resistant to lytic reactivation than wild-type. The loss of both isoforms, on the other hand, consistently triggered elevated Zebra expression with concomitant significant decreases in LMP1 expression, a protein that is essential for immortalization *in vitro* [214,251]. Since this was not observed in B cells infected with either Δ2A or Δ2B viruses, it is possible that either LMP2A or LMP2B may be able to provide the maintenance function for viral latency *in vitro*.

It is possible that strong lytic induction was not observed due to weak stimulation of BCR signaling, and may contribute to some of the phenotypes observed for BCR stimulation experiments. Previous studies have shown that a wide variety of reagents can induce lytic reactivation, and may differ in their ability to stimulate the lytic cycle in our experimental system, which relied on successful triggering of BCR signaling with a cocktail of anti IgM/IgG/IgA antibodies. Therefore, the use of chemical lytic inducers, such as histone deacetylase inhibitors or protein kinase C activators, could induce a stronger lytic signal that may allow for a better understanding of the role of LMP2A in the maintenance of viral latency in early B cell infections with recombinant virus.

In addition to experiments examining the stability of latency in the presence of lytic inducing reagents, we also examined latent and lytic gene expression during natural progression of early EBV infection *in vitro*. Inclusion of lytic genes (Zebra) revealed a lack spontaneous lytic induction for both wild-type and LMP2 KO virus-infected B cells, which corroborates previous data that showed transient lytic gene expression did not lead to lytic cycle induction *in vitro* [45,195]. Our results also demonstrate that LMP2A does not play a critical role in maintenance of latency during early infection *in vitro*. The lack of significant differences in the expression of other EBV latent genes between wild-type and LMP2 KO virus-infected cells indicates that the recombinant viruses were subject to the same regulatory controls governing the expression of these genes as wild-type virus. Also, this was another illustration of the stability of viral latency in these cells, even in the absence of LMP2. The only transcript with significantly different levels was LMP2B; the levels of LMP2B transcript were significantly elevated in Δ2A virus-infected B cells. The increased LMP2B expression observed may imply the existence of an unknown indirect regulatory mechanism requiring LMP2A. It is as likely that the effect is an artifact of the release of the LMP2B promoter from transcriptional repression caused by the drop in through-transcription from the upstream-mutated LMP2A promoter that allows improved RNA polymerase initiation.

Although no statistically significant differences in expression were detected for the majority of latent genes between wild-type and LMP2 KO viruses, observed slight differences in gene expression could be due to heterogeneity within the B cell population and among donors. This heterogeneity could contribute to differences in proliferation rates among primary B cells infected by different recombinant viruses. While variability in the virus stocks could account for variations in gene expression or observed phenotypes from other experiments, all virus stocks

were tested for titers and genome copy number/GIU ratios [217], and only stocks with comparable genome copy#/GIU ratios were used. Therefore, differences in the titers of our virus stocks should not be great enough to account for the effects noted in our experiments.

7.5 CONCLUSION

In conclusion, EBV infection leads to activation and proliferation of B cells that result in efficient production of LCLs **(Figure 30, Wt panel)**. Our kinetic analysis shows that LMP2A plays critical roles in activation, proliferation, and survival of infected primary B cells during the early stages of infection (Δ2A vs wt infection). The early effects observed for Δ2A-infected B cells were not due to loss of stable latency or altered latent gene expression, but appeared to effectively decrease the probability that an LCL would be established **(Figure 30, ΔLMP2A panel)**. LMP2B did not seem to play significant roles in the kinetics of B cell activation, proliferation, survival or regulation of gene expression or lytic induction, and LMP2B-deficient EBV was as capable of inducing primary B cell activation and establishment of LCLs as wild-type EBV from purified B cells **(Figure 30, ΔLMP2B panel)**. The loss of both protein isoforms caused the lowest levels of B cell activation, proliferation and survival, and the least stable expression of latency genes following lytic stimulation, which resulted in the lowest probability of establishing LCLs **(Figure 30, ΔLMP2A/ΔLMP2B panel)**. Our studies have confirmed the advantage of LMP2A expression in early B cell infection and LCL formation *in vitro*, and have suggested a subtle but clear role for LMP2B in the maintenance of viral latency. The exact mechanisms by which LMP2 elicits these effects on infected B cells require further investigation.

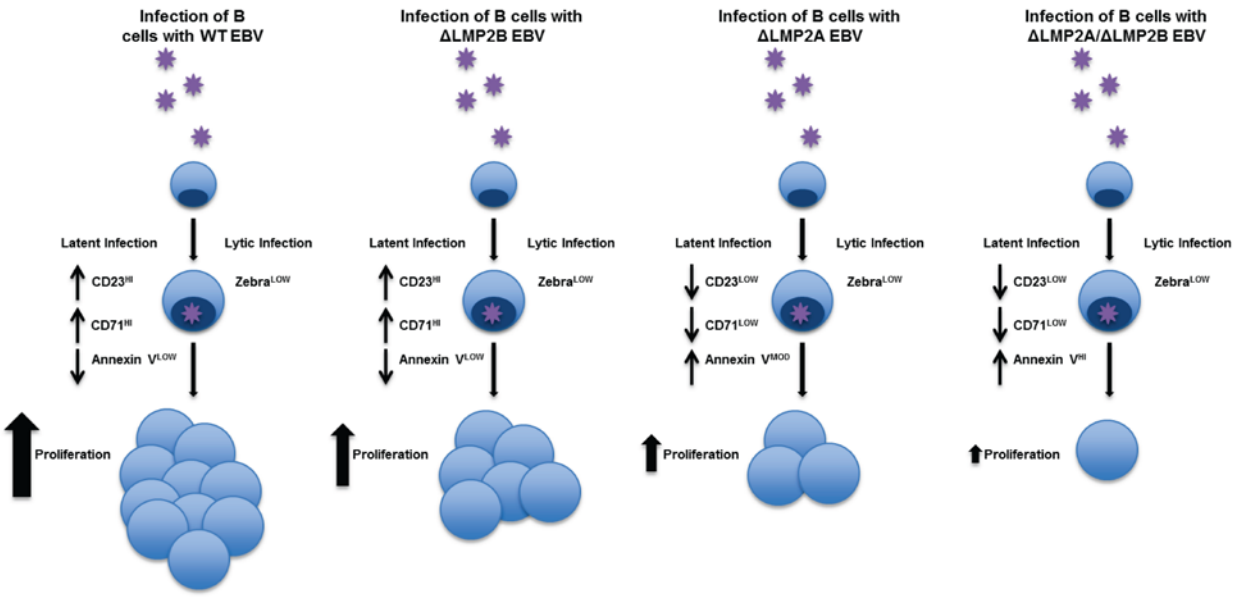

Figure 30. Schematic representation of role for LMP2 in early infection leading to B cell proliferation.

Loss of LMP2B expression does not significantly affect the processes of activation, proliferation or protection from apoptosis during early infection. However, loss of LMP2A drastically reduces the ability of EBV to induce efficient activation and proliferation, and the ability of the virus to protect infected cells from apoptosis is reduced. Interestingly, loss of both isoforms results in an environment in which activation, proliferation, and protection from apoptosis are severely reduced, leading to almost complete loss of LCL formation.

7.6 PUBLIC HEALTH SIGNIFICANCE

Epstein-Barr virus is a ubiquitous virus that is known to infect approximately 95% of the adult population worldwide, and has been associated with an increasing number of malignancies and autoimmune disorders. For most of these disorders, the exact link to EBV in disease pathogenesis is not clear, and requires further investigation into possible risk factors for disease development. Most of the earlier studies in the field have focused on the ability of EBV to

transform, or immortalize, B cells *in vitro*, a superb model system for studying EBV-associated tumors and the functions of latent genes in tumorigenesis. During Latency III in LCLs, EBV restricts gene expression to a set of nine latent genes. Four of these nine genes were determined to play an integral role in B cell transformation *in vitro*: EBNA2, LMP1, EBNA3A and EBNA3C. Surprisingly, of these four genes, only LMP1 is expressed in a large number of human malignancies *in vivo*. Though the *in vitro* LCL system is somewhat artificial due to the use of recombinant virus systems in the majority of the studies, the results have provided a plethora of important information about the functions, tumorigenic or not, of these various latent genes.

Our work focused primarily on the protein products of the LMP2 gene, LMP2A and LMP2B, and, specifically, their roles in early B cell infection and immortalization *in vitro*. There has been much speculation over the exact role of this gene in these processes, with some studies proclaiming the gene is dispensable for immortalization and others demonstrating an essential role for the gene in this process. Interestingly, LMP2A, along with EBNA1, is routinely detected in large numbers of EBV-associated malignancies of B cell origin, as well as epithelial cell origin. This persistence of LMP2A expression may indicate that this gene is a significant risk factor in EBV-associated tumorigenesis. Additionally, LMP2B expression is often found in NPC, and may represent an important risk factor for development of that particular EBV-associated malignancy.

Therefore, we generated complete recombinant EBV with mutations in the LMP2 genes, which contrast with the experimental approach of the aforementioned previous studies, and infected B cells in order to better understand the function of the two isoforms in tumorigenesis within the context of a controlled *in vitro* system. We discovered a critical role for LMP2A in B cell activation and proliferation during early B cell infection, which directly impacted the ability

of EBV to induce LCL formation. Unfortunately, our data do not clearly elucidate any possible roles for LMP2B in early infection. However, the ability to transform infected B cells *in vitro* is not impeded by the loss of LMP2B, indicating that this protein may not play an important role in tumorigenesis. Although these results exist within the context of an *in vitro* system, it is possible that LMP2A may be critical for tumorigenesis of B cells *in vivo* in diseases such as PTLD, which can mirror *in vitro* derived LCLs. In the future, therapeutics designed to target this gene may provide transplant patients with improved treatments for PTLD, and subsequently, higher rates of remission.

8.0 FUTURE DIRECTIONS

In this study, we have demonstrated that expression of LMP2A augments early activation and proliferation of EBV-infected B cells, leading to efficient LCL formation. Previous studies have disputed the role of LMP2 in B cell transformation, but these studies utilized systems with either high levels of wild-type contamination or incomplete viruses [205-208]. Our studies have focused on the role of the two isoforms in early infection, as well as effects on subsequent LCL establishment. In this way, the work demonstrates an important initial step in our understanding of the role of LMP2A and LMP2B in early EBV infection of B cells, as well as their functions in the EBV life cycle.

The next logical step for this research study would include the expansion of our understanding of the underlying molecular mechanisms for the observed effects on activation, proliferation and apoptosis. Our initial work has revealed a critical role for LMP2A in early B cell infection. Additional research is needed to understand how LMP2A affects these cellular processes. For instance, the molecular mechanisms for LMP2A-associated proliferation of B cells are not clear, especially those involved during early infection. Many earlier studies of LMP2A-associated signaling pathways utilized overexpression systems in BL cell lines, such as BJAB, which could provide conflicting information due to the activation of cellular oncogenes before infection with EBV. Therefore, the LCL system developed for our recombinant viruses would provide important information of the signaling pathways involved directly in

tumorigenesis. Another interesting direction for our proliferation studies would be to investigate a possible role for LMP2A in DDR attenuation, which allows for LCL outgrowth [195]. Perhaps the loss of LMP2A expression affects the ability of EBV to attenuate DDR and allow for efficient LCL formation. Our data also suggested that LMP2A is important for B cell activation. The molecular mechanisms for B cell activation by the viral proteins LMP1 and EBNA2 have been elucidated [107]. However, the mechanisms of LMP2A-induced activation are not known, as this protein was not previously shown to be involved in this process. It would be interesting to explore LMP2A signaling pathways and compare to those provided by the BCR within the context of early B cell infection.

Our research has shown that LMP2A plays an important role in the protection of infected B cells from apoptosis during early infection. The next step for this work could include more detailed study of the differences in pro-apoptotic and anti-apoptotic protein expression in LMP2A/2B KO virus-infected B cells. The loss of LMP2B alone did not significantly affect apoptosis levels, though loss of both proteins resulted in significantly elevated numbers of apoptotic B cells, possibly indicating some level of involvement for this protein in the anti-apoptotic process. It would also be interesting to look into the possible apoptotic pathways that are affected by these two proteins. LMP2A and LMP2B share a large percentage of their sequences; however the difference in their initiating exons is significant. The presence of the CSD for LMP2A allows this protein to produce its own signal, directly modulating existing signaling pathways within infected B cells and epithelial cells. LMP2B does not encode a CSD, and, therefore, must elicit effects indirectly via interactions with proteins that are capable of initiating cell signaling pathways. Thus, it is possible for these two proteins to affect cell survival

in very different ways. The knowledge of these pathways could further explain the role of these two proteins in early B cell infection.

Further study into the PBMC and purified B cell immortalization study is warranted. At this point we do not understand the reasons for differences in immortalizing potential for our recombinant EBV viruses between these two experimental approaches. Furthering our knowledge in this area could provide important information regarding the role of bystander cells in controlling early EBV infection *in vivo*, since natural infection occurs in the midst of various cell types and tissues. Also, further study in this area could aid in the refinement of *in vitro* culture conditions for the study of EBV infection. *In vitro* systems are usually considered artificial, and may not mirror the conditions occurring in an infected person. Adjustments to these systems are necessary to remain relevant research tools.

The role of LMP2 in the stabilization of viral latency remains unclear. The use of stronger lytic inducing agents for future studies may help to shed light on the roles of these proteins in the maintenance of latency. It is very difficult to assess lytic induction in the *in vitro* LCL system because very few B cells undergo lytic cycle induction. Therefore, exploring better ways to induce a larger population of B cells to switch to the lytic cycle could aid in our understanding of viral genes involved in this process. Also, it would be interesting to study the possible molecular mechanisms involved in the transition from latency to lytic cycle induction within the context of our LCL system. This may help provide answers for the significant increase in proliferation in Δ2B virus infections. We were unable to fully explain this phenomenon in the current study due to lack of appreciable differences in apoptosis, and latent and lytic gene expression.

Finally, additional future studies could include the use of inducible systems for LMP2A and LMP2B expression at different time points during infection of B cells with LMP2 KO viruses. The rescue of LMP2A KO LCLs could help explain at what time point post-infection LMP2A is critical for LCL outgrowth. It is possible that LMP2A is most important for early B cell infection, but may not be necessary for maintenance of established LCLs, which could account for the discovery of LMP2 negative LCLs. Our *in vitro* infection system has allowed for the study of LMP2 in a variety of cellular and viral processes.

APPENDIX

ABBREVIATIONS USED IN THIS DOCUMENT

AIDS	Acquired Immunodeficiency Syndrome
APC	Allophycocyanin
ATM	Ataxia–telangiectasia-mutated protein
B2M	β-2 microglobulin
BAC	Bacterial artificial chromosome
BART	BamHI A rightward transcripts
BCR	B cell receptor
Bim	Bcl-2-interacting mediator of cell death
BL	Burkitt's lymphoma
BSA	Bovine serum albumin
CAEBV	Chronic Active EBV
CBP	CREB-binding protein
CD	Cluster of differentiation
CFSE	5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester
CNS	Central nervous system
Cp	BamHI C-region promoter
CR	Complement receptor

CSD	Cytoplasmic signaling domain
CSF	Colony stimulating factor
CTAR	C-terminal activation region
CtBP	Carboxy terminal binding protein
CTL	Cytotoxic T killer cells
CyA	Cyclosporin A
DDR	DNA Damage Response
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DS	Dyad symmetry
EA	Early antigen
EBER	EBV-encoded noncoding RNA
EBNA	Epstein-Barr virus nuclear antigen
EBNA-LP	Epstein-Barr virus nuclear antigen leader protein
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetra-acetic acid
EGFP	Enhanced green fluorescence protein
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated protein kinase
FACS	Fluorescence-activated cell sorting
FAM	6-carboxyfluorescein
FBS	Fetal bovine serum
FDC	Follicular dendritic cell

FR	Family of repeats
GC	Germinal center
GIU	Green Inducing Unit
gp	Glycoprotein
GSK	Glycogen synthase kinase
HDAC	Histone deacetylase
HEK	Human embryonic kidney cells
HEPES	hydroxyethyl piperazineethanesulfonic acid
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HL	Hodgkin's lymphoma
HLA	Human leukocyte antigen
HRS	Hodgkin Reed-Sternberg cells
HSP	Heat shock protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Infectious mononucleosis
IR	Internal repeats
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK	Janus kinase
JNK	c-Jun N-terminal protein kinase

kDa	Kilodalton
KO	Knockout
KSHV	Kaposi's sarcoma-associated herpesvirus
LCL	Lymphoblastoid cell lines
LCV	Lymphocryptoviridae
LMP	Latent membrane protein
MAPK	Mitogen-activated protein kinase
MFI	Mean Fluorescence Intensity
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
mRNA	messenger RNA
NK	Natural killer cell
NPC	Nasopharyngeal carcinoma
OriP	Origin of plasmid replication
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Programmed death
PE	Phycoerythrin
PFA	Paraformaldehyde
PI3-K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PKR	Protein kinase RNA-activated

PML	Promyelocytic leukemia
PTLD	Post-transplant lymphoproliferative disease
Qp	BamHI Q-region promoter
RDV	Rhadinovirus
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription PCR
sIg	Soluble immunoglobulin
STAT	Signal transducer and activator of transcription
TAMRA	Tetramethylrhodamine
TIL	Tumor-infiltrating lymphocytes
TM	Transmembrane domain
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAF	TNFR-associated factors
TRADD	TNFR-associated death domain
TR	Terminal repeats
VCA	Viral capsid antigen
Wp	BamHI W-region promoter
Wt	Wild-type
XBP	X-box binding protein
ZEBRA	BZLF1-encoded broadly reactive activator

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