CELLULAR PATHWAYS LEADING TO PATTERNS OF LYTIC EPSTEIN-BARR VIRUS REACTIVATION IN IMMORTALIZED B CELL LINES

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ABSTRACT

Lymphoblastoid cell lines (LCLs) are created by culturing lymphocytes from the peripheral blood and adding Epstein-Barr virus (EBV), a ubiquitous human herpesvirus which infects, activates, and transforms B cells. These cell lines are used for genotyping, as targets for cytotoxic cells, and as models for EBV immortalization of B cells, particularly post-transplant lymphoproliferative disease (PTLD) in which EBV-immortalized cells proliferate in the absence of a cytotoxic T-cell response. Studies have shown more diversity in LCLs than would be expected from cell lines that are often treated as interchangeable. It is not known how their diversity in factors like morphology, growth factor production, or cellular gene expression influences the EBV life cycle. In this study I investigated connections between LCLs' cellular and viral phenotypes, categorizing them as either low in EBV copy number or fluctuating within a high range.

As measured by lytic EBV replication and viral gene expression, LCLs showed high or low lytic permissivity, with permissivity defined as the likelihood that a cell will switch from stable latent infection into the lytic EBV life cycle. Permissivity was not affected by blocking the late events of the lytic cycle. I used flow cytometry to

characterize 19 aspects of LCL surface phenotype, but found little association with lytic permissivity. Microarrays and PCR were used to identify genes expressed at higher levels in non-permissive LCLs, including transcription factors that maintain B cell lineage. Unfolded protein response (UPR) genes and the UPR protein Grp94 were expressed at higher levels in permissive LCLs. A drug was used to investigate effects of the UPR on permissive and non-permissive LCLs that had been maintained for short or long periods of time. The UPR enhanced permissivity, causing more cells to enter the lytic cycle, but this did not lead to lytic replication. This study enhances our knowledge about EBV life cycles by giving us new information about host factors that contribute to the lytic switch. This data about LCL diversity has public health relevance to the diversity of PTLD cases, since identifying risk factors for PTLD is a significant part of care for EBV-positive transplant recipients.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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1.0 INTRODUCTION

1.1 CLASSIFICATION OF EBV

Epstein-Barr virus (EBV) is a human gammaherpesvirus with a tropism for B cells and epithelial cells. Like other herpesviruses, it consists of a nucleoprotein core, surrounded by a capsid of 150 hexons and 12 pentons, which is in turn surrounded by a complicated tegument and an outer lipid envelope containing at least ten viral glycoproteins as well as some cellular proteins acquired by budding from host cells. Viral particles are about 200nm in diameter and contain a single linear genome of about 185kb. This is classified as a Type C genome among herpesviruses, meaning it can be divided into a unique short segment (U1) and a mostly unique long segment (U2-U5), separated by iterations of the 3.1kb major internal repeat (IR1). At both ends of the genome there are 538bp tandem terminal repeats (TR) [1].

EBV is unique in that EBV infection, in the absence of other stimuli, can induce proliferation and transformation of human and other primate B cells. Under natural conditions of infection it is restricted to humans. Like many herpesviruses, EBV can cause symptomatic infection once in the host's lifetime, upon primary infection, and then remains latent in a host's cells for decades. The virus was first identified in cases of Burkitt's lymphoma (BL) from an African region where BL is endemic, and was found to

be a previously unidentified herpesvirus [2, 3]. It was also the first human virus known to immortalize human cells, and thus the first virus believed to have oncogenic properties in humans, though not the first virus known to be oncogenic [4]. The avian alphaherpesvirus MDV was established as the agent of the malignancy known as Marek's Disease or visceral lymphomatosis at around the same time that EBV was determined to be associated with BL [5].

EBV was the first large DNA virus to be sequenced, and was determined to be part of the *Lymphocryptovirus* genus of gammaherpesviruses. EBV is also known as human herpesvirus 4 (HHV-4), and is the type specimen of *Lymphocryptovirus* [1]. KSHV/HHV-8, the other human gammaherpesvirus, is a member of the *Rhadinovirus* genus. LCVs are distinguished from rhadinoviruses by genetic traits such as genome size (e.g. HHV-8 has a 160kb genome) and differences in their repertoire of such proteins as glycoproteins, cytokine homologues, and anti-apoptotic proteins (e.g. rhadinoviruses tend to contain a viral IL-6 homologue, while EBV contains a viral IL-10) [6]. There are many EBV homologues that infect other primate species, including some that cause malignancies, such as CalHV-3 of marmosets; herpesvirus papio of baboons; and rhesus LCV 1 and 2, which differ just like EBV-1 and EBV-2 [7, 8].

There are two EBV subtypes, EBV-1 and EBV-2, which are very closely related, differing mostly in the sequence and structure of the EBNA2, EBNA3A, EBNA3B, and EBNA3C genes. EBV-2 historically occurs mostly in equatorial Africa and Papua New Guinea, with EBV-1 the dominant subtype in the rest of the world. EBV-2 is overrepresented in certain Western populations, e.g. homosexual HIV-positive males. EBV-1 and EBV-2 are thought to be similar in their infectivity and association with

disease, although the EBNA2 gene from EBV-2 appears to be less efficient at transforming cells and inducing them to proliferate. Although some commonly-used cell lines like Jijoye contain EBV-2, most scientific work on EBV, including this study, uses EBV-1 [9, 10].

The EBV genome is found in the linear form in infectious virus particles, but in a circular episome form in latently infected cells. Upon cell entry, circularization takes place at the terminal repeats (TR) which form the ends of the linear genome. Circularized episomes contain negatively supercoiled DNA, are associated with histones and localize to metaphase chromosomes. Latent replication of these episomes, using the cellular replication machinery, occurs alongside replication of the cellular genome, once per episome per cell division. During mitosis each episome is tethered to a host chromosome by the EBV protein EBNA1; however, the replicated episomes are not divided equally between the daughter cells[11, 12]. Lytic replication occurs by the rolling-circle model typical of herpesviruses, using virally encoded replication machinery and a circular plasmid with minimal nucleosomes or supercoiling as the template, producing multi-genome concatamers as replication intermediates. The concatamers are then cut at the terminal repeats and packaged into virions [13].

The processes of circularization and linearization of the genome, as it switches between lytic infectious virion and latent episome, lead to variability in the size of the genome via variability in the length of the terminal repeats. One promoter for the important EBV oncogene latent membrane protein 1 (LMP1) is located close to a terminal repeat, and it has been shown that LMP1 levels are inversely correlated with the upstream TR number [14]. Transcripts for LMP2A/2B also cross the TR region

between exons, though TR number is not known to influence LMP2A or LMP2B activity. Most EBV-associated malignancies and neoplasias consist of latently infected cells whose circular EBV episomes are replicated by the cellular machinery, and have a fixed number of TRs suggesting that they contain a clonal strain of virus. An exception is the AIDS-associated tongue lesion known as oral hairy leukoplakia (OHL), which depends on ongoing lytic virus production and superinfection of the epithelial cells, and consequently is found to contain genomes with a variety of different TR sizes [15].

Another contributor to variability between EBV strains, both in EBV-1 and EBV-2, is the number of copies of the major internal repeat in the W region of the genome [16]. The EBNA leader protein (EBNA-LP) contains two exons in this region and consequently is of variable length, which affects the transactivation activity of EBV transcription factors EBNA-LP and EBNA-2 [17]. In addition to these sources of diversity between strains, the B95-8 strain of EBV has an 11.8kb deletion, in which the I region of the genome is truncated and the I' and I'' regions are absent. B95-8 was the reference strain used in the initial sequencing of EBV, and consequently another wild-type strain had to be sequenced later to fill in the gap [18, 19]. B95-8 is used as the standard EBV strain in countless experiments, including those in this study.

1.2 NATURAL HISTORY OF INFECTION

1.2.1 Primary infection and persistence

EBV typically enters the body through an oral route, through contact between infected saliva and the mucosal epithelium of the oropharynx. The virus can replicate lytically in epithelial cells, and also pass through the mucosa directly into the secondary lymphoid organs of Waldeyer's ring (e.g. tonsils, adenoids). Primary infection in children is mild or asymptomatic, but in adolescents or adults it usually leads to infectious mononucleosis (IM). This involves the proliferation of newly infected and activated tonsillar B cells, which express immunogenic EBV proteins – followed by a greater proliferation of effector T and NK cells which kill off the B cells [20].

Symptoms of acute IM (lasting 1-3 weeks from onset of symptoms) include fever, pharyngitis, and swollen lymph nodes during the overwhelming proliferation of lymphocytes. Hepatosplenomegaly and jaundice are possible as the new cells die off and are metabolized. Debilitating fatigue and malaise often lasts for months after the initian symptoms are resolved, and antibody responses (seroconversion) usually persist for the rest of a person's life [20]. Chronic active EBV infection (CAEBV) is defined as severe illness lasting more than six months, accompanied by abnormally high levels of antibodies against lytic antigens or low levels of antibodies against EBNAs. Canonical CAEBV involves chronic hepatitis, splenomegaly, lymphadenitis, or other major organ involvement; high EBV titers in affected tissues; and often impaired T or NK cell cytotoxicity [21]. Although CAEBV was first described as an ongoing IM-like proliferation

of B cells, most cases in Asia show proliferation of EBV- infected T or NK cells. It is not known how non-B lymphocytes are infected by EBV, but it is known that this infection occurs to mature T or NK cells and is not the result of hematopoietic stem cells becoming infected and differentiating into lymphocytes [22].

Throughout the lifespan of a healthy EBV carrier, latent virus is found in a small percentage of B cells. There are two B-cell compartments which contain latently EBV-infected cells: quiescent memory B cells in the bloodstream, and proliferating CD10+CD77+ germinal center B cells (centroblasts) in the follicles of tonsils or other lymphoid organs. When symptoms of IM are detected, the percentage of EBV+ cells in the peripheral blood is already declining exponentially, and it continues to decrease during the decades after IM is resolved [23, 24]. Healthy carriers intermittently shed infectious virus in saliva; this occurs when latently infected memory cells traffic to Waldeyer's ring and differentiate into antibody-secreting plasma cells (PCs), which is accompanied by activation of lytic replication [25]. Most of the infectious virus that is produced is made by epithelial cells rather than by PCs [26].

1.2.2 Cell entry and exit

Unlike other lymphotropic human herpesviruses (HHV-8 and the betaherpesviruses CMV, HHV-6 and HHV-7), EBV is not proposed to interact with heparan sulfate on cell surfaces, and consequently its tropism is severely restricted. One marker of B cells is high surface expression of CD21, also known as complement receptor 2 (CR2), which is part of the CD19 co-receptor complex that promotes survival

of B cells activated by stimulation of surface immunoglobulin [27]. Some neutrophils are $CR2^{high}$ and can be infected by EBV, but this leads to an abortive infection because neutrophils express high levels of the death receptor Fas, and EBV LMP1 upregulates expression of Fas ligand, leading to apoptosis [28].

Attachment to B cells is mediated by interaction of CR32 with the abundant EBV glycoprotein gp350/220. This leads to cellular activation, contributes to pro-survival signaling through CD19 and the PI3K/Akt pathway, and stimulates endocytosis via non-clathrin-coated vesicles. Once endocytosed, a complex of EBV glycoproteins gH, gL and gp42 mediate membrane fusion, partially via gp42 interaction with MHC class II. The capsid is transferred to the nucleus and the linear genome is released. Within 16 hours of virus-cell contact, newly circular episomes can be detected and are being used as templates for latent gene expression [27]. EBV survival in B cells depends on inactivation of apoptotic pathways immediately after cell entry, which is mediated by transient expression of two viral Bcl-2 homologues [29].

Within 24 hrs of EBV infection, resting B cells show expression of the BZLF1 protein, which is called Z-encoded broadly reactive activator (ZEBRA) and serves as the primary lytic switch gene. ZEBRA is homologous to K-bZIP of HHV-8, but is unlike K-bZIP in that when expressed in the absence of any other lytic genes it can induce a switch from latency to lytic replication [30]. Latent viral genes are quickly expressed upon EBV infection, along with cellular activation markers like CD23 and CD44, and the germinal center marker CD10, indicating that EBV is inducing the cell to enter the cell cycle and prepare for differentiation [31].

The production of infectious virus does not proceed immediately after infection of resting B cells with wild-type EBV. Studies differ in the kinetics of virus production by newly infected cells, ranging from 3 days before infectious virus is produced (and 5 days before enough virus is secreted to produce secondary infection and transformation of other cells in culture) to more than 9 days before any secreted virus is seen [32]. This long life cycle is related to the complex structure and multi-step lytic cycle of herpesviruses. Kalla *et al.* also suggest that there is a need to experience latent genome replication and methylation before the lytic cycle can naturally be induced. In lymphocryptoviruses like EBV [32]. There are three categories of lytic genes: immediate early (IE), delayed early, and late. The two IE genes BZLF1 (ZEBRA) and BRLF1 (Rta) are transcription factors that reinforce each other's expression and induce a cascade of delayed early viral proteins. One of the first genes expressed after ZEBRA and Rta induction is BMLF1, an abundantly expressed and immunogenic protein that serves as an mRNA export factor, essential for large-scale synthesis of the full array of lytic genes. Other delayed early genes include the viral DNA replication machinery. Lytic DNA replication is then necessary for production of late viral proteins including all of the structural proteins that make up the viral particle [30, 33].

EBV release proceeds in a manner similar to that of other herpesviruses [34, 35]. The viral genome enters the capsid through a dodecameric complex of Portal (BBRF1) protein. The capsid complex is enveloped by fusion with the inner nuclear membrane, and de-enveloped upon fusion with the outer nuclear membrane. Nuclear egress depends on disassembly of the nuclear lamina by kinases including cellular PKC and the Cdk1 homologue BGLF4, which is the only EBV-encoded protein kinase [36]. Once

in the cytoplasm, the capsid becomes complexed with an amorphous network of tegument proteins, which includes viral gene products important for events following cell entry. Cellular proteins like actin, tubulin, cofilin, Hsp90, and Hsp70 are also incorporated into the tegument network [37]. Finally, the tegument-coated capsid buds into regions of the trans-Golgi network, acquiring a lipid envelope containing numerous viral glycoproteins. Secretory vesicles traffick mature virions to the cell membrane, where the infectious particles are released.

Infection of epithelial cells is less well characterized and does not involve CR2. The receptor interactions necessary for attachment to epithelial cells are unclear, although the envelope protein complex BMRF2/BDLF2 binds to α5β1 integrins on the basolateral surface of these cells. Fusion with epithelial cells which do not express MHC class II is also dependent on gH and gL, but is actually inhibited by viral gp42. Virions produced by MHC II^+ cells end up with more gp42 sequestered by the virus-producing cell and less gp42 in the viral envelope, making the virions more infectious for MHC II^- cells. EBV produced in epithelial cells is up to 100-fold more infectious to B cells than EBV produced in B cells, while EBV produced in B cells is up to 5-fold more infectious to epithelial cells [27]. However, much of the EBV spread through the epithelium is by passage of virus between neighboring cells, rather than through production of free virus into the supernatant [38].

1.2.3 Establishment of latency in B cells

Upon entering a B cell, EBV produces latent proteins which contribute to activation and survival of the cell. Newly-infected cells produce the full complement of

latent proteins – EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, LMP1, LMP2A, and LMP2B – along with the abundant untranslated EBV-encoded RNA (EBER-1 and -2) transcripts, and BamA region transcripts (BARTs) whose significance is unknown. EBNA1, EBNA2, EBNA3A, EBNA3C, LMP1, LMP2A, and EBERs are considered indispensable for efficient B cell immortalization. This latency program, in which EBV gene expression is sufficient to induce proliferation and immortalization, is known as **Latency III** or the "growth program" of gene expression. *In vivo* this program is found in newly infected naïve B cells and "bystander" memory B cells in the tonsils. Cytotoxic responses against these proteins, particularly epitopes on EBNA2 and EBNA3 proteins, usually mean infected cells are killed off [10, 33].

Table 1. EBV latency programs.

Pattern	EBV gene expression	In healthy carriers	In malignancies
Latency 0	*EBERs;* possibly LMP2A	circulating quiescent memory B cells	--
Latency I	*EBERs; BARTs;* EBNA1; possibly LMP2A; BARF1 in epithelial cells	circulating quiescent memory B cells, when dividing	Burkitt's lymphoma; gastric carcinoma in epithelial cells
Latency II	*EBERs; BARTS;* EBNA1; LMP1; LMP2A; LMP2B; BARF1 in epithelial cells	germinal center B cells	Hodgkin's disease; DLBCL; NPC in epithelial cells
Latency III	*EBERs; BARTs;* EBNA1; LMP1; LMP2A; LMP2B; EBNA2; EBNA3A; EBNA3B; EBNA3C	newly infected, proliferating B cells in tonsils/adenoids	PTLD; other lymphoproliferations of immunodeficiency

A small number of Latency III-expressing cells downregulate these proteins and express the **Latency II** program, which has also been called the "default program". This includes EBNA1, LMP1, LMP2A, LMP2B, BARTs, and EBERs. This combination of

genes is characteristic of EBV-infected cells in the germinal centers (GCs), which undergo normal affinity maturation and differentiation into memory cells [23]. A germinal center is a region of the lymphoid follicle in a tonsil or other secondary lymphoid organ, which takes shape during a T-cell-dependent immune response. The GC is populated by activated B cells which, when cycling through the follicle, encounter follicular dendritic cells (FDCs) presenting their cognate antigen. In the “dark zone” of the GC these B cells are induced by signals from helper CD4 T cells to proliferate as centroblasts, while undergoing somatic hypermutation of their germline DNA which alters the affinity of their surface immunoglobulins. They then cease proliferating, becoming centrocytes, and are driven by CXCL13 to move to the “light zone”, where they are selected for their ability to bind antigens presented by FDCs. Most apoptose, but the highest-affinity cells emerge from the follicle as memory or plasma cells[39, 40]. *In vivo*, LMP1 and LMP2A are ligand-independent substitutes for the external signals which induce B cells with high-affinity surface Igs to survive this process. However, it seems that in the natural course of infection, these viral proteins do not enable cells which would otherwise be killed off to survive [41].

EBV-infected cells that go through the germinal center reaction appear to have been under the same selection pressure as EBV-negative cells, as determined by the pattern of germline mutations [42]. These cells become long-lived memory B cells and are the reservoir for EBV in the peripheral blood. They express the **Latency 0/Latency I** program, which was previously called the “latency program”. For most of these cells’ existence, they are circulating in a resting state, producing no EBV proteins, but can be identified as EBV+ by EBER RNA expression (Latency 0). During the occasional cell

divisions, the EBNA1 protein is produced (Latency I) and tethers the viral episome to a host chromosome [23]. These cells are perceived by the body as normal memory cells and undergo the same rates of homeostasis as EBV-negative memory cells [24]. LMP2A mRNA has also been detected at low levels in the peripheral blood, in the absence of other latent transcripts beyond EBNA1 and EBERs, but the role of LMP2A in quiescent cells is unknown [43, 44].

1.2.4 EBV-associated cancers

EBV can transform primate B cells upon infection, turning quiescent primary cells into proliferating and activated cell lines. Only two EBV latent proteins are classified as oncogenes – that is, tumorigenic when expressed in the absence of other EBV genes: LMP1 and BARF1. LMP1 is a CD40 mimic found in Latency II and Latency III, and BARF1 is a soluble CSF-1 receptor usually associated with the lytic EBV program.

EBV has been suggested as a cause for certain rare tumors (e.g. T-cell AILD, and immunodeficiency-associated leiomyosarcoma). However, it is mostly strongly associated with the lymphomas and carcinomas described here. Aside from NK/T-cell lymphoma, in which EBV is strongly correlated with tumors in extranodal nasal locations, these are all diseases of the B cells or epithelial cells [45, 46]. Primary effusion lymphoma, plasmablastic lymphoma and DLBCL are tumors consisting of large B cells, which often contain EBV and show irregular production of LMP1 and/or LMP2A, but are probably not caused by EBV [10, 47-50]. EBV is thought to have a role in

causing Burkitt's lymphoma, Hodgkin's disease, post-transplant lymphoproliferative disease, nasopharyngeal carcinoma, and gastric carcinoma.

1.2.4.1 Burkitt's lymphoma (BL)

BL is a lymphoma with germinal center (GC) B cell characteristics: somatic antibody gene rearrangement, though usually without isotype switching; high levels of the BCL6 transcriptional repressor; and CD10+CD77+ surface phenotype. Unlike GC centroblasts in healthy carriers, EBV expresses a Latency I profile (only EBNA1 and untranslated RNAs); rare cases containing other EBNAs are associated with EBV integration into the host chromosome, or EBNA2-negative strains of virus. BL is found extranodally more often than most lymphomas, and characterized by a chromosome 8 translocation that constitutively turns on the gene for the c-*myc* oncogene. This translocation is usually between MYC and an Ig gene, and is usually t(8:14), but t(2:8), and t(8:22) also occur. BL has endemic and sporadic forms. It is endemic in boys in Papua New Guinea and central Africa, in whom more than 95% of cases are EBV+. The sporadic form is associated with childhood though not as strongly, and only about 30% of cases are EBV+. Malaria has a mitogenic effect on B cells in Waldeyer's ring, and an immunosuppressive effect on T cells, which may lead to more B cells that ought to be killed off by T cell selection, and a longer lifespan for these cells during which they can express EBV proteins and possibly re-circulate into the dark zone for further DNA rearrangement [46, 51].

Clearly, the process of somatic hypermutation in the germinal center predisposes cells to occasional translocations that lead to BL or other tumors. The fact that EBV is associated specifically with BL implicates EBV latent proteins in tumorigenesis and maintenance of the tumor, and there have been many hypotheses about how this can happen. EBV+ BL cell lines contain more of a “mutator phenotype”, with higher rate of chromosome instability, than EBV- BL lines [52]. Initial EBV infection of B cells leads to overexpression of the SHM enzymes AID and DNA polymerase-η. EBV genes EBNA2, EBNA3C (both may disrupt the mitotic spindle checkpoint), and LMP1 (may inhibit the ATM DNA damage repair complex) all likely predispose cells to aberrant translocations [53, 54]. These proteins are not found in normal cases of BL, but may be implicated in both EBV+ and EBV-negative BL by “hit and run tumorigenesis”, or an initial transforming event followed by loss of the virus from the transformed cells.

EBV also helps sustain BL after oncogenesis, as can be determined by the lack of EBV-negative B cells in BL lesions (some EBV-negative cells would be predicted by our knowledge of the dispersal of EBV episomes during cell division) [11]. When EBNA1 is expressed without LMP1 (as in BL), it reduces HLA I loading and presentation of viral antigens on the cell surface. EBNA1 also contributes to chromosomal instability by upregulating enzymes that produce reactive oxygen species. This could lead to the subsequent translocations that reinforce c-*myc* activity, such as silencing the p53 tumor suppressor pathway. As for the untranslated RNAs, EBERs have been shown to upregulate Bcl-2 and IL-10, but this effect is not observed in BL, so the role of EBV may be limited to “hit and run” events and the effects of EBNA1 [51, 53].

1.2.4.2 Hodgkin's disease (HD)

Like BL, HD seems to be derived from GC-experienced B cells that have undergone affinity maturation. Classical HD is a treatable lymphoma mostly localized to lymph nodes or the spleen. HD is more common in men than in women, and EBV+ cases (25-50% of total cases) are even more likely to be male. EBV positivity is associated with older patients; a history of IM symptoms upon initial infection; and HD incidence in developing countries. The malignant cells in HD are large Hodgkin/Reed-Sternberg cells (HRS cells) which were at first thought to be granulocytes or macrophages based on size, shape and expression of a confusing array of lineage markers. Sequencing of their Ig rearrangements showed that they are descended from B cells. HRS cells are CD30+ and CD15+, unlike most B cells; lack B cell markers like CD19 and CD20; and often have antibody sequences that are "crippled" by unsuccessful SHM, meaning they are descended from B cells that would not normally survive as centroblasts in the GC. Even those with functional Ig rearrangements do not express Ig on the cell surface. They do retain surface proteins needed to interact with T cells, including CD40 and CD80 [46, 55-57]

HRS cells make up only 0.1-10% of the total cells in the lesion, and in most cases are surrounded by infiltrating activated lymphocytes, making it hard to characterize HD's genetic abnormalities. Latency II expression is found in the infected HRS cells. There is less evidence for EBV-induced "hit and run" etiology for HD than there is for BL. Patients who experienced IM symptoms upon primary EBV infection are at higher risk for EBV+ HD but probably not for EBV-negative HD [46, 56]. EBV-negative cases have much higher incidence of some factors, like excessive receptor

tyrosine activity and blockage of A20(TNFAIP3) function, which activate NFκB and STAT pathways. This indicates that there are mechanisms for inducing HD that have nothing to do with EBV. HRS cells depend on the HD milieu to proliferate, and are replenished by circulating B cells, but those cells also have HRS genotypes and likely come from the same progenitor cells [56-58].

Because the HD lesion contains so many non-tumor cells, it is considered more the result of a self-sustaining immune microenvironment than the result of endogenous immortalizing events within the cells. In populations where it tends to be EBV-negative, HD is more easily associated with genetic factors, like autoimmune diseases and polymorphisms in cytokine genes [59, 60]. In EBV+ cases the virus is likely contributing to the milieu that surrounds the HRS cells and prevents resolution by the immune system. This milieu contains IL-21 and IL-21R, which lead to constitutive activation of STATs, proliferation factors like IL-10, BAFF and APRIL, and cytokines that attract Th2 and Treg cells. EBV EBNA1 may be upregulating the Treg chemoattractant CCL20; LMP1 induces IL-10 production. EBV infection may also be upregulating HD growth factors autotoxin and LPA. The combination of LMP1 and LMP2A activate numerous signal transduction pathways described **below** [56, 57]. A recent genetic comparison of EBV+ and EBV- HD found more signs of activated T cells, Th1 responses, and antiviral immunity in EBV+ cases [61].

The differentiation of HRS cells away from a B-cell phenotype seems to follow the downregulation of many B cell factors that are repressed by Notch. In mouse models, EBV LMP2A constitutively activates the Notch pathway, which otherwise is usually associated with T cells rather than B cells [62]. LMP2A can also downregulate

expression of common B-cell lineage factors PU.1 and EBF1, and upregulate proliferative and anti-apoptotic genes like Ki-67 and survivin, albeit in transgenic mouse B cells [63]. HRS cells often have overexpression of Notch and Id2, another non-DNA-binding transcription factor that blocks the action of important B cell transcription factors like Pax5 and E2A(TCF3) [56].

1.2.4.3 Post-transplant lymphoproliferative disorder (PTLD)

Although cells expressing the Latency III program are killed off in most people, these cells can grow out in immunocompromised individuals. AIDS patients, people with primary immune disorders, and non-transplant patients given immunosuppressive drugs [e.g. methotrexate] often develop lymphomas but they are not uniformly EBV+. However, in transplant recipients, PTLD caused by Latency III-expressing B cells is a frequent side effect of immunosuppression [46]. In bone marrow recipients the PTLD is usually donor-derived and the prognosis is usually very poor and treatable only by donor T lymphocyte infusion or *ex vivo*-cultured EBV-specific CTLs. In solid organ recipients the PTLD is far more likely to be treatable, often by simply reducing the immunosuppression [64].

PTLD is a diagnosis that comprises several types of lesion. Polymorphic PTLD is the most common form, which can be either monoclonal or polyclonal, and includes B cells in apparently different stages of development, even when the cells have been determined to be monoclonal by sequencing their Ig rearrangements. Polymorphic PTLD is most often EBV-associated and Latency III-expressing. Monomorphic PTLD

includes tumors that are also found in immunocompetent people (e.g. DLBCL, B-ALL, B-CLL, Burkitt's lymphoma, and T-cell lymphomas), and are more likely to involve chromosomal abnormalities than EBV-induced cell proliferation. There are also HD-like PTLDs, and some that are not necessarily malignant, but involve overproduction of plasmablasts or resemble the proliferation of EBV-infected cells found in IM. These are defined as hyperplasias rather than tumors, and are also associated with Latency III [65, 66]. They usually manifest soon after transplantation, and can be controlled by reduction in immunosuppression, but may be followed months or years later by a PTLD that is monoclonal and/or has chromosomal abnormalities [67].

In solid-organ transplant recipients, risk for PTLD is related to the type of organ transplanted, the age of the recipient, and the EBV serostatus of the donor and recipient. Children are at higher risk, as they are more likely to be seronegative before the transplantation and then to become EBV-infected either by the donor organ or by natural contact soon after the transplant. Intensity of immunosuppression, and cumulative exposure to immunosuppression, is also a key risk factor. T-cell-specific immunosuppression is the biggest risk factor, particularly therapeutic monoclonal antibodies leading to cytotoxic clearing of T cells. Calcineurin inhibitors are the next most risky form of immunosuppression, while mycophenolate mofetil and mTOR inhibitors (rapamycin, sirolimus) actually contribute to the prevention of tumors [65, 66]. Most cases of PTLD involve lymph nodes but about half have multiple-organ involvement, especially liver, lungs, and CNS. In children, the risk ranges from 1-8% among kidney recipients to 15-20% among intestinal recipients. A higher risk is related to more aggressive immunosuppressive regimens, and more lymphoid tissue in the

donor organs [65, 68]. Increased monitoring for EBV activity and cytokines in transplant recipients has led to earlier detection of PTLD, and reduced use of T-cell-specific immunosuppression (cyclosporine, OKT3) has led to reduced incidence [66, 69].

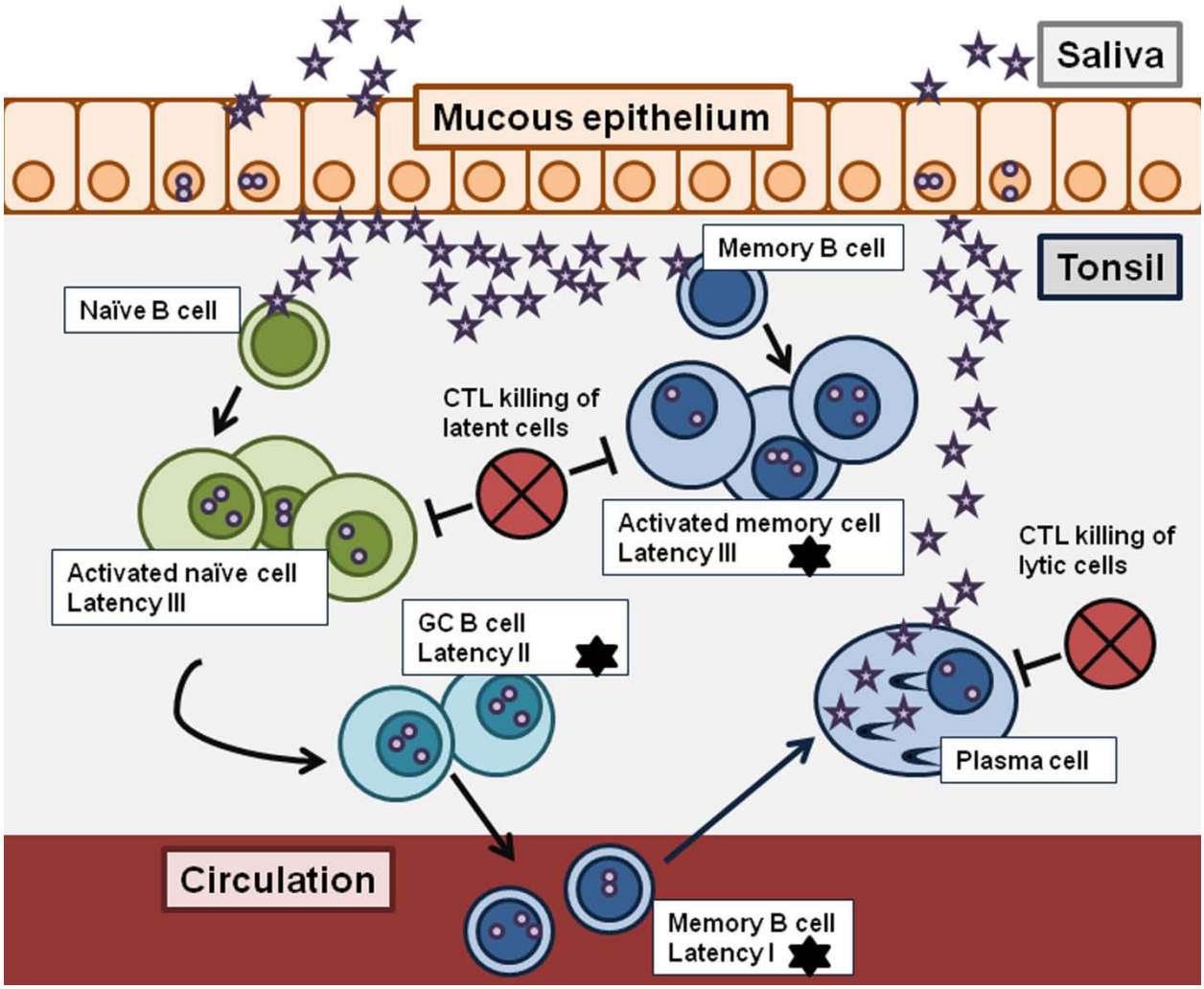

Figure 1. The cycles of EBV infection and persistence. EBV enters the tonsil at the mucous epithelium, where infectious virus is amplified. EBV infects naïve or memory B cells, activating them to proliferate. EBV+ naïve cells can go through a germinal center reaction and exit to the peripheral circulation as resting memory cells. If these memory cells differentiate into antibody-secreting plasma cells they traffick to the tonsil and become a site of lytic virus production. **Black stars** indicate points in B cell differentiation where GC-experienced cells may give rise to tumors (BL from Latency I cells; HD from Latency II; PTLD from Latency III).

1.2.4.4 EBV-associated carcinomas

Nasopharyngeal carcinoma is a disease of the mucosal epithelium, most prevalent in East Asia, and especially among Cantonese Chinese. Non-keratinizing NPC and undifferentiated NPC are nearly always EBV-positive, although some squamous-cell NPC are EBV-negative. Like HD, NPC is characterized by an immune-suppressive microenvironment, with a high number of regulatory T cells and a surprising lack of cytotoxic T cells against latent EBV targets. Elevated anti-EBV antibody, specifically of the IgA isotype, is diagnostic of NPC. This includes antibody against lytic viral antigens, indicating that although many cases lack detectable virion production, at least the early stages of reactivation are taking place [70, 71]. The microenvironment contains inflammatory cytokines that function as tumor growth factors and are downstream of lytic EBV reactivation, including IL-1α, IL-6, and IL-8 – in addition to the anti-inflammatory IL-10 induced by LMP1, and a lytic viral IL-10 homologue [71-73].

Although the EBV strains found in NPC cases of Chinese people, even second-generation immigrants to North America, are typically a different strain from those found in European-American NPC cases, it seems that the most important risk factors are environmental, specifically consumption of salt-preserved foods (fish, vegetables, eggs) containing chemicals such as nitrosamines that can reactivate EBV and cause DNA damage [74, 75]. EBV+ NPC is highly prevalent among the Inuit of the Arctic, who have a high intake of nitrosamines from diet and tobacco consumption [76]. Other risk factors include exposure to wood dust and burning incense sticks, and SNPs in DNA repair genes. Extensive genetic analysis has also been done to find connections to certain HLA alleles, chromosomes, and tumor suppressor genes like GADD45G [74, 77].

Gastric carcinoma is associated with EBV as well. Although only about 10% of cases worldwide are EBV+, this is far higher than the rate of EBV+ esophageal or colon cancer. EBV+ gastric carcinoma is not endemic in any population, and is actually more common in European and Hispanic populations than populations like the Chinese who are at highest risk for NPC, suggesting a different etiology [78-80]. One review suggests that EBV is likely to infect the stem cell layer of distrupted gastric epithelium, and then have its genome hypermethylated as part of an antiviral response. Carcinogenesis may develop if host genes are also hypermethylated in this process [81].

EBV+ gastric carcinoma cases produce much more IL-1β, but not other growth factor cytokines, even though there is an inflammatory microenvironment just as in NPC [82]. EBV+ cases are particularly likely to have multiple carcinomas, mucosal atrophy, or massive lymphocyte infiltration. EBV+ cases show surprisingly few chromosomal abnormalities, with effects on cancer genes more often mediated by hypermethylation. As with NPC, EBV+ gastric carcinoma is more common in men; and different populations show great discordance in age-dependence. Studies with results ranging from significant association between EBV+ gastric carcinoma and men under 50, to a total lack of EBV+ gastric carcinoma in men under 60, are described in reviews by Herrera-Goepfert *et al.* and Uozaki *et al.* [81, 83], indicating that the demographic causes of gastric carcinoma are not yet known.

The latency programs in EBV-infected epithelial cells are slightly different from those in B cells. NPC and gastric carcinoma usually express EBNA1, LMP2A, EBERs, and the soluble protein BARF1, which in B cells is considered to be a lytic protein [84]. NPC also expresses LMP1, and *in vitro* studies suggest NPC could be treated by

blocking LMP1 with siRNA or chemicals like arsenic trioxide [77]. BARF1 functions as an oncogenic growth factor *in vitro*, and is a CSF-1 receptor homologue that prevents the antiviral cytokine CSF-1 from inducing macrophage differentiation and interferon production. Some cases of NPC, and most gastric carcinoma, show LMP2A expression in the absence of LMP1. Since these two genes seem to be induced by different signaling pathways in Latency II B cells (unlike Latency III), it is logical that the totally different milieu of the mucosal epithelium can support LMP2A expression without LMP1.

1.3 VIRUS-CELL INTERACTIONS

1.3.1 Latent genes

1.3.1.1 LMP1

Latent membrane protein 1 (LMP1) is the only latent EBV protein considered to be an oncoprotein. It is most easily described as a ligand-independent homologue of the B cell coreceptor CD40, which provides activating and survival signals in the absence of CD40 ligand. It has an N-terminal cytoplasmic tail (residues 1-23), six transmembrane domains (24-186), and a long C-terminal cytoplasmic domain (187-386). In the latter domain, there are two C-terminal activation regions (CTARs) that activate NFκB transcription factor pathways. CTAR-1 is required for B cell immortalization and contains binding sites for several TNFR-associated factors (TRAFs) which convey the NFκB-activating signal, while CTAR-2 does so by interacting with TNFR-associated death domain (TRADD). CTAR-2 induces a smaller array of NFκB isoforms and is not requred

for B cell immortalization. Among the NFκB-independent roles for these regions, CTAR-1 greatly enhances EGFR production, downregulates $p27^{KIP1}$, and activates ERK1/2 and PI3K-Akt kinase pathways, while CTAR-2 activates p38/MAPK and JNK/AP-1 signaling cascades. Between these two regions is a series of proline-rich repeats, which has been designated CTAR-3 because it binds JAK3 and thus activates some members of the STAT family of transcription factors [85-87].

LMP1 is not functionally interchangeable with CD40; it does not depend on trimerization and localization to lipid rafts for signaling, and uses TRAFs 3 and 5 rather than TRAFs 1 and 2 [88, 89]. Transgenic LMP1 does substitute for CD40 signaling to allow functional B cell development in mice, but cells expressing the LMP1 transgene overexpress activation markers and induce less inflammation. LMP1 also induces excessive isotype switching which may be related to IgG1+ cells having increased lifespan and less dependence on BCR signaling for survival than IgM+ cells. Both LMP1 and CD40 (if induced to signal constitutively rather than only upon activation) downregulate BCL6 and thus block GCs from forming [90-92]. LMP1 is produced abundantly but also has a high rate of turnover. This implies an important role as well for the short N-terminal domain since that is the initial site of ubiquitination necessary for degradation [93].

1.3.1.2 LMP2A

LMP2A functions as a ligand-independent mimic of BCR (B cell receptor) signaling in the absence of a functional B cell receptor, inducing B cells to become

activated and proliferate. If BCRs are present, LMP2A serves to block their pro-apoptotic signals. This occurs partially by excluding BCRs from the lipid rafts where they get cross-linked, which blocks activation of many downstream mediators of BCR signaling – and partially by attracting ubiquitin ligases to degrade kinases like Lyn [94]. Like LMP1, LMP2A activates the PI3K-Akt kinase pathway, leading to inactivation of GSK-3β, and subsequent accumulation of β-catenin, an important oncogene [95]. The PI3K-Akt pathway also helps LMP2A inhibit TGF-β-induced caspase activity, providing another anti-apoptotic effect. Effects of LMP2A that have been seen mostly in epithelial cells (e.g. carcinoma cell lines) include activating the ERK/MAPK and JNK/MAPK pathways, possibly increasing cell motility and proliferation, and also inactivating STAT3 and NFκB, repressing LMP1 expression [96]. LMP2A is both an activator and a substrate of ERK MAP kinase in B cells [97].

LMP2A consists of a long N-terminal cytoplasmic signaling domain (CSD) and a short C-terminal cytoplasmic tail, separated by twelve transmembrane domains. The only known function of the C-terminal region, and certain inner loops on the transmembrane domains, is to allow LMP2A to cluster with other proteins. These regions are highly palmitylated on cysteines, but that does not seem to be necessary for its clustering function [98, 99]. The N-terminal CSD contains numerous tyrosine residues including an immunoreceptor tyrosine-based activation motif (ITAM) similar to those in the BCR complex. This can substitute for the BCR and confer survival signals to cells that would otherwise not survive, although that does not seem to actually happen in immunocompetent EBV carriers [42].

Recently some investigators have become convinced that a low level of LMP2A expression is typical of the Latency I profile of gene expression – in BL, gastric carcinoma, and the resting B cells that form the normal blood reservoir for EBV [84, 100]. Longnecker and colleagues suggest that LMP2A enhances the immortalizing effect of c-*myc* in two ways that make BL cases better able to proliferate. First, by enhancing production of pro-survival Bcl proteins like Bcl-xL, thus counteracting the p53 pathway that is induced by c-*myc*; and second, by activating the ERK/MAPK pathway which degrades the pro-apoptotic Bim protein that is also induced by c-*myc* [100, 101]. Rechsteiner et al. hypothesize that the occasional LMP2A+ B cell found in healthy patients is a cell which has encountered its cognate antigen and undergone abortive BCR signaling, requiring LMP2A to block the BCR from activating the cell and reactivating lytic EBV [102].

1.3.1.3 LMP2B

LMP2B is identical to LMP2A but without the first exon, and thus without the N-terminal signaling domain. It is expressed from a bidirectional promoter that also drives LMP1 expression, and not from the LMP2A promoter. LMP2B has long been suggested to be a negative regulator of LMP2A, aggregating with LMP2A to sequester LMP2A from the lipid rafts, possibly by keeping it in perinuclear regions rather than on the cell surface [103]. It is believed to be expressed at levels inversely proportionate to the levels of LMP2A, and it inhibits the CSD of LMP2A from being phosphorylated. When overexpressed in EBV-infected cells, LMP2B increases the amount of BCR-induced

lytic reactivation, overriding the LMP2A blockade. However, when expressed in BJAB cells containing no other EBV proteins, LMP2B can globally block the phosphorylation that follows BCR crosslinking [104-106]. LMP2B, but not LMP2A, colocalizes with CD19, a part of the BCR complex that has regulatory effects on BCR signaling [103].

LMP2B does share some functions with LMP2A, despite lacking any signaling domain. Both proteins target interferon receptors IFNAR1 and IFNGR1 for degradation, leading to a global blockade of interferon signaling in infected epithelial cells; and both proteins induce adhesion and spreading of epithelial cells, through an unknown mechanism that is PKC-, PI3K-, ERK-, and PLC-γ-independent [107, 108]. Recently it has been found that EBV-negative BJAB cells expressing LMP2B have increased expression of many pro-apoptotic proteins, but also constitutive serine phosphorylation of these proteins that counteracts their apoptotic effect [106].

1.3.1.4 EBNA1

EBNA1 is a protein distinctive to lymphocryptoviruses. It is found in all known LCVs, while its equivalent in rhadinoviruses (e.g. HHV-8) is LANA1, which shares some EBNA1 functions but has no sequence homology. EBNA1 has a C-terminal domain, similar to the DNA-binding domain of papillomavirus protein E2, which mediates homodimerization and binds recognition sites in *OriP*, the EBV latent origin of DNA replication. EBNA1 binding induces a strong bend in episomal DNA at *OriP*, assisting in replication of the episome by host machinery [33]. The N-terminal domain of EBNA1 has AT hooks that bind metaphase chromosomes, and also interacts with

chromosomes indirectly through the cellular protein EBP2, allowing episomes to be efficiently assorted between daughter cells during cell division [109].

As a protein that is found in all forms of EBV latency in dividing cells, EBNA1 is one of the primary potential targets for EBV immune recognition. Between the C-terminal and N-terminal domains are glycine-alanine repeats (GAr) which inhibit proteasomal degradation of EBNA1 and its presentation by HLA I. EBNA1 has an unusually low rate of protein translation from mRNA, making it underrepresented in the pool of DRiPs (defective ribosomal products) that are responsible for much of the antigens that are presented by MHC I. This effect may be mediated by a high purine:pyrimidine ratio in the Gly-Ala region, leading to a slow rate of translation [110].

EBNA1 is expressed from three promoters. During Latency III, EBNA1 is expressed from long transcripts from the C and/or W promoters (Cp and Wp), which may also encode any of the other EBNA genes. During Latency I, it is expressed from the Q promoter (Qp), whose only protein product appears to be EBNA1 [33]. EBNA1 downregulates its own production, by inhibiting mRNA translation through an unclear mechanism [111], and by directly binding and repressing Qp during both Latency I and Latency III [112]. Finally, interaction between EBNA1 and the de-ubiquitinating enzyme USP7 leads to changes in the expression of MDM2 and p53, causing an anti-apoptotic effect. P53 is a tumor suppressor protein turned on in B cells after activation and proliferation, to stop the cell cycle and initiate apoptosis in the case of DNA damage or other stresses [110].

1.3.1.5 EBNA2

The most significant transcription factor in EBV latency is EBNA2. The acidic activation domain of EBNA2 recruits a large transcription complex including TFIIB, TFIIH, and p300/CBP [113]. EBNA2 can be described as a homologue for the intracellular region of Notch (Notch-IC), which lacks a DNA-binding domain but acts to induce transcription of a wide range of cellular genes, largely through cooperation with cellular transcription factors PU.1 and RBP-Jκ/CBF-1. RBP-Jκ serves as a repressor which recruits HDACs to its targeted promoters; binding by EBNA2 or Notch-IC turns it into an activator. However, EBNA2 is not known to share Notch's ability to counteract B cell differentiation by degrading transcription factors [33].

EBNA2 is one of the first proteins produced upon EBV entry into a cell, inducing expression of other Latency III genes, cellular activation markers like CD23 and CD21/CR2, and oncogens c-*myc* and c-*fgr*. Two studies have used microarrays to investigate the cellular genes whose expression is influenced by EBNA2 [114, 115]. Interestingly, EBNA2 represses the promoter for the Ig heavy chain locus on chromosome 14, explaining why Burkitt's lymphoma cell lines with Ig-µ:c-*myc* translocations are driven into growth arrest, rather than Latency III, by the sudden induction of EBNA2 [116].

EBNA2 is essential for transformation of B cells. The lower efficiency with which EBV-2 transforms primary B cells is due to differences in the EBNA2 sequence compared to EBV-1. EBNA2 induces expression of anti-apoptotic molecules like Bcl-2, and it inhibits apoptosis by binding and sequestering Nur77. Although it drives cells into the G1 and then the S phase of the cell cycle, by upregulating c-*myc*, c-*fgr*, cyclin D1,

and cyclin E1, EBNA2 also seems to enhance p53's induction of $p21^{WAF1}$, which induces S phase arrest. This effect on $p21^{WAF1}$, combined with EBNA2-induced downregulation of MAD2 and securin, can lead to unstable mitotic spindle complexes, premature anaphase and chromosomal instability. This may provide another mechanism for "hit and run" tumorigenesis [54].

Expression of LMP1, LMP2A and LMP2B is induced by EBNA2 during Latency III. EBNA2 activation of LMP2A and Cp/Wp depends on RBP-Jκ, while activation of the LMP1/LMP2B promoter depends on interaction with both PU.1 and RBP-Jκ. EBNA2-independent production of LMP1 or LMP2A is unusual and is usually associated with Latency II. There may be an auto-regulatory pathway in which LMP2A sustains its own expression by upregulating Notch [117], while LMP1 expression in HD and NPC has been associated with IL-10 and/or IL-21 secreted in the immediate milieu of the neoplasia [118, 119].

1.3.1.6 EBNA-LP

EBNA-LP (EBNA5) and EBNA2 are the first proteins expressed in newly infected cells. EBNA-LP is a coactivator for many of the genes induced by EBNA2. The name EBNA-LP is short for EBNA leader protein, because it is encoded by the upstream "leader" region of the large mRNA transcripts that can also encode any other EBNA gene. It has an unusual structure consisting largely of two domains of 22 and 44 amino acids which are encoded by the major internal repeat (W1 and W2 exons), leading to heterogeneity in the protein's size and structure [33].

Very early after infection, EBNA-LP disrupts the PML bodies of the nuclear matrix, removing Sp100 from these complexes. Sp100 and EBNA-LP both act as coactivators of EBNA2 at this stage, leading to overexpression of other EBV latent genes. Within 8 days of infection both EBNA-LP and Sp100 are concentrated in the PML bodies rather than being dispersed throughout the nucleus, and EBNA2 activation of latent genes is more restricted as cells become lymphoblasts expressing Latency III phenotypes [17, 120, 121]. Independent of its role as an EBNA2 coactivator, EBNA-LP acts anti-apoptotically by reducing the level of p53-dependent cell death. EBNA-LP binds the cyclin-dependent kinase inhibitor p14ARF and directs both p14ARF and p53 to complexes which lead to their proteasomal degradation [122]. EBNA-LP binds to another important tumor suppressor, Rb, but its effects on Rb are unknown.

1.3.1.7 EBNA3A, -3B and -3C

These three genes all encode large proteins (135-165 kDa) which are expressed at low levels, have long half-lives and contain a high percentage of the immunodominant epitopes found in healthy EBV carriers. EBNA3A and EBNA3C are necessary for EBV transformation of B cells, while EBNA3B is dispensible. The EBNA3 genes have limited sequence homology and each consists of a short 5' exon and a long 3' exon. All three contain a proline-rich domain and complicated amino acid repeat motifs which are unique to each protein [123, 124]. They share the ability to counteract some of EBNA2's upregulatory effects, by binding RBP-Jκ and restricting expression of LMP2A and the C promoter which encodes all EBNA open reading frames. However, all

three can disrupt at least one G_2/M cell cycle checkpoint [125], and EBNA3C in particular can also induce gene expression through RBP-Jκ and other transcription factors. There is significant redundancy among the EBNA3 genes, as studies deleting either EBNA3A or EBNA3C, while leaving the rest of the viral latency profile intact, have found no effects on CR1, c-*myc*, LMP, or EBNA gene expression [126].

EBNA3C (EBNA6) interacts with RBP-Jκ and HDAC1 and thus can upregulate CD21/CR2. It also enhances expression from the LMP1 promoter, by coactivating EBNA2 and by interacting with PU.1 through its basic leucine zipper (bZIP) domain. EBNA3C coactivation of EBNA2 target genes depends on interaction with SUMO proteins, which may then deactivate HDACs or enhance histone acetylation by recruiting p300/CBP [127]. Neither the SUMO-interacting domain nor the bZIP is found in other EBNA3 proteins. EBNA3C thus enhances EBNA2's effects in several ways, while still counteracting EBNA2 by repressing the viral C promoter.

The RBP-Jκ binding region is probably the most essential EBNA3C domain for inducing cell proliferation, through upregulating proteins like integrin α4 and the TCL1 proto-oncogene. TCL1 is a transcription factor associated with GC B cells, and is downregulated by EBNA2 and LMP1 in some lymphomas, causing them to exit the processes of affinity maturation and antibody rearrangement. Many genes have been found to be downregulated by EBNA3C, including Jagged1 (a major ligand for the EBNA2 homologue Notch), and cell cycle regulators $p16^{INK4A}$ and $p27^{KIP1}$ (cyclin-dependent kinase inhibitors 2A and 1B) [128-130]. EBNA3C is associated with other phenotypes that reinforce cell cycle progression, including hyperphosphorylation and degradation of Rb, and enhanced cyclin A-dependent kinase activity, which increases

the amount of free E2F1, a transcriptional activator of S phase entry [131, 132]. EBNA3C also interacts with the cell stress-associated protein Gadd34, possibly inhibiting downstream apoptotic effects of the unfolded protein response, and was seen to induce aberrant cell division [130, 133].

EBNA3A (EBNA3) cooperates with EBNA3C in repressing the pro-apoptotic gene Bim (Bcl2-interacting mediator of cell death). Bim is induced by overexpression of c-*myc*, so this effect of the EBNA3 genes further enhances the oncogenic effect of c-*myc* deregulation and may explain the discovery of EBNA3A and EBNA3C in some unusual cases of Burkitt's lymphoma [126]. EBNA3A and EBNA3C also share the ability to bind the broadly acting co-repressor CtBP. In doing so they resemble the Drosophila gene *Hairless* in their ability to repress RBP-Jκ by binding both it and CtBP [124]. In fact, RBP-Jκ is the human homologue of Drosophila gene *Su(H)* or *Suppressor of Hairless*.

Unlike EBNA3C, EBNA3A is known to regulate chaperone complexes. It upregulates production of chaperones Hsp70 (HSPA1A) and Hsp70B/B' (HSPA6), and co-chaperones Hsp50 and Bag3 – and also physically interacts with these proteins, which form a complete Hsp70 chaperone complex [134]. EBNA3A also binds part of the TCP-1 chaperonin complex, and XAP-2, a factor involved in Hsp90 chaperone activity. EBNA3A relocates these chaperone complexes to the nucleus, and they may be important for maintaining correct folding of EBV genes with unstable tertiary structure. XAP-2 is also known to bind the oncogenic X antigen of hepatitis B virus [135].

One recent study showed that proliferating cell lines can be derived using an EBNA3A-deleted virus, as long as pre-activated B cells from Waldeyer's ring are used

rather than resting B cells from the peripheral blood. This study found that EBNA3A deficiency increased the rate of apoptosis and vastly increased the production of the cell cycle regulator $p16^{INK4A}$ (like to EBNA3C deficiency), while decreasing the amount of many proliferation-associated proteins like lymphotoxin and S100A4/metastasin. However, one of the other genes enhanced by EBNA3A was the tumor suppressor Rb. Of the genes regulated by EBNA3A, 16.2% were regulated in the opposite direction by EBNA2, while 9.1% were regulated in the same way by EBNA2 [136].

EBNA3B (EBNA4) has been less characterized, as it is apparently non-essential for inducing transformation and proliferation of B cells. One study found that EBNA3B appears to greatly repress expression of the genes ENTH4 (epsin 4), TTF2 (an RNA polymerase II transcription termination factor), and most interestingly, CXCR4. CXCR4 is a receptor for the chemokine CXCL12/SDF-1, known to be downregulated by HHV-6, -7, and EBV. Its expression on the cell surface is important for retaining immature B cells in the bone marrow. Though its role in the trafficking of mature B cells is unclear, it may help them escape the lymphoid organs into the periphery [137]. EBNA3B is also unique in possessing an unusual motif called a stonin homology domain [123].

For each EBNA protein, a schematic of the important domains and amino acids for its protein-protein interactions and its effect on cells can be found in the review by Johannsen et al. [138].

1.3.1.8 BARTs and microRNAs

The BamHI A region of the EBV genome contains several putative open reading frames, though the rightward RNA transcripts from this region are usually untranslated and thus just called “BamA rightward transcripts” (BARTs). Various BARTs are found in most EBV diseases and latency types, but expression varies widely and unpredictably between cell lines, and the protein products of the ORFs have not been found *in vivo*. The putative ORFs include the A73, RPMS1, and RK-BARF0 proteins, which if artificially expressed can (respectively) interfere with Src kinases, bind to RBP-Jκ, and bind to Notch proteins [139-141].

BARF0 transcripts of unknown function are specifically associated with latency in epithelial cells. This is typical of both the “Latency I” of gastric carcinoma cases and the “Latency II” of NPC. The BARF1 protein is also often detectable in these cases. BARF1, or the viral soluble CSF1 receptor, is considered an early lytic gene in B cell infection, but has been seen in both gastric carcinoma and NPC, in the absence of any other lytic genes [142, 143]. The mRNA for BARF0 and other BART mRNAs also contains several microRNAs of unknown function, many of which come from a single long and stable intron [144]. The only other known EBV microRNAs are in the BamH region of the genome, are encoded in Latency III B cells as part of the EBNA transcripts, and seem to be involved in breakdown and turnover of those transcripts [145].

1.3.1.9 EBER RNAs

EBER1 and EBER2, or “EB virus-encoded small RNAs”, are non-encoding transcripts, without 5’ caps or poly(A) tails, and contain several stem-loop structures [146]. They are highly abundant and almost always found in EBV-infected cells *in vivo*, making them good histological markers for FISH to identify whether a lesion is EBV-positive. EBERs are predominantly found in the nucleus, but can bind to the cytoplasmic kinase PKR and interfere with antiviral interferon signaling. It seems that under normal circumstances they actually inhibit interferon-induced apoptosis by some mechanism downstream of PKR. They are not essential for immortalization but do enhance the production of growth factors IL-10, IL-9 and IGF-1. IL-10 upregulation seems to occur by binding RIG-1, a common antiviral detector of double-stranded RNAs. EBERs also interact with the large ribosomal subunit L22, and the La protein which helps RNA polymerase III produce small RNAs such as the EBERs themselves. EBER1 and EBER2 are highly conserved among EBV homologues from other primate species, and are homologous to the VAI/VAII RNAs that are important for adenoviruses [147, 148].

Surprisingly, EBERs have recently been seen secreted by Latency I and Latency III B cells, in complexes with La protein. This activates the dsRNA-detecting receptor TLR3, which in combination with RIG-1 may induce significant levels of type I interferon and proinflammatory cytokines in some cases of lymphoma or CAEBV [149].

1.3.2 Lytic reactivation

Lytic reactivation is generally induced *in vitro* by the addition of histone deacetylase (HDAC) inhibitors, which enhance acetylation of the histones associated with the EBV episome. The Z and R promoters, for the genes encoding lytic switch proteins ZEBRA and Rta, both contain ZEBRA response elements and AT-rich binding sites for Sp1/Sp3 factors and MEF2D. During latency MEF2D recruits HDACs to these promoters, leading to condensed chromatin and gene silencing. Adding HDAC inhibitors like TPA and butyrate leads to Zp and Rp activation by directly influencing HDACs; B cell receptor signaling has the same effect, through phosphorylating MEF2D which causes it to associate with histone acetyltransferases [HATs] rather than HDACs [150]. ZEBRA and Rta are necessary to induce the cascade of viral gene expression necessary for lytic replication. ZEBRA is expressed first, upstream of Rta, in physiological systems including BCR signaling [151].

There are many drugs that induce lytic reactivation. TPA and butyrate act directly on the promoter, although TPA-driven reactivation is also dependent on activating PKC kinase pathways. Demethylating agents like azacytidine induce lytic reactivation and cessation of the cell cycle by making CpG sites in Zp and Z-responsive promoters more accessible [30]. Valproic acid leads to both histone acetylation and demethylation of the lytic promoters [152], while 5-fluorouracil, *cis*-platin, taxol, doxorubicin, methotrexate, gemcitabine, dexamethasone, and rituximab induce the lytic cycle through a variety of signal transduction pathways [153, 154]. Consequently, EBV reactivation is sometimes a complication of chemotherapy [155]. One group has done a trial of azacytidine in NPC

and AIDS-lymphoma patients, to drive infected B cells out of proliferation and into the lytic cycle. *In vitro* this drug causes lytic induction and cell death, but it had no effect *in vivo* [156].

Therapy for EBV infection sometimes includes inhibitors of late lytic replication (e.g. acyclovir), which block virus production but do not affect the progression of existing tumors. Inducing the initial lytic events, while blocking actual virus production with acyclovir, has been suggested as a way to inspire cytotoxic immunity against a tumor without enabling the virus to spread, and has had good results *in vitro* and in mouse models [152-154, 157]. Retinoic acid, a form of vitamin A, both inhibits the growth of cancer cells and restricts lytic reactivation by binding to nuclear retinoic acid receptors and ZEBRA, but also seems to specifically encourage the differentiation of memory B cells into PCs and is a promising component of chemotherapy for MM [158, 159].

There is a comparatively small number of proteins known to influence activation of the Z promoter. EBV LMP1 and cellular CD40 signaling both repress lytic reactivation, and EBV LMP2A represses lytic reactivation in a manner negatively regulated by LMP2B [105, 160]. After BCR signal transduction, lytic reactivation occurs because of MEF2D dephosphorylation, because JNK MAPK signaling activates c-Jun/ATF-2, and because p38 MAPK signaling activates CREB/ATF-1 [30, 161]. These CREB/ATF factors bind to the CRE-like ZII motif in the Z promoter, which is also bound by AP-1 transcription activators. The Z promoter is activated by Smad proteins after TGF-β stimulation, which may be one way that ZEBRA reinforces its own production (in addition to directly binding its promoter). Sμbp-2 and ZEB1(TCF8) are two transcription factors that repress the Z promoter, although it is likely that TGF-β/Smad signaling turns

ZEB1 into an activator [162, 163]. The Z and R promoters both have potential sites for the negative regulatory factor YY-1, and the R promoter is induced by EGR1(Zif268), which in turn is induced by ZEBRA [30, 164].

Two recent studies [165, 166] added XBP-1 to this list. XBP-1 and Blimp1 are the primary proteins that induce B cells to exit either a proliferating or quiescent phenotype and terminally differentiate into antibody-secreting plasma cells (PCs). XBP-1-mediated induction of ZEBRA expression confirms a series of associations between B cell terminal differentiation and lytic reactivation which began in the mid-1980s. *In vitro*, Crawford and Ando [167] found that in LCLs, the cells producing lytic antigens were overwhelmingly positive for the plasma cell marker PC-1/ENPP1. Wendel-Hansen et al. [168] found that a non-proliferating minority of cells within an LCL contained high levels of cytoplasmic Ig and low levels of latent EBV antigens. These findings fit into the model for *in vivo* virus propagation over decades, in which latently infected resting memory cells become activated, traffic from the peripheral blood to the secondary lymphoid organs and differentiate into PCs while also undergoing lytic reactivation [23].

The relationship between PC differentiation and lytic reactivation is not necessarily a one-way path in which XBP-1 is upstream of ZEBRA. At least one factor, IL-21, can induce XBP-1 expression and other PC factors while also leading to lytic induction via a JAK/STAT pathway unrelated to PC differentiation [169]. No lytic DNA is normally found in the peripheral blood, indicating that the later steps of lytic replication occurs after plasmablasts have trafficked to lymphoid organs [24]. However, ZEBRA's binding to Pax5, a TF that counteracts XBP-1 and Blimp1 activity, seems likely to inhibit Pax5 activity and thus encourage PC differentiation [170].

1.4 LYMPHOBLASTOID CELL LINES

In vitro EBV infection is a convenient way to produce B-lymphoblastoid cell lines (LCLs), even from small, residual and poorly preserved blood samples [171]. These cell lines can be used as targets for assays of cytotoxic T cells, as models for immortalization and senescence, or as sources of DNA for genotyping. Some investigators have used LCLs as sources of RNA for profiling individuals' gene expression, as an attractive alternative to the limited cell number and potentially degraded sample quality of using primary cells [172]. Isogenic LCLs can also be created using the donor's own strains of EBV – simply by inducing latently infected primary B cells to produce infectious virus *ex vivo*, which infects and transforms other B cells in the culture. Isogenic LCLs have been used to identify the strain of EBV responsible for a disease – for example, to see whether a case of PTLD was caused by the organ donor's or the transplant recipient's infection [173].

LCLs are often used as models for PTLD [174], as they have similar morphology and phenotype involving high expression of activation and adhesion markers, and grow readily in SCID/hu mice. LCLs have been characterized for properties such as time to clonality; rate of episome multiplication; frequency of Ig isotypes; patterns of DNA methylation in contrast to those of primary cells; and the rates of genetic instability and selection that sometimes lead to truly tumorigenic cell lines [175-180].

In general, LCLs are considered to be more similar to CD38- CD23+ PTLDs derived from germinal center-experienced "bystander" B cells than the CD38+ CD23- PTLDs that originate from centroblasts [181]. LCLs are phenotypically more

heterogeneous than they are presumed to be by many studies that use them as interchangeable reagents in immunological assays. There is a consensus profile of LCLs (CD10- CD19+ CD20+ CD21+ CD23+ CD30+ CD40+ CD40L- CD77- Fas+ OX40- OX40L-, IL-6 secreting), accompanied by unpredictable and variable CD23 and CD38 expression. The consensus is that they express most or all of the Latency III genes, and usually a background level of spontaneous lytic reactivation despite negligible release of infectious virus into the supernatant [182-185]. The level of expression of latent genes is expected to be uncorrelated to lytic activity, and also uncorrelated to the number of latent episomes, though this has not been shown for all latent genes [186].

One study described a CD19-CD20- subgroup, which produced less soluble Ig and negligible amounts of the paracrine growth factors IL-6 and IL-10 [187]. The surface markers that are expected to be most variable on LCLs are proteins upregulated after activation, like CD38 and CD23 [188-190]. These findings suggest that LCLs should not be used as if interchangeable in clinical studies, particularly those that depend on coculture with other cells – just as we do not consider all endothelial cell lines, or pancreatic cell lines to be interchangeable. Different LCLs can have varying effects on other cells in the culture for reasons including diversity in growth rate and tendency to aggregate; diversity in cytokine secretion; and diversity in the ability to bind and stimulate cognate T cells.

It has been observed that LCLs usually achieve clonality or biclonality within four months of establishment; given the diversity in lineage of the B cells that can be transformed by EBV, and the epigenetic factors that influence gene expression, this necessarily implies that LCLs are not representative of the B-cell population from the

donor whose cells were used to establish the LCL [175, 191]. Monoallelic transcription at seemingly random sites also influences their gene expression, implying significant diversity even between LCLs derived from the same blood draw [192]. Rather than serving as surrogates for gene expression of a given blood donor, they may be more useful as models for *diversity* in B cell gene expression.

We have known for almost 40 years that LCLs differ in their capacity for lytic induction – both spontaneously and in response to stimuli [193, 194] – but not the sources of this diversity. Hypotheses about combating EBV disease by manipulating its reactivation are incomplete without knowing what cellular factors are inducing or repressing this reactivation. Therefore, we need to use modern techniques to learn more about the variables that influence the life cycle of the virus harbored in these cells.

2.0 STATEMENT OF THE PROBLEM

In this study we address the unexplored diversity of EBV-immortalized B-lymphoblastoid cell lines in terms of virus-cell interaction. These cell lines are established in the laboratory but resemble cases of post-transplantation lymphoproliferative disease that express the “Latency III” profile of EBV latent gene expression. In addition to being employed as sources of DNA for characterizing individuals’ genotypes, they are used as models for lymphocyte transformation and as targets for autologous cytotoxic T cells. Studies have shown diversity in their cell surface phenotype, their production of growth factors, and their ability to be induced by stimuli to switch from Latency III into the lytic life cycle that produces infectious virus. However, it is hard to describe each LCL’s phenotype before using it, and it is not clear which aspects of the phenotype are relevant for a given study, so they are often treated as interchangeable.

Many studies which use LCLs to look at the effects of gene deletions or mutations use only one or two LCLs to show the control phenotype. LCLs are also often seen to vary widely in expression of EBV genes with no particular pattern or explanation of the diversity. The diversity of LCLs for EBV gene expression has rarely been studied using a large sample size, and such studies were more common in past years when

sera against “EBNA” (EBV nuclear antigen), “EA” (early antigen), and “VCA” (viral capsid antigen) were used to characterize cells for EBV latency or stages of the lytic cycle. Using quantitative reverse transcriptase-PCR to measure production of the full complement of EBV latent genes in a representative sample of LCLs will add to our knowledge of the Latency III phenotype. This is especially likely when information about latent EBV gene expression is combined with measurement of lytic reactivation, host gene expression profiling, and phenotypic information about each LCL as determined by expression of cell surface markers.

Our laboratory has used LCLs as cell lines containing various mutant and deletant strains of EBV, and we have often measured the ability of different EBV variants to transform B cells by observing whether, and how effectively, they induce LCLs to grow out. In these experiments we have become aware that data based on LCL creation needs to be replicated with cell lines from multiple donors, as LCLs derived from different individuals (even using the same strain of virus) show different patterns of growth and EBV activity. Also, in studies of transplant recipients who are immunosuppressed and at high risk for PTLD, we have observed that some EBV-infected cells have a “high-copy” phenotype (> 10 EBV genomes/cell, compared to less than 5 and usually 1 or 2 in normal EBV+ peripheral blood cells). Patients at higher risk for PTLD have more high-copy cells, which also have aberrant cell surface phenotypes, including a high rate of surface immunoglobulin-negative (Ig-null) cells.

We hypothesized that LCLs could also be categorized into high-EBV-copy and low-EBV copy phenotypes, and that this might correlate with cell surface phenotype. We also hypothesized that much of the diversity of LCLs in their tendency to switch to the

lytic cycle could be explained by diversity in host factors, including expression of transcription factors that influence both EBV lytic reactivation and B cell differentiation into plasma cells. With both these ideas in mind, we believed both goals could be addressed by studying a large population of LCLs, with the diversity of the population maximized by having each LCL from a distinct blood donor. The following experimental components were proposed and completed.

SPECIFIC AIM ONE: Analyze EBV latency, lytic gene expression, and cellular phenotype in a subset of EBV-immortalized LCLs. We are aware of the diversity among these seemingly similar cell lines, in aspects including expression of activation markers, production of growth factors, and level of EBV reactivation in response to stimuli like BCR cross-linking. Using a group of LCLs all established using the same virus strain, we categorized them by their profile of spontaneous lytic EBV activity and latent gene expression, hoping to find evidence for robust subsets of LCLs that can be categorized by their patterns of EBV replication. We also used 8-color flow cytometry to characterize LCLs by expression of cell surface markers, with the goal of being able to predict a cell line's EBV activity by using flow cytometry to learn about its lineage or activation state.

SPECIFIC AIM TWO: Measure host gene expression and identify cellular pathways which contribute to EBV lytic reactivation. There are several factors known to influence lytic reactivation, including BCR signaling, drugs that affect the chromatin, and direct induction of lytic switch gene ZEBRA by transcription factors like

Smad proteins and XBP1. Using microarrays and quantitative RT-PCR we sought to describe host gene expression patterns in LCLs of different phenotypes. We focused on looking for host pathways that induce or repress lytic reactivation, and used protein measurements and an ER stress-inducing drug to describe the effects of one such pathway, the unfolded protein response.

Most of the data in **Aim One (p. 59)**, and some of the data in **Aim Two (p. 83)** – have been published in the journal *Virology* [195]. Other data in the second aim will be presented at the 14th Biennial Conference of the International Association for Research on Epstein-Barr Virus and Associated Diseases, and will be part of a future submitted manuscript.

3.0 MATERIALS AND METHODS

3.1.1 Establishment of LCLs and cell culture.

Lymphoblastoid cell lines were established by suspending PBMCs in RPMI-1640 including 20% FBS and tacrolimus, to which was added supernatant from the B95-8 virus-producing cell line. After 10-15 days of incubation the viral supernatant was removed and immortalized cells were maintained for at least four weeks before being used; they were then grown in RPMI-1640 including 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin.

A total of 62 LCLs from 62 different donors were studied. These lines had been established for two separate immunological studies. The collection of pediatric LCLs was established during a study of post-transplant lymphoproliferative disease, and the adult LCLs were established during a HIV-related study. After determining the average genome copy number/cell along with other characteristics, cell lines with > 200 copies/cell were classified as permissive for spontaneous lytic virus induction.

LCLs were split every 3-6 days, depending on density. Every split was preceded by cell counting using a Neubauer hemacytometer, and LCLs were all split to $4x10^5$ cells/ml. For some, dividing to concentrations below $1x10^5$ cells/ml led to very slow growth or cell death, so counting before splitting was necessary. For RNA

measurements, protein measurements, and flow cytometry, proliferating LCLs were always used, with proliferating defined as 2 days after being split to $4x10^5$ cells/ml. For DNA measurements to characterize LCLs by number of EBV genomes/cell, samples were taken more frequently, including times when the cells were at high density, though never more than 5 days after being split (see **Figure 4**).

For inhibiting lytic viral replication, acyclovir was added to the media at a concentration of 22.5 µg/ml. For inducing the unfolded protein response, thapsigargin was added to the media at a concentration of 500 nM. TPA (2 nM) and sodium butyrate (500 µM) were also used in studies of cells induced to lytic reactivation. All these chemicals were obtained from Sigma.

3.1.2 Cell lysis and DNA PCR.

For DNA measurement, cell pellets were lysed in 10mM Tris [pH 7.6] containing 50mM KCl, 2.5 mM $MgCl_2$, 1% Tween 20, and 0.1 mg/ml proteinase K. Lysis was done for 60 min. in a 55ºC waterbath, followed by inactivation of proteinase K by incubating for 15 min. in a boiling waterbath. For measuring the number of EBV genomes, we detected a sequence in the BLLF1 gene encoding viral gp350 (forward primer 5'-GTATCCACCGCGGATGTCA-3'; reverse primer 5'-GGCCTTACTTTCTGTGCCGTT-3'; and probe 5'FAM-TGGACTTGGTGTCACCGGTGATGC-TAMRA-3'). For normalizing to the amount of cellular DNA, we detected a sequence in human GAPDH [196].

For GAPDH PCR, lysates were diluted 1:10 into the same buffer without proteinase K. For gp350 PCR, lysates were diluted further (1:50) into the same buffer, without proteinase K, and including a background of 25 cell equivalents/ul of DNA from

the EBV-negative DG75 cell line. Lysates of the DG75 cell line were used as standards for GAPDH DNA, while lysates of the Namalwa cell line, each of which contains two integrated copies of the EBV genome, were used as standards for EBV DNA.

DNA PCR was quantitative real-time PCR performed on an ABI 7500 thermal cycler. Each PCR reaction was a 50ul volume containing 20 µl of DNA lysate, and 30 µl of PCR master mix. This mix contained 0.6X PCR Buffer II; 0.5% AmpliTaq Gold (Applied Biosystems); 0.8% ROX reference dye (Invitrogen); 1.5 mM $MgCl_2$; 200 µM of each dNTP [dATP, dCTP, dGTP, dTTP]; 400 nM forward primer, 400 nM reverse primer; and 200 nM probe (primers from Integrated DNA Technologies). The program was 2 minutes at 50ºC; 10 minutes at 95ºC; 40 cycles of (15 seconds at 95ºC, 1 minute at 60ºC); and 5 minutes at 60ºC. The reaction mix uses only 60% of the recommended amount of PCR Buffer II and $MgCl_2$ because DNA lysis buffer makes up 40% of the reaction.

3.1.3 Cell lysis and RNA PCR.

RNA was extracted from cell pellets using the TRIzol process (Invitrogen) and resuspended in DEPC-treated water. RNA was then DNAse-treated using a Zymo Research kit, and transcribed into cDNA using MultiScribe reverse transcriptase (ABI). For samples that were used in large-scale analysis of cellular gene expression with small volumes of cDNA (Low-Density Array), a High-Capacity cDNA Archive Kit (ABI) was used to transcribe cDNA, producing a greater number of cell equivalents per volume.

For each viral transcript, the standards used were purified PCR product containing a known number of copies of the target sequence. The buffer used to dilute these standards was 10 mM Tris [pH 7.6] including 0.1% Tween 20, with a background of 25 cell equivalents/ul of DNA from the DG75 cell line. Viral gene expression was expressed as mRNA copies/cell; the number of cells was determined by measuring β_2-microglobulin cDNA, using a predesigned primer set (ABI). For β_2-microglobulin measurement, the standards used were dilutions of cDNA from the IB4 cell line, each containing a known number of cell equivalents.

All RT-PCR was quantitative real-time PCR performed on an ABI 7500. Each PCR reaction was a 25ul volume containing 2µl of cDNA template, and 1X PCR Buffer II; 0.5% AmpliTaq Gold (Applied Biosystems); 0.8% ROX reference dye (Invitrogen); 2.5 mM $MgCl_2$; 200 µM of each dNTP [dATP, dCTP, dGTP, dTTP]; 400 nM forward primer, 400 nM reverse primer; and 200 nM probe (primers from Integrated DNA Technologies). The program was 2 minutes at 50ºC; 10 minutes at 95ºC; 40 cycles of (15 seconds at 95ºC, 1 minute at 60ºC); and 5 minutes at 60ºC.

3.1.4 Viral PCR targets.

EBV mRNA targets included the latent genes EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, LMP1, LMP2A, LMP2B, and the lytic genes ZEBRA and BMLF1. Also quantified were the numbers of transcripts from the C, W, and Q promoters. Qp is a latent promoter, but a measurement of its transcripts also detects transcripts from the lytic F promoter. For both Cp and Wp, two alternative splices were measured – a

nonproductive splice that encodes EBNA1, -2, -3A, -3B, or -3C; and a *productive* splice that encodes EBNA-LP as well as one of the other EBNA proteins. The amount of EBNA-LP mRNA can be measured as the sum of the Cp and Wp productive splices. All EBV transcripts except BMLF1 were quantified with primers crossing an mRNA splice site, detecting cDNA but not genomic DNA. Primer sequences are found in **Table 2**.

3.1.5 Flow cytometry.

For surface staining, proliferating cells were washed in cold PBS, and chilled for 1 hour in FACS buffer (HBSS including 2.0% BSA, 5 mM EDTA, and 0.1% sodium azide) to block, followed by filtering through a 100 µm nylon mesh cell strainer (BD Falcon). Cells were stained in a cocktail of 8 antibodies at known concentrations in 30-100 µl FACS buffer. Primary staining was in the dark, on ice for 30-60 minutes. This was followed by two washes in FACS buffer. Washes during FACS staining consisted of centrifuging for 3 minutes at 8ºC, at 1200 rpm in a Beckman SX4250 rotor for 5ml tubes, or 1800 rpm in a Beckman S2098 rotor for 96-well U-bottom plates. Secondary incubation was with streptavidin-Pacific Orange (Molecular Probes) to fluorescently label the proteins stained with biotin-linked antibodies. Incubation with streptavidin-PO was also on ice in the dark for 30-60 minutes. After two more washes cells were brought up in FACS buffer containing 1% PFA, to fix for at least 30 minutes. If cells were to be stored for more than 1 night (up to 3 nights), they were then spun down and brought up in FACS buffer with 0.1% PFA to minimize bleaching of APC and other fluorophores.

Table 2. Sequences of primers used for quantitative RT-PCR of EBV transcripts.

Transcript	Primers (5' to 3' sequence)	
EBNA1	forward	GAT TCT GCA GCC CAG AGA GTA GTC
	reverse	TCG TCA GAC ATG ATT CAC ACT TAA AG
	probe	TCG TCG CAT CAT AGA CCG CCA GTA GAC
EBNA2	forward	TAA CCA CCC AGC GCC AAT C
	reverse	GTA GGC ATG ATG GCG GCA G
	probe	CAC CAC GTC ACA CGC CAG TGC TGG GT
EBNA3A	forward	GAT TCT GCA GCC CAG AGA GTA GTC
	reverse	CTT CTT CCA TGT TGT CAT CCA GG
	probe	CCC GGC CTG TCC TTG TCC ATT TTG
EBNA3B	forward	GAT TCT GCA GCC CAG AGA GTA GTC
	reverse	CCA CGC TTT CTT CAT TAT TCA GGT
	probe	TAG ACC GCC AGT AGA CCT GGG AGC AGA
EBNA3C	forward	GAT TCT GCA GCC CAG AGA GTA GTC
	reverse	CCA GGG TCC TGA TCA TGC TC
	probe	AAG ACC CAC CAT GGA ATC ATT TGA AGG A
LMP1	forward	TCA TCG CTC TCT GGA ATT TG
	reverse	TCC AGA TAC CTA AGA CAA GTA AGC AC
	probe	AGC ACA ATT CCA AGG AAC AAT GCC TGT C
LMP2A	forward	CTA CTC TCC ACG GGA TGA CTC AT
	reverse	GGC GGT CAC AAC GGT ACT AAC T
	probe	TGT TGC GCC CTA CCT CTT TTG GCT GGC G
LMP2B	forward	CGG GAG GCC GTG CTT TAG
	reverse	GGC GGT CAC AAC GGT ACT AAC T
	probe	TGT TGC GCC CTA CCT CTT TTG GCT GGC G
C2:W1 LP-	forward	TCC TGC ACG TGA GCA TCC T
	reverse	TTC TAC GGA CTC GTC TGG GTT
	probe	TGA AGG CCC TGG ACC AAC CCG
C2:W1 LP+	forward	TCC TGC ACG TGA GCA TGG G
	reverse	TTC TAC GGA CTC GTC TGG GTT
	probe	TGA AGG CCC TGG ACC AAC CCG
W0:W1 LP-	forward	CCA GGA GTC CAC ACA AAT CCT A
	reverse	TTC TAC GGA CTC GTC TGG GTT
	probe	TGA AGG CCC TGG ACC AAC CCG
W0:W1 LP+	forward	CAG GAG TCC ACA CAA ATG GGA
	reverse	TTC TAC GGA CTC GTC TGG GTT
	probe	TGA AGG CCC TGG ACC AAC CCG
BZLF1	forward	TTC CAC AGC CTG CAC CAG T
	reverse	AGC AGC CAC CTC ACG GTA GT
	probe	CAA CAG CCA GAA TCG CTG GAG GAA TGC G
BMLF1	forward	CCT ACC TCG GCA TCG TTT GT
	reverse	TCC GCG TCG CCT TTT GT
	probe	TGA CTG TCT TGT CCT GTA GGT CCC ACT TCT
Fp-Qp	forward	CTT GAA AAG GCG CGG GAT A
	reverse	GCG GTC TAT GAT GCG ACG AT
	probe	CCA AAC GCT CAT CCC AGG GAA GC

*All probes were conjugated with 5' FAM and a 3' TAMRA quencher.

For intracellular staining, proliferating cells were spun down, washed once in cold PBS, filtered through a cell strainer, and brought up in 100 µl Cytofix/Cytoperm (BD) to fix and permeabilize. After 30 minutes incubating at room temperature, cells were washed twice in FACS buffer with 0.1% saponin. Staining was done in FACS/saponin for 30-60 minutes in the refrigerator [4ºC], with antibodies labeled with Alexa Fluor-conjugated Zenon goat f(ab')2 fragments (Molecular Probes). Stained cells were washed twice in FACS/saponin and brought up in FACS containing 0.1% PFA for storage of up to 3 nights.

The following mouse α-human monoclonal antibodies were used: PE-α-IgG (clone G18-145, BD); PE-α-CD138 (clone M115, BD); PE-α-BR3 (clone 11C1, BioLegend); PE/Cy5.5-α-CD19 (clone SJ25-C1, Caltag); PE/Cy7-α-HLA-DR (HLA II) (clone L243, BioLegend); APC-α-IgM (clone MHM-88, BioLegend); Alexa647-α-CD23 (clone D3.6, BioLegend); Alexa647-α-CD30 (clone MEM-268, BioLegend); APC/Cy7-α-CD20 (clone 2H7, BioLegend); APC/Alexa750-α-CD27 (clone CLB-27/1, Caltag); Pacific Blue-α-HLA-A,B,C (HLA I) (clone W6/32, BioLegend); Pacific Blue-α-CD45 (clone HI30, BioLegend); Pacific Blue-α-CD69 (clone FN50, BioLegend); biotin-α-IgD (clone IA6-2, BD); biotin-α-CD38 (clone AT13/5, AbD Serotec); and biotin-α-CD40 (clone LOB7/6, AbD Serotec).

We also used PE/Cy7-conjugated rat α-mouse B220 (BD), and FITC-conjugated polyclonal goat antibodies (Biosource) specific for human IgA and for all human Ig's (pan-Ig). Antibodies against viral proteins included mouse α-ZEBRA (clone BZ.1, Santa Cruz), and mouse α-gp110 (clone 5B2, Abcam). The samples were measured in a BD FACSAria, and data was analyzed using FACSDiva and FlowJo software.

3.1.6 Gene expression microarrays.

RNA was isolated using the TRIzol process (Invitrogen) and resuspended in DEPC-treated water. Technicians at the University of Pittsburgh Genomics and Proteomics Core Laboratories (GPCL) transcribed the RNA into cDNA, which was hybridized to Illumina HumanRef-8 v2 BeadChip arrays. Software used to analyze the data included caGEDA (http://bioinformatics2.pitt.edu/GE2/GEDA.html) to do efficiency analysis and get J5 scores; DAVID (http://david.abcc.ncifcrf.gov/) and Ingenuity Pathways Analysis (Ingenuity) to get information about gene ontologies, pathways and biological roles of each gene of interest; and GSEA (http://www.broad.mit.edu/gsea/) to get information about entire sets of genes, rather than individual genes, that were upregulated in each sample population.

$$J5_i = \frac{\bar{A}_i - \bar{B}_i}{\frac{1}{m}\sum_{j=1}^{m} |\bar{A}_j - \bar{B}_j|}$$

Figure 2. J5 measures differential expression of a gene in absolute terms. For a set of ***m*** genes (in this case, m=20589), sample population **A** is compared to sample population **B**. For each gene ***i***, we take the difference between the mean expression level in A and the mean expression level in B. We then divide this difference by the average of all such differences across the set of genes.

DAVID analyses were begun on March 28, 2009, with the DAVID 2008 Knowledgebase that had been updated March 2008. We used Entrez gene symbols to identify genes, and searched using the “HUMAN” database. We were looking for all

gene ontologies that contained more than 5 total genes and at least 1 gene that was determined to be differentially expressed in our study. GSEA analyses were begun on March 19, 2008, with the following settings: 1000 permutations; genes identified by HUGO gene symbol with *collapse dataset* set to false; weighted enrichment score normalized by gene set size; gene sets from GSEA databases c2.all.v2.5.symbols.gmt (curated) and c3.all.v2.5.symbols.gmt (motif); and gene sets of < 15 or > 500 genes filtered out. The J5 score **(Figure 2)** is a measure of the scale of difference of the sample group means for a given gene relative to the average difference of all the genes on the array [197].

3.1.7 RT-PCR arrays.

RNA was isolated using the TRIzol process and reverse-transcribed using the High-Capacity cDNA Synthesis kit (ABI); cDNA was mixed with TaqMan Gene Expression Master Mix and applied to custom-made 384-well plates (Low-Density Arrays, ABI) containing primers for 92 genes of interest and 4 endogenous controls. Seven permissive and seven non-permissive LCLs were measured. For four LCLs, RNA was taken on two dates and measurements were averaged together; the rest were measured on one date. Five were assessed both with and without acyclovir, to reaffirm that viral replication has a negligible influence on the cellular gene expression across the whole cell line; the rest were only measured without acyclovir. PCR was performed on an ABI 7900HT at the GPCL, and data was analyzed using SDS2.1 software (ABI), Microsoft Excel, and Minitab 15. Results from RT-PCR arrays were quantified using the

$2^{-\Delta\Delta Ct}$ method (for each sample, taking the difference between each experimental gene's cycle of detection (Ct) and that of an endogenous control gene, and then comparing this difference to the difference in a reference sample).

3.1.8 Relative Quantitation of XBP-1u and XBP-1s mRNAs.

XBP-1 cDNAs were generated by random hexamer RT, and amplified using the ABI 7300 Real-time PCR System, SYBR green qPCR master mix reagent (Fermentas), and the following primers: XBP-1 forward: 5'-GTTGAGAACCAGGAGTTAAG-3'; XBP-1 reverse 5'-GAGAAAGGGAGGCTGGTAAG-3' to generate 357nt (XBP-1u) and 331nt (XBP-1s) amplicons. Amplifaction conditions were 95 degrees for 20 seconds, followed by 21 cycles of 95°C for 5 seconds and 60°C for 30 seconds. To quantitate relative XBP-1 splicing, dissociation curve analysis was performed post-run, according to the manufacturer's instructions. Relative XBP-1 splicing was calculated by comparing peak heights from the unspliced (Tm 85.7 degree) and spliced (Tm 82.7 degree) peaks, and measured as the ratio of XBP1s/(XBP1s+XBP1u).

3.1.9 Protein gel electrophoresis and Western blot.

Protein lysis buffer was 20mM Tris [pH 8.0] including 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.08% sodium deoxycholate, 10% glycerol, 0.1 mg/ml DNase I, and protease inhibitors [10 mM NaF, 1 mM Na_3VO_4., 0.5 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml pepstatin A]. Cells were pelleted and washed 3X in cold PBS before lysing. Each sample was resuspended to a

concentration of ~1x10^4 cells/µl, and lysed for 30 minutes on ice, with occasional flicking to mix samples. Lysates were sonicated with a Cole Parmer Ultrasonic Processor, at continuous amplitude of 50, for 15 seconds, and spun down in a chilled centrifuge, to remove the pellet and save the supernatant. Loading buffer was then added to a final concentration of 75 mM Tris-HCl [pH 6.8], 1% SDS, 1% glycerol, and 667 mM β-mercaptoethanol – and denatured the proteins in a 70ºC waterbath for 15 minutes. Lysates were kept in a -20ºC freezer, and run on preliminary gels for GAPDH so that we could standardize the quantity of protein between samples.

Protein lysates were run on 10% Bis/Tris gels using NuPAGE SDS/MOPS running buffer (for most proteins) or NuPAGE SDS/MES running buffer (for ID2, an especially small protein), in a NuPAGE apparatus at 200V for 50 minutes. We used Benchmark Prestained Protein Ladder (Invitrogen) to monitor electrophoresis and transfer, and MagicMark XP Western Protein Standard (Invitrogen) as a ladder visualizable upon scanning.

After running, the top part of the gel (containing the wells) and the bottom part of the gel (which is thicker than the rest, to fit into the premade gel casing) were cut off with a razor. Proteins were transferred from gel to PVDF membranes using a Hoefer TE70XP semi-dry apparatus, for 130 minutes at a steady current of 0.6-0.8 milliamps/cm^2 of gel. Normally the gels are ~7cm by ~8cm and we use 40 milliamps per gel. Transfer buffer was 25 mM Tris, 0.1% SDS, and 190 mM glycine, dissolved in water – and then mixed with 20% methanol. PVDF membranes had been equilibrated for 15 seconds in methanol, followed by 2 minutes in water, followed by at least 5 minutes in

transfer buffer/methanol. Each gel-membrane combination was sandwiched between six sheets of blotting paper soaked in transfer buffer/methanol.

Membranes were blocked in PBS/0.05% Tween 20 with 3% milk, and stained with antibodies in PBS/0.05% Tween 20 with 0.5% milk. Antibodies used for Western blot staining were rabbit α-Grp94 (sc-11402, Santa Cruz Biotechnology); rabbit α-EBF1 (sc-33552, Santa Cruz); rabbit α-ID2 (a gift of William Walker, Department of Cell Biology and Physiology / Magee Women's Research Institute); and mouse α-GAPDH (sc-47724, Santa Cruz), followed by Alexa 488-conjugated goat secondary antibodies. Staining was visualized using a Fuji FLA-2000 scanner. Bands were quantified using Image Gauge / Image Reader software, by drawing rectangles of equal size around each band, measuring the signal intensity, and subtracting the background signal intensity. For each band, background signal intensity was measured by drawing rectangles of the same size around background regions of the blot (in the same lane, above and below the band in question).

3.1.10 Immunofluorescence assay and quantification.

Cells were spun onto slides using a cytospin, fixed in PBS containing 1.5% PFA, blocked in Superblock (Pierce) containing 3% BSA and 0.1% saponin, and stained in Superblock containing 0.5% BSA and 0.1% saponin. PBS containing 0.1% saponin was used as rinse buffer. Fixing lasted 10 minutes at room temperature, blocking lasted 30 minutes at 4ºC, and staining lasted 60 minutes at room temperature. Primary staining used rabbit α-Grp94 (sc-11402, Santa Cruz), mouse α-ZEBRA (sc-43904, Santa Cruz)

or mouse α-gp110 (clone 5B2, Abcam); nonspecific staining was observed by using normal mouse IgG (sc-2025, Santa Cruz) and normal rabbit IgG (sc-2027, Santa Cruz). All primary staining was with antibodies at a concentration of 1 µg/µl. Secondary staining used Alexa 488-conjugated goat α-mouse and Alexa 594-conjugated goat α-rabbit (Molecular Probes), at a 1:2000 concentration. Vectashield mounting medium containing DAPI was used to visualize nuclei.

Cells were imaged using a Nuance VIS-Flex filter system (CRi), followed by unmixing of the spectra in Nuance 2.4 software. The auto-threshold function was used to identify regions of interest (minimum 300 connected pixels) in each field by Alexa 594 intensity. All regions consisting of one intact cell were quantified for Grp94 content (Alexa 594 intensity) by taking the measure of total signal (counts).

4.0 SPECIFIC AIM ONE:

ANALYZE EBV LATENCY, LYTIC GENE EXPRESSION, AND CELLULAR PHENOTYPE IN A SUBSET OF EBV-IMMORTALIZED LCLS

4.1 ABSTRACT

B-lymphoblastoid cell lines are used as sources of DNA for genotyping, as sample B cells from a given person, as targets for immune cells cultured *ex vivo* for therapeutic purposes, or as models for EBV immortalization of B cells. They vary in cell surface phenotype, size and shape, secretion of growth factors, rate of growth, survival at low densities in culture, and gene expression profile, but it is not known how any of these types of heterogeneity influences the EBV life cycle. In this part of the study we started with 62 LCLs and looked at their EBV DNA content, categorizing them as high-copy or low-copy. In a subset of 22 LCLs we used RT-PCR to measure expression of all EBV genes that code for latent-cycle proteins, and some genes of the lytic cycle, and confirmed that high DNA content indicated a high level of lytic reactivation from latency. High permissivity for lytic reactivation accompanied lower expression of latent genes in general, except the BCR-signalling mimic LMP2A. Lytic permissivity was a stable property: in some LCLs less than 1/1000 of cells were producing lytic proteins at any time, and others had as many as 5% lytic cells. We used 8-color flow cytometry to look

at 19 surface markers for B cell lineage, activation or Ig isotype in 19 of these LCLs, but observed little connection between surface phenotype and permissivity.

4.2 RESULTS

4.2.1 Levels of EBV DNA/cell vary within a characteristic range in both adult- and juvenile-derived LCLs.

We hypothesized that LCLs vary in permissivity for EBV reactivation in a manner influenced by the ontology of the cells that gave rise to the LCL. We measured the EBV DNA content in 62 independently-established LCLs which could be divided into two groups (adult or pediatric) depending on the age of the B cell donor. In both groups the majority of cell lines had < 200 copies of EBV DNA/cell with some ranging above 500 copies/cell, when measured at a single timepoint **(Figure 3)**. LCLs derived from healthy adult donors had a mean value of 254.4 (95% CI 151.7 – 357.2) and a median value of 124.0. LCLs derived from the peripheral B cells of pediatric transplant candidates had a mean of 193.4 copies of EBV DNA/cell (95% CI 132.6 – 254.2) and a median value of 123.8. LCLs from adult and pediatric donors were not statistically different in EBV DNA content, either in this cross-sectional measurement or in longitudinal observations (not shown).

Permissive and non-permissive LCLs were also subcloned at limiting dilutions and measured for EBV DNA/cell to confirm that their lytic permissivity phenotype was shared by the daughter cell lines **(Figure 3C)**. In addition to resembling their parent lines in morphology and growth rate, all sublines from the low-copy lines were low-copy like the parent, and only three of 22 sublines from one of the high-copy lines were

measured as low-copy. That subcloning did not affect the phenotype was expected, since all our LCLs had been growing in culture for at least three months prior to use in these experiments. This is in agreement with the study of Ryan *et al.* [175] showing that LCLs reach clonality or stable biclonality within two months of establishment. However, as our aim was to characterize a set of LCLs that were representative of those widely used in clinical and scientific assays, our cell lines were not all clonal. Normally, LCLs are not subcloned or investigated for clonality before being used in the laboratory.

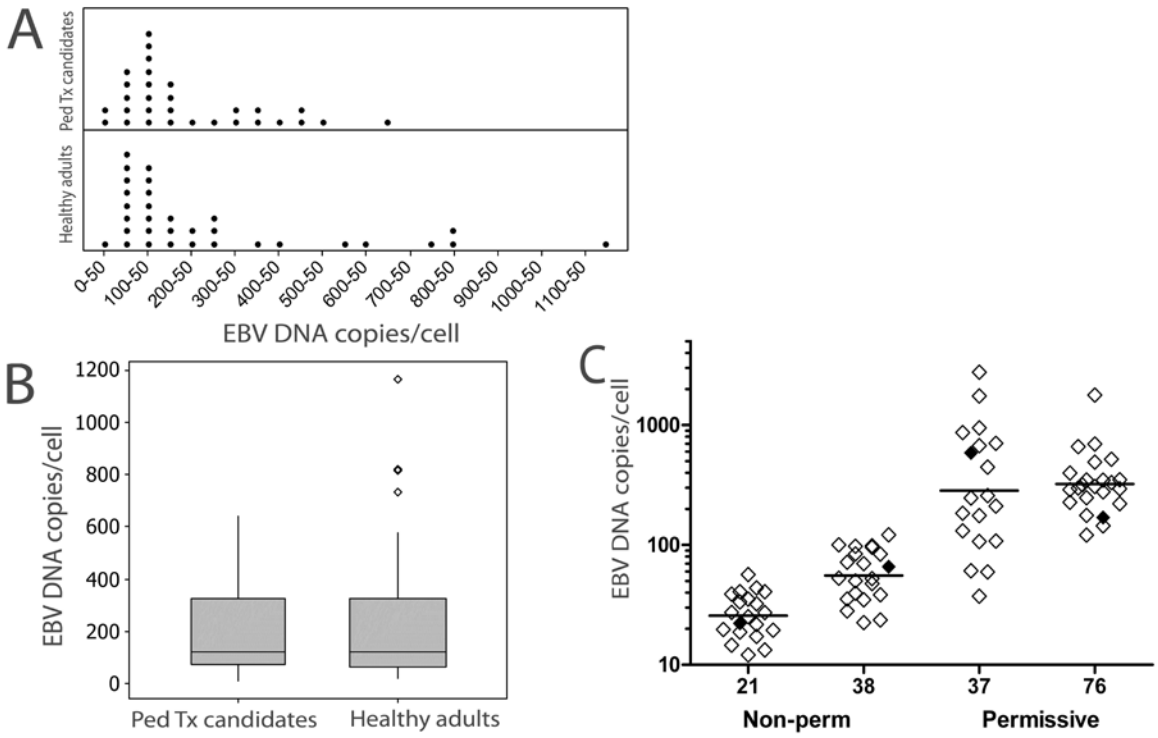

Figure 3. Sets of proliferating LCLs from pediatric and adult donors have comparable levels of EBV DNA content. LCLs generated from pediatric transplant candidates (n=32) and from healthy adults (n=30) were maintained for > 2 mo and measured for EBV DNA content two days after being split to equal densities. **(A)** In each group, the majority of cell lines had < 200 copies of EBV DNA/cell, while some ranged above 500 copies/cell. **(B)** Boxes show the 25th and 75th percentiles; diamonds represent values outside 1.5x the interquartile range. **(C)** Four LCLs were subcloned, with subclones allowed to diverge for six weeks before being measured for EBV DNA/cell. White diamonds represent subclones (mean of 2 timepoints), while black diamonds represent parent lines (mean of 7 timepoints). Bars indicate geometric means of subclones (n = 18-22) for each LCL.

4.2.2 EBV DNA/cell fluctuates within a characteristic range and does not depend on a continuing cycle of virus reactivation.

It has been previously shown that within two months post-infection the number of latent EBV episomes per cell in LCLs commonly increases from 1 or 2 per cell to dozens through mechanisms not related to classical episome maintenance (replication once per cell cycle in S phase from *oriP*) [198]. Since most LCLs undergo some level of spontaneous lytic reactivation, and this lytic permissivity varies amongst LCLs [199, 200], when LCLs are used in therapeutic protocols they are routinely cultured in the presence of acyclovir (ACV), to suppress the production of infectious virus [201]. ACV is a nucleoside analogue that inhibits herpesviral DNA polymerase and prevents lytic, but not latent, viral replication. To investigate whether the high levels of EBV DNA in some LCLs were the consequence of lytic reactivation, we grew LCLs in the presence of ACV. When grown with ACV, all LCLs had their levels of EBV DNA/cell reduced below 100 **(Figure 4A)**. Thus, for LCLs maintained without lytic inducers or lytic-inhibiting drugs, the amount of EBV DNA/cell is a marker for lytic permissivity of the population.

As LCLs grow in culture and undergo spontaneous lytic replication, a typical LCL might be expected to gradually increase its number of episomes/cell as a result of re-infection by virus in the supernatant. Alternatively, as the cells in each line that are disposed to enter a lytic replication cycle reactivate and die off, the cell line might decrease over time in its lytic permissivity. Neither trend was observed, as can be seen over the course of several weeks **(Figure 4A)**. When grown without ACV, each LCL with greater than a baseline level of lytic permissivity showed levels of EBV DNA/cell that fluctuated significantly but stayed within a certain range. The peaks and valleys

seen on a graph of the fluctuation did not correlate with the days at which the cells were fed with fresh media, and there was no correlation between EBV DNA/cell and density of cells in culture. Although the level of EBV DNA/cell in each LCL fluctuated from day to day, there was no steady trend upward or downward over time.

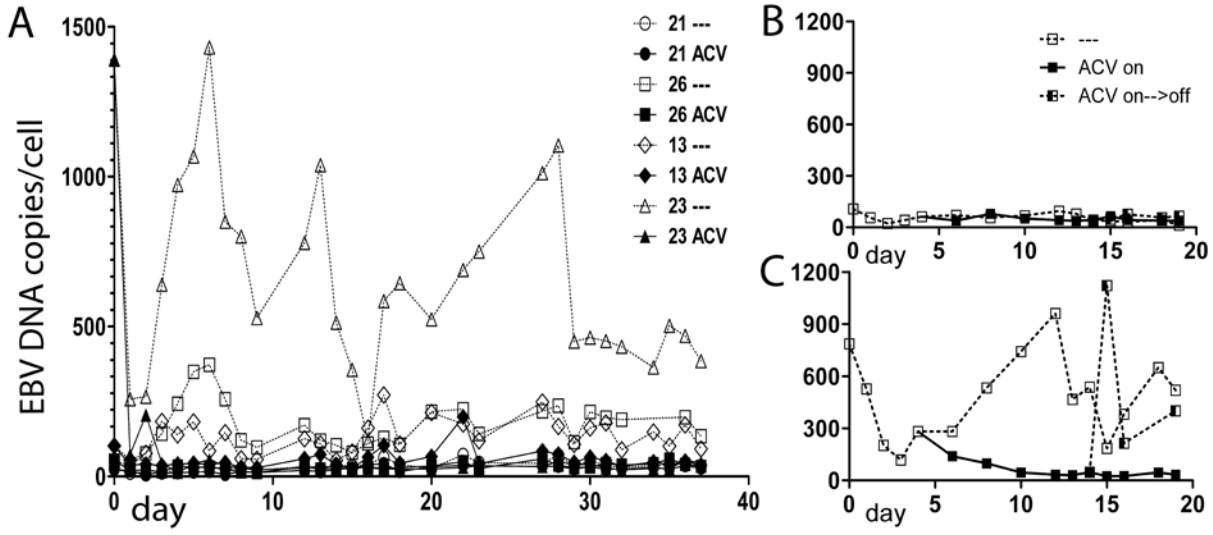

Figure 4. In each LCL, EBV DNA/cell fluctuates within a certain range and is reduced to a baseline level by the antiviral drug acyclovir. (A) Four LCLs were grown in complete media and passed regularly to equal densities. The number of EBV genomes/cell in each LCL did not remain stable or steadily increase over time. In the presence of acyclovir EBV DNA/cell reached a stably low level, with no effect on cellular morphology. Non-permissive LCL21 **(B)** and highly permissive LCL23 **(C)** were grown in media without ACV from days 0 to 20. On day 4, each LCL was split into two flasks, with or without ACV. On day 14, each LCL growing under ACV was split into two flasks, with or without ACV. Upon removal of ACV, LCL23 returned to its high-copy phenotype.

Cell lines with consistently high levels of EBV reactivation (so-called permissive LCLs) might be engaged in a self-reinforcing reactivation cycle that could be broken by the addition of acyclovir. To test this, we cultured permissive and non-permissive LCLs for 14 days in ACV and then removed it and continued culture passage. Non-permissive LCLs, represented by LCL21 **(Figure 4B)**, maintained the low-copy phenotype before, during and after the application of ACV. Permissive LCLs, represented by LCL23

(Figure 4C), had their EBV DNA/cell reduced to baseline level when grown in ACV, but immediately after the removal of ACV returned to their high-copy phenotype. This suggests that the rate at which spontaneous EBV lytic reactivation takes place in an LCL is influenced not by the number of viral episomes or by ongoing virus production, but by cellular factors whose expression is a characteristic of that cell line.

4.2.3 All LCLs show a Latency III pattern of gene expression with a detectable level of lytic reactivation.

To characterize LCLs for their lytic permissivity, and to confirm that they had comparable Latency III profiles of viral gene expression, we quantified 15 viral lytic and latent mRNA transcripts using real-time RT-PCR. For a subset of LCLs ($n \geq 21$), we measured the level of EBV DNA/cell on five days, with RNA extracted on the third day. Every LCL expressed transcripts for the full complement of EBV Latency III genes **(Figure 5)**. Every LCL also expressed detectable levels of the transcripts for three early lytic genes (BZLF1, which produces the lytic switch protein ZEBRA; BMLF1, which produces the abundant nuclear EB2 protein; and the lytic F promoter, which represented the majority of transcripts detected using primers specific for sequences in the downstream Q region). For all three of these transcripts, the variance among LCLs was greater than for any of the latent transcripts.

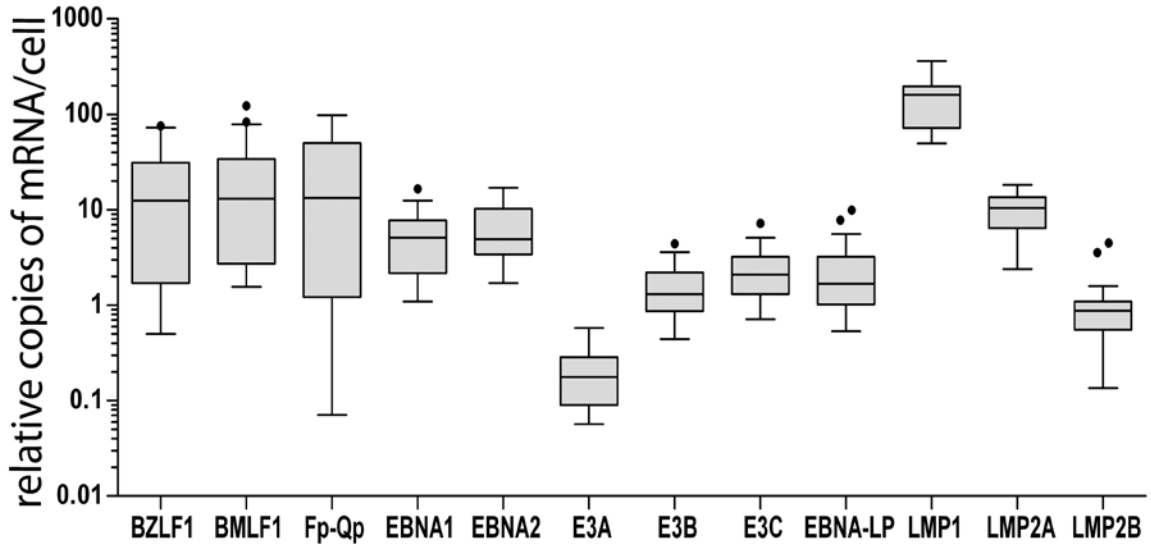

Figure 5. Viral Gene Expression in LCLs. Cell Lines (n ≥ 21) were measured for EBV DNA and RNA content after growing for > 3 mo. Levels of mRNA/cell were measured using real-time RT-PCR. Each of nine latent transcripts and three lytic transcripts was detectable in every LCL measured.

4.2.4 The expression of lytic EBV genes in an LCL correlates positively with its permissivity for lytic reactivation.

We measured the relative expression levels of all six EBNA genes and two LMP genes in this subset of LCLs (n ≥ 21). The expression of these lytic mRNAs correlated positively with the number of EBV genomes in an LCL **(Figure 6A-C)**. More specifically, there is a division between those with < 200 EBV genomes/cell, which all have very low levels of lytic mRNAs – and those with > 200 EBV genomes/cell, which all have high levels of lytic mRNAs. The linear regressions in Figure 4 show significant correlation, though they are not significant if only the permissive LCLs (> 200 EBV genomes/cell) are included in the regression.

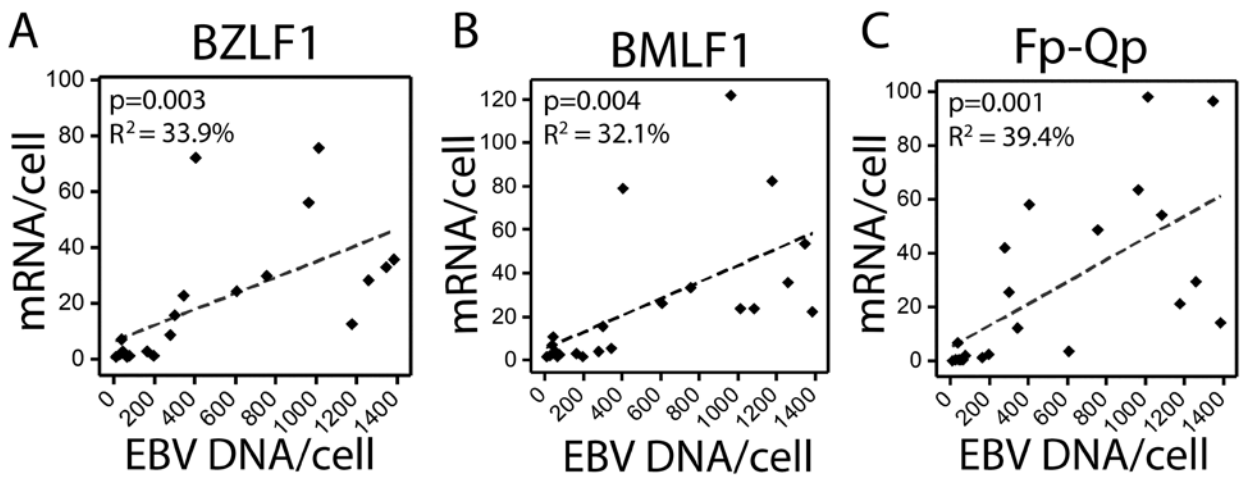

Figure 6. Permissivity for EBV lytic reactivation correlates with EBV DNA content. Levels of EBV DNA/cell (mean of 5 timepoints) were plotted against levels of EBV mRNA (1 timepoint, taken 2 days after cells were split to a common density) representing the lytic transcripts BZLF1/ZEBRA **(A)**; BMLF1/SM **(B)**; and F promoter **(C)**. Statistics shown are the p-value derived from Student's *t* test, and the coefficient of variation adjusted for sample size.

More significant linear correlations were found between the number of EBV genomes/cell and the percentage of cells expressing early (ZEBRA) or late (gB/gp110) lytic viral proteins. For each of these LCLs, intracellular staining and flow cytometry was used to detect cells that have tripped over into the immediate early stage of lytic replication (ZEBRA+ cells) **(Figure 7A)**, and also cells that have progressed through the early stages and into the production of lytic genomes and late viral proteins (ZEBRA+/gB+ double positive cells) **(Figure 7B)**. For each LCL the ratio of ZEBRA+ cells which are also gB+ is between 10% and 30%, indicating that although LCLs vary in permissivity for ZEBRA induction, they are similar in their ability to undergo the full cycle of lytic replication once ZEBRA is induced.

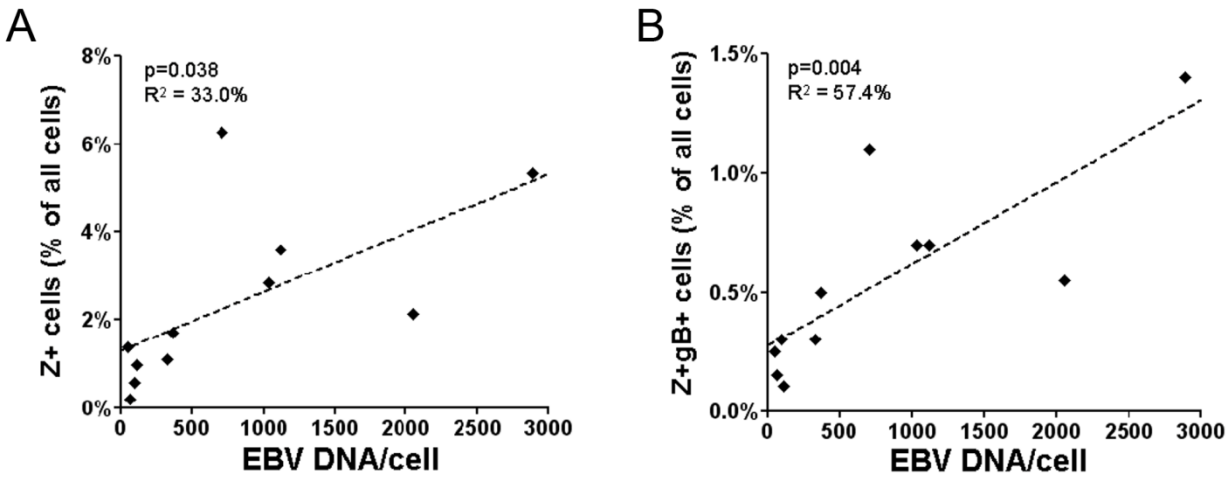

Figure 7. LCLs with high levels of EBV DNA contain more cells undergoing lytic replication. LCLs (n=11) were measured for EBV DNA content using real-time PCR, and flow cytometry was used to detect expression of the immediate early protein ZEBRA **(A)** and the late lytic protein gp110/gB **(B)**. DNA/cell levels are averages of 5 timepoints; ZEBRA+ percentages are averages of 3 or 4 timepoints; ZEBRA+/gB+ double-positive percentages are averages of 1 or 2 timepoints.

4.2.5 The expression of latent EBV genes in an LCL correlates negatively or weakly with its permissivity for lytic reactivation.

We also measured the relative expression levels of all six EBNA genes and two LMP genes in the same subset of LCLs (n ≥ 21). All eight were expressed in every LCL; the most abundant transcript was LMP1, and the least abundant was EBNA3A (**Figure 5**). **Figure 8** shows the level of mRNA for each latent gene plotted against the level of EBV DNA/cell. For EBNA1, -2, -3A, -3B, -3C, -LP, LMP1, and LMP2B, the relationship is similar. Each one shows a slightly negative, non-linear relationship, and any apparent correlation is attributable to a minority of non-permissive LCLs that contain high levels of the transcript in question. Each one also has a different set of LCLs that seem to be

overexpressing it; the correlation is not due to a few non-permissive cell lines that overexpress all of these genes. Only EBNA3A **(Figure 8D)** and EBNA3C **(Figure 8F)** generated a significant negative linear regression ($p < 0.05$) between gene expression levels and EBV DNA/cell. Based on the data in **Figures 6-8**, we designated LCLs as either “permissive” or “non-permissive” for spontaneous lytic reactivation. Non-permissive LCLs were those with less than 200 copies of EBV DNA/cell, which also had highly restricted expression of lytic mRNAs and proteins. Permissive LCLs were those with more than 200 copies of EBV DNA/cell, which also had more production of lytic mRNAs and proteins, and more restricted expression of most latent mRNAs. At least 5 days of DNA measurement was necessary to categorize an LCL for spontaneous permissivity.

The only latent gene product whose expression positively correlated with the level of lytic EBV DNA/cell was LMP2A **(Figure 8H)**. This relationship is not as strong as the correlation between EBV DNA content and BZLF1 and BMLF1 gene expression, and it resembles the relationship between EBV DNA content and other latent genes, in that non-permissive LCLs show a wider range of expression levels than permissive LCLs. No inverse relationship was detected between LMP1 and LMP2A expression, even though LMP1 is known to repress lytic reactivation in its role as a CD40 mimic [160], while LMP2A signaling can downregulate the expression of LMP1 [202].

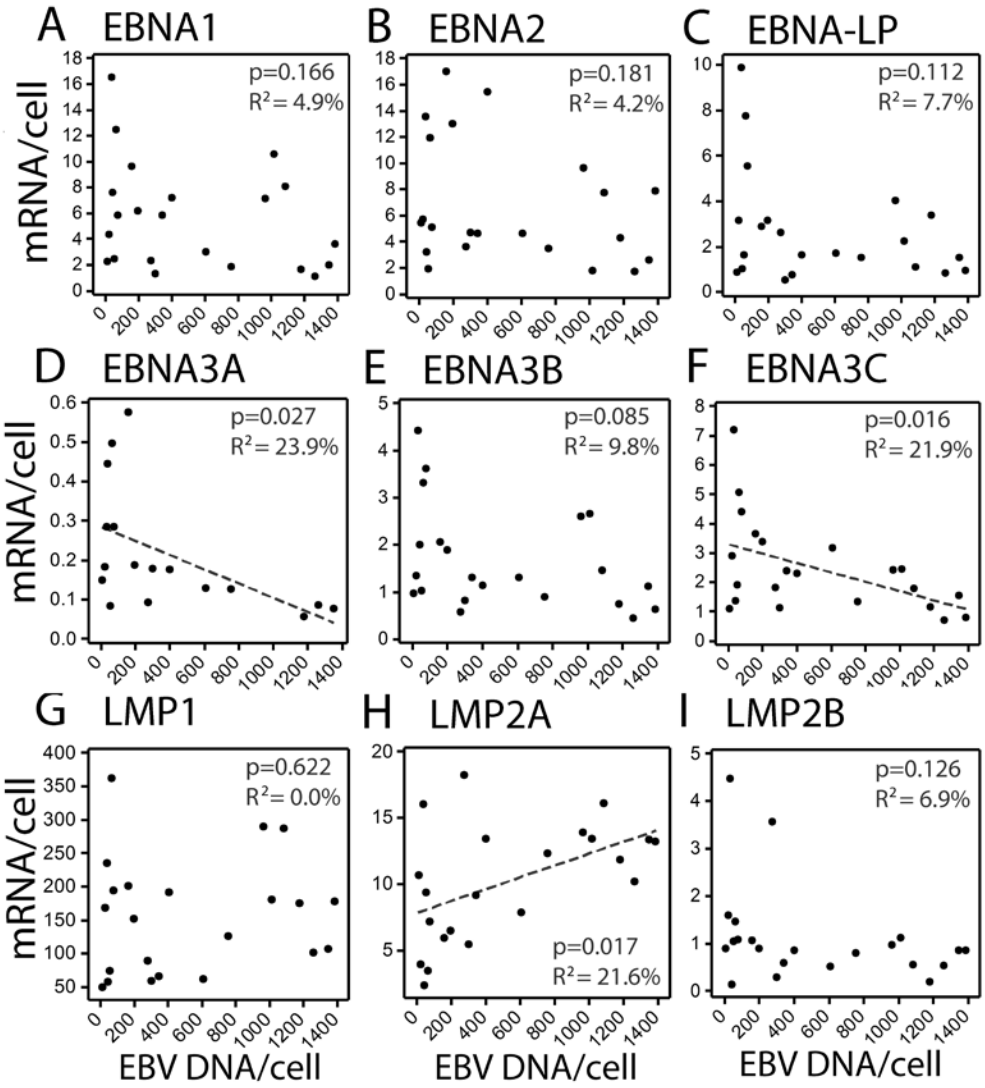

Figure 8. No strong correlation exists between EBV latent gene expression and EBV DNA content. LCLs (n ≥ 17) were measured for EBV DNA and RNA content after growing for > 3 mo, as described in Fig. 3. Gene expression was measured for EBV nuclear antigens 1 **(A)**, 2 **(B)**, leader protein **(C)**, 3A **(D)**, 3B **(E)**, and 3C **(F)**, and EBV latent membrane proteins 1 **(G)**, 2A **(H)** and 2B **(I)**. Statistics shown are as in Figure 4. Linear regressions are shown when $p < 0.05$.

4.2.6 The C and W promoters are both active in LCLs.

After months in culture LCLs may be expected to have completed the shift from using the W promoter (Wp) to using the C promoter (Cp) for expressing EBNA genes. RT-PCR primers were designed to detect the 5' ends of polycistronic EBNA transcripts and amplify RNAs with C_2 to W_1 splices (representing transcription from Cp), or W_0 to W_1 splices (representing transcription from Wp). Importantly, alternative splices between these exons determine whether the mRNA contains an EBNA-LP start codon (LP+) or no EBNA-LP start codon (LP-) Therefore, two sets of 5' primers were used to distinguish LP+ from LP- transcripts **(Table 2)**. EBNA-LP expression level was estimated by adding together the detected levels of C_2:W_1 and W_0:W_1 LP+ splices. LP- splices were presumed to encode at least one downstream EBNA protein, but not EBNA-LP.

The RNA levels for the 5' ends of EBNA transcripts (sum of the Cp and Wp cDNAs) were similar to the RNA levels for the 3' ends of EBNA transcripts (sum of the EBNA ORF cDNAs). When the sum of C2:W1 and W0:W1 splice cDNAs is plotted against the sum of EBNA ORF cDNAs, a linear trendline closely resembles the function (x=y) **(Figure 9A)**. This concordance suggested that the efficiency of reverse transcription did not grossly favor some transcripts over others.

Although C transcripts were more abundant than W transcripts, all four targets were detected in every LCL **(Figure 9B)**. There was no correlation between time in culture and having a greater ratio of C promoter to W promoter activity. W and C promoters appear to share the property of being more highly expressed in non-permissive LCLs, and this relationship reaches statistical significance for two of four

transcripts **(Figure 9B)**. In agreement with the findings of Elliott et al. [203], our data indicate that the Wp is never totally silenced even after more than 6 months in culture.

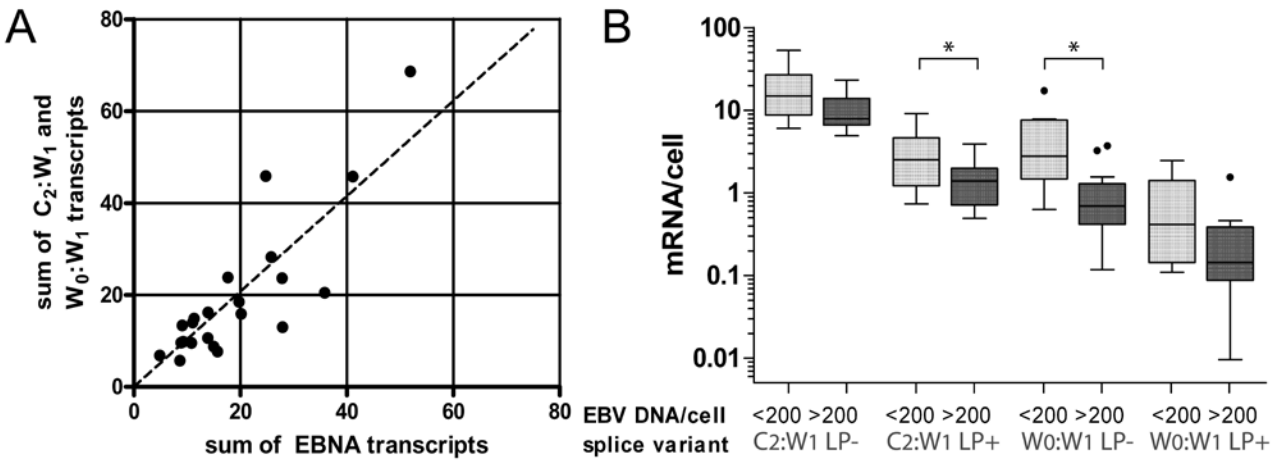

Figure 9. Both W and C promoters are actively making EBNA transcripts in all LCLs. LCLs (n=22) were measured for EBV DNA and RNA content after growing for > 3 mo, as described in Fig. 3. We detected polycistronic EBNA transcripts representing four splice variants – either C_2:W_1 or W_0:W_1, and either containing or not containing an EBNA-LP start codon. **(A)** For each LCL, the sum of EBNA1, -2, -3A, -3B, -3C, and -LP transcripts is plotted on the x-axis. The sum of C_2:W_1 and W_0:W_1 transcripts is plotted on the y-axis. **(B)** Non-permissive LCLs (< 200 EBV genomes/cell) contain more transcripts of each splice variant, as with all latent transcripts except LMP2A.

4.2.7 Inhibition of lytic replication does not affect spontaneous BZLF1 induction.

Varying levels of lytic replication in LCLs might not be directly related to the levels of ZEBRA induction, but instead correlate more closely with the level of success in translating that initial lytic switch into full-fledged viral replication accompanied by production of late lytic proteins. To characterize spontaneous reactivation at the level of individual cells rather than in pooled populations, we used flow cytometry to detect the number of cells in each cell line expressing lytic virus proteins, and the level of

expression of these proteins, when grown with or without acyclovir. Just as with lytic mRNA expression, there was a linear relationship between the number of EBV lytic genomes in a cell line and the number of cells producing both early (ZEBRA) and late (gp110) lytic proteins **(Figure 7)**. ZEBRA expression is strongly self-reinforcing, meaning that ZEBRA+ cells should be easily told apart from ZEBRA-negative cells, rather than there being a continuum between detectable and undetectable. EBV gp110 is abundant and, unlike other herpesvirus gB homologues, is localized to intracellular membranes rather than the cell surface, making it a good marker for late lytic activity by FACS or IFA [204]. The reactivating cells in non-permissive LCL38, though less numerous than those in permissive LCL01, stain just as brightly for ZEBRA and gp110, indicating that although LCLs vary in the fraction of cells that switch into lytic reactivation, the cells that do so produce similar levels of lytic proteins **(Figure 10)**.

Permissive LCLs, maintained in the presence of ACV for 4 weeks, were compared to the same LCLs grown with no antiviral drugs. ACV greatly reduced the production of gp110, but had no effect on either the number of cells producing ZEBRA or the level of ZEBRA produced in those cells **(Figure 10A)**. Flow cytometry analysis for two permissive LCLs grown with or without ACV at four timepoints found that ACV did not affect the number of cells producing ZEBRA **(Figure 10C)**, but did reduce the number of $ZEBRA^+$ $gp110^+$ double positive cells, expressed both as a percent of the total cell population **(Figure 10D)** and as a percent of the $ZEBRA^+$ cells **(Figure 10E)**. This further indicates that the “high-copy” status of an LCL is a stable phenotype characterized by greater permissivity for BZLF1 activation, and is independent of whether lytic replication of the genome actually occurs.

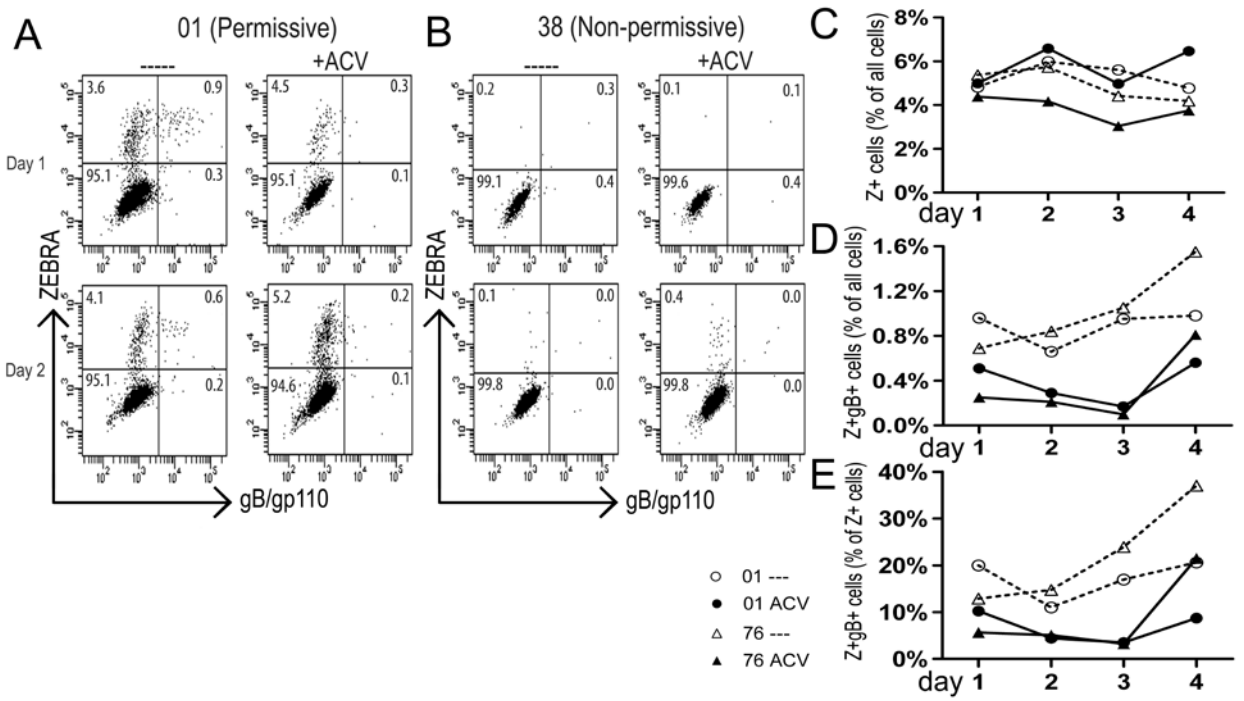

Figure 10. Permissive LCLs contain more cells expressing lytic EBV proteins. (A, B) Permissive LCL01 and non-permissive LCL38 were stained for expression of the immediate early protein ZEBRA(Z) and the late viral protein gB/gp110. In cells grown without acyclovir, the proportion of cells expressing Z and/or gB is greater in 01 than in 38. Data is representative of 9 LCLs at 4 timepoints. **(C, D, E)** Quantification of flow scatterplots. Two permissive LCLs, grown with or without ACV, were stained on 4 days each for ZEBRA and gB expression. **(C)** shows the ratio of ZEBRA-positive cells to total cells at each timepoint. **(D)** shows the ratio of ZEBRA/gB-double positive cells to total cells. **(E)** shows the ratio of ZEBRA/gB-double positive cells to ZEBRA-positive cells. Even long-term treatment with ACV only reduces the number of cells expressing late lytic genes – not the % of cells initiating lytic reactivation.

4.2.8 Profiling LCLs by their display of surface markers.

4.2.8.1 Establishment of antibody panels.

We hypothesized that high rates of lytic permissivity might be associated with LCLs of a non-memory cell lineage, or with those that experience more spontaneous differentiation toward PC status. Also, LCLs with reduced lytic reactivation might have

lost certain aspects of the B cell phenotype, such as surface immunoglobulin expression. Panels of fluorescently labeled antibodies were used to characterize the LCLs for numerous cell surface markers.

Markers investigated with the “Lineage/Activation” panel of antibodies included CD138, CD38, CD27, B220, CD23, CD69, and CD30. CD138 (SDC1/Syndecan-1) is a proteoglycan that interacts with the extracellular matrix, found on differentiated plasma cells, post-GC B cells, and HRS cells with post-GC (LMP1+) phenotype [205, 206]. CD38 is a plasma cell marker, is also highly expressed throughout the GC reaction, and is expressed at moderate levels in both stimulated and unstimulated B cells from the peripheral blood. CD38 is downregulated by EBNA2 but upregulated by c-*myc* [206-208]. CD27 is a memory cell marker which usually appears when IgD expression is blocked after cells leave the GC. CD23 is the receptor for CD70, and is a coactivator for cells to differentiate into plasma cell status. Most plasma cells express CD27, although cases of multiple myeloma are often made up of $CD27^{low}$ $CD138^{low}$ PCs [206, 209].

The α-B220 antibody was actually a rat monoclonal against the murine CD45 isotype B220, which is a pan-B cell marker in mice; this antibody identifies naïve (mature, CD27-, not terminally differentiated) B cells in the bloodstream, and is useful in distinguishing malignancies from normal B cells in some regions of the lymph node [210, 211]. CD23 is an IgE receptor that is upregulated as soon as EBV infects a B cell, is induced by EBNA2 and LMP1, and is expressed at variably high levels in activated lymphocytes but downregulated in plasma cells [189, 212]. CD23 and CD69 are both activation markers that are downregulated in BL [181]. Like CD27, CD30 is a TNFR family member (with ligand CD153), and it confers NFκB activation. CD30 is the marker

used to identify HRS cells; it is not found on normal naïve, mature, or GC B cells, but is found at low levels in activated cells and plasma cells [213-215].

Markers investigated with the “pan-B cell markers” panel of antibodies included HLA class I and class II; and CD19, CD20, BAFFR/BR3, CD45, and CD40, all of which should be on all B cells except terminally differentiated plasma cells. CD19 is a part of the BCR complex; CD20 is of unknown function; and BR3 is a receptor for the growth factors BAFF and APRIL; all three are highly expressed on naïve, mature, and GC B cells, distinguishing B cells from T cells [216, 217]. CD45 was once called the “leukocyte common antigen” and comes in several isoforms which have different extracellular domains but a common cytoplasmic domain. The cytoplasmic domain dephosphorylates several tyrosine kinases, making CD45 a coactivator in BCR signaling as well as other activation cascades in other cell types [218, 219]. CD40 is a ligand-dependent signaling molecule mimicked by LMP1 which induces activation and proliferation and also represses EBV lytic reactivation. Unlike CD19, CD20, or CD45, CD40 is retained on most HRS cells [215].

We also used monoclonal antibodies against surface Ig isotypes IgM, IgD, IgG and IgA – and to identify Ig-null B cells, a reagent that stains all human antibodies (goat pan-Ig). Several peripheral blood samples from normal donors were stained with these panels of antibodies, and the gating strategy for determining a donor’s isotype is shown in **Figure 11A**. An example of the distinction between $CD27^{high}B220^{low}$ memory cells, and $CD27^{low}B220^{high}$ naïve cells, is shown in **Figure 11B**.

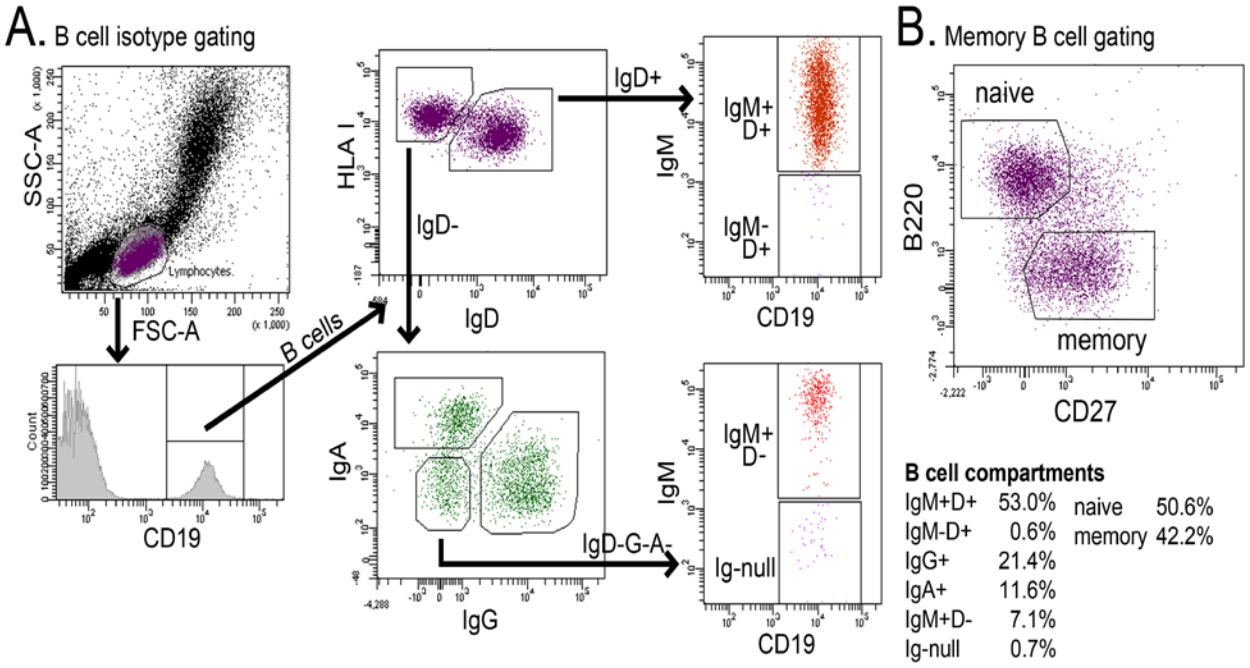

Figure 11. Gating strategies for B-cell antibody panels. (A) A sample from an adult donor was stained with the antibodies against IgA, IgG, CD19, HLA II, IgM, CD27, HLA I, and IgD [see Materials and Methods for fluor conjugates]. Gating for lymphocyte morphology is followed by gating for CD19 to identify B cells. B cells are gated based on IgD and HLA I to identify IgD+ and IgD- cells. IgD+ cells are measured for IgM to identify naïve (IgM+D+) cells. IgD- cells are measured for IgA and IgG to identify class-switched (IgG+ or IgA+) memory cells. IgD-A-G- cells are measured for IgM, using the gate developed with IgD+ cells, to identify Ig-null (IgD-A-G-M-) cells. **(B)** The same donor's cells were stained with the antibodies against pan-Ig, CD138, CD19, B220, CD23, CD27, CD69, and CD38. Identifying cells that are both $CD27^{bright}$ $B220^{dim}$ makes the identification of $CD27^{bright}$ (memory B) cells more reliable.

4.2.8.2 Correlations of LCL surface phenotype with EBV lytic activity.

Nineteen LCLs were maintained without ACV and measured for all of these markers. Although there were examples of LCLs having low levels of one surface marker or another (CD19, CD40, CD45, HLA II), and they varied in intensity of CD38, CD27, and CD138, there was no coherent phenotypic pattern (e.g. enhanced PC

differentiation) that could be associated with EBV reactivation. Linear regression of each surface marker suggested a negative correlation between EBV DNA content and the percent of cells positive for CD45 (p=0.006, R^2adj=33.3%), CD38 (p=0.020, R^2adj=23.5%); and CD40 (p=0.031, R^2adj=20.0%), and a positive correlation between EBV DNA content and the percent of cells positive for B220 (p=0.017, R^2adj=25.0%).

When we used mean fluorescence intensity of the cells, rather than percent positive, as the metric, the only correlation suggested by linear regression was a positive one between B220 and EBV DNA content (p=0.035, R^2adj=19.0%). The five linear regressions found to be significant to $p < 0.05$ are shown in **Figure 12A-E**. The fact that none has an adjusted R^2 value of more than 33.3% suggests that none of these markers are predictive for EBV DNA content. Also these surface phenotypes were not connected with each other, as we performed linear regressions among CD45, CD38, CD40 and B220 and found that only CD38 and CD45 showed a correlation (p=0.015, R^2adj=25.8%) **(Figure 12F)**.

Table 3 shows that there was no association between EBV DNA content and class-switched or IgM^+ IgD^+ status, and that none of the LCLs were of the Ig-null phenotype sometimes found in cases of PTLD [220, 221]. For six cell lines examined with and without ACV, the drug had little or no effect on surface marker expression. Of the 18 markers observed in that experiment **(Figure 13)** most LCLs had higher median fluorescent intensity (MFI) for CD69 when grown with ACV, and higher MFI for CD23 when grown without ACV. However, these differences are not significant when compared to the differences between LCLs, and may be effects of the complicated compensation calculations inherent in quantifying cells with 8 fluorescent markers at

once. These data confirm that long-term inhibition of EBV lytic replication does not impact the cellular phenotype.

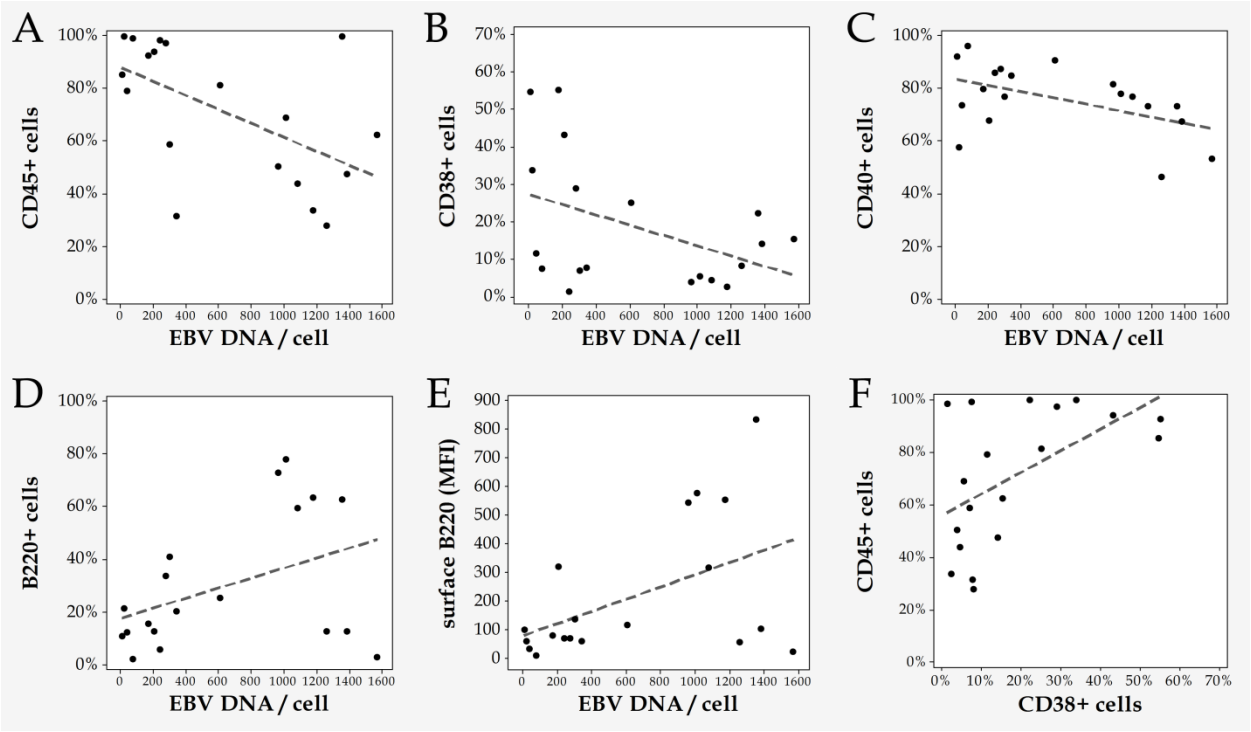

Figure 12. LCLs do not show cell surface marker phenotypes which correlate with EBV DNA content. 19 LCLs were measured for 19 surface markers and for EBV DNA/cell. **(A-C)** CD45, CD38, and CD40 were the only markers which, when quantified as % of cells staining positive, were negatively associated with EBV DNA content. None were significantly associated when quantified as mean fluorescent intensity. **(D-E)** B220 was the only marker positively associated with EBV DNA content, measured as either % of cells staining positive or as MFI. **(F)** Of these four surface markers, only CD45 and CD38 were correlated with each other (p=0.015, R^2adj=25.8%).

Table 3. Cell surface phenotype is not significantly correlated with permissivity for EBV reactivation.

		Isotypes					MHC		pan-B cell		
LCL	**DNA /cell**	**IgA**	**IgM**	**IgD**	**IgG**	**pan-Ig**	**HLA II**	**HLA I**	**CD19**	**CD20**	**BR3**
		FITC	APC	Pacific Orange	PE	FITC	PE-Cy7	Pacific Blue	PE-Cy5.5	APC-Cy7	PE
QW	171.4	/	+++++	+	++	+++++	+++++	+++++	+++++	+++++	+++++
7	1384.4	-	+++++	/	+++	++++	+++++	+++++	/	++++	+++++
14	1081	-	++++	-	++	+	+++++	+++++	+++++	++++	+++++
33	1570.9	-	++++	-	++	+	+++++	+++++	+++++	+++++	+++++
20	8.7	/	++++	++	++++	+++++	+++++	+++++	+++++	+++++	+++++
17	40.5	-	++++	/	++++	+++++	+++++	+++++	+++++	+++	+++++
37	240.1	-	+++	-	/	-	++++	+++++	+++++	+++++	+++++
26	606.1	-	++	+	++++	+++	+++++	+++++	+++++	++++	+++++
21	21.4	-	-	-	+++++	+++++	+++++	+++++	+++++	+++++	+++++
38	76	-	-	-	++++	++	+++++	+++++	+++++	+++++	+++++
72	274.9	-	-	-	+++++	+++	+++++	+++++	+++++	+++++	+++++
10	301.4	-	-	-	++++	+++	+++++	+++++	+++++	++++	+++++
23	342.5	-	-	-	+++	++++	+++++	+++++	+++++	+++++	+++++
6	963.5	-	-	-	+++++	++++	+++++	+++++	+++++	+++++	+++++
3	1014.6	-	-	-	++++	++++	+++++	+++++	+++++	+++++	+++++
TR	1177.1	-	-	-	++++	+++++	+++++	+++++	+++++	+++++	+++++
32	1259.6	-	-	-	++++	++	+++++	+++++	+++++	+++++	+++++
76	1357.6	-	-	-	+++	+++	+++++	+++++	+++++	+++++	+++++
77	207.9	+++++	-	-	/	++++	++	+++++	+++++	++++	++++

85-100% positive: +++++ **15-30% positive:** +
65-85% positive: ++++ **5-15% positive:** /
45-65% positive: +++ **0-5% positive:** -
30-45% positive: ++

Table 3 (contd.)

		pan-B cell		Activation / Lineage						
LCL	**DNA /cell**	**CD40**	**CD45**	**CD30**	**CD27**	**B220**	**CD23**	**CD69**	**CD138**	**CD38**
		Pacific Orange	Pacific Blue	A647	APC-A750	PE-Cy7	A647	Pacific Blue	PE	Pacific Orange
QW	171.4	++++	+++++	+++	+++++	+	+++++	++	+	+++
7	1384.4	++++	+++	+	+	/	+++++	+	++	/
14	1081	++++	++	++	+++	+++	+++++	+	+	-
33	1570.9	+++	+++	++	+++	-	++	+	++	+
20	8.7	+++++	+++++	+++	+++	/	+++++	++++	+	+++
17	40.5	++++	++++	+	+	/	+++++	/	+	/
37	240.1	+++++	+++++	+++	+++	/	+++++	+	/	-
26	606.1	+++++	++++	+++	+++	+	+++++	/	++	+
21	21.4	++	+++++	++++	++++	+	+++++	+	+	++
38	76	+++++	+++++	+++	+++	-	++++	/	++	/
72	274.9	+++++	+++++	++++	++++	++	+++++	++	++	+
10	301.4	++++	+++	+++	++++	++	+++++	/	/	/
23	342.5	+++++	++	++++	+++++	+	++++	/	/	/
6	963.5	++++	+++	+++	+++++	++++	+++++	/	+	-
3	1014.6	++++	++++	+++	++++	++++	+++++	/	+	/
TR	1177.1	++++	++	+++	+++++	+++	+++++	/	/	-
32	1259.6	+++	+	+	++++	/	+++++	+	/	/
76	1357.6	++++	+++++	++++	+++++	+++	+++++	-	+	+
77	207.9	++++	+++++	++++	++	/	+++++	/	+	++

85-100% positive:	+++++	**15-30% positive:**	+
65-85% positive:	++++	**5-15% positive:**	/
45-65% positive:	+++	**0-5% positive:**	-
30-45% positive:	++		

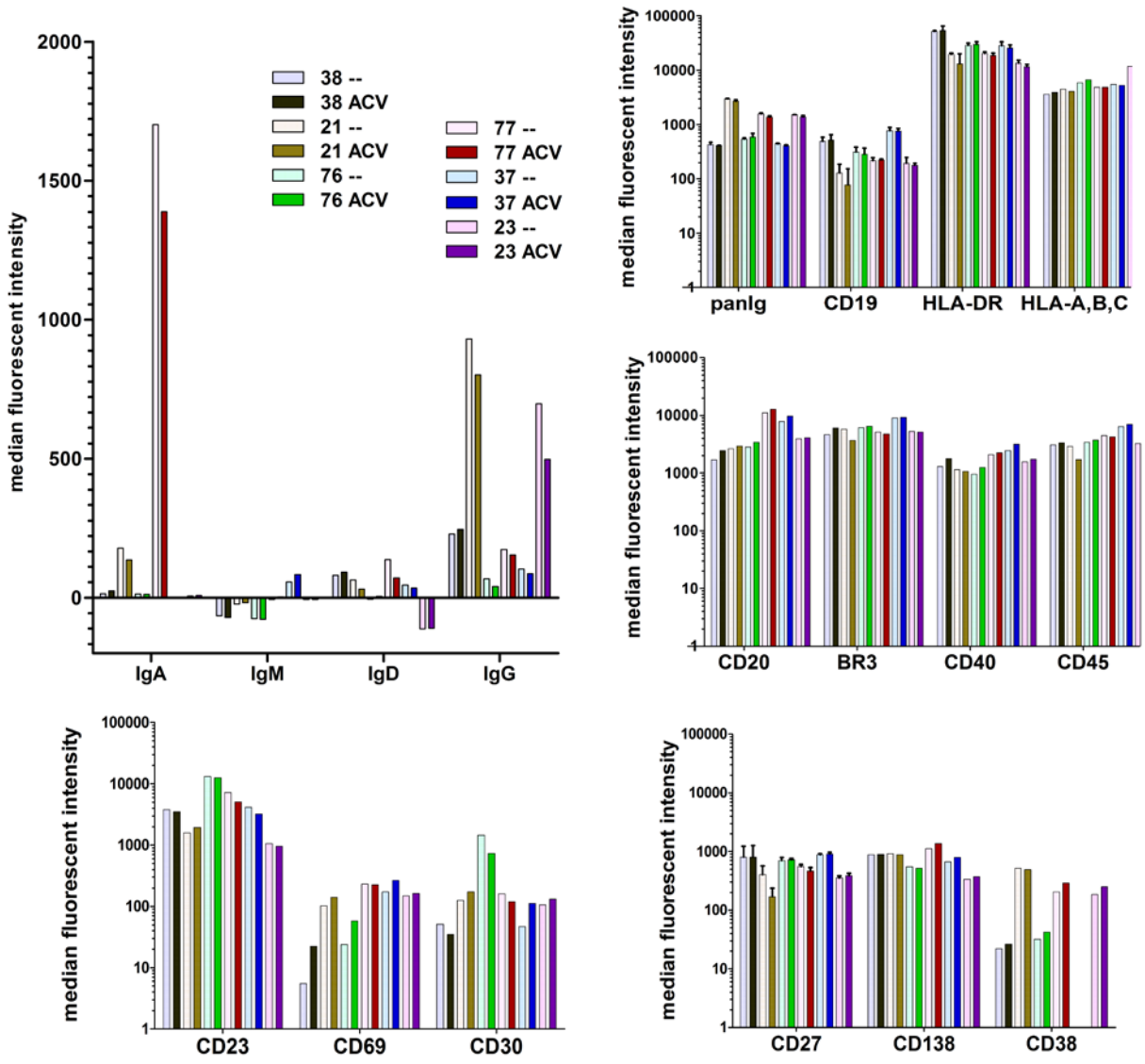

Figure 13. Culturing LCLs in acyclovir does not affect their B-cell phenotype. Six LCLs were cultured for ~2 mo with or without ACV. We measured them for the 18 surface markers shown here on the x-axes, and for each surface marker, subtracted the MFI of isotype-control-stained cells from the MFI of cells stained with the specific antibody to get the MFI shown here on the y-axes. For each of the LCLs (gray: LCL38 / gold: LCL21 / green: LCL76 / red: LCL77 / blue: LCL37 / purple: LCL23), the darker bar indicates cells cultured with ACV and the lighter bar indicates cells cultured without ACV.

5.0 SPECIFIC AIM TWO:

MEASURE HOST GENE EXPRESSION AND IDENTIFY CELLULAR PATHWAYS WHICH CONTRIBUTE TO EBV LYTIC REACTIVATION.

5.1 ABSTRACT

In Aim One it was established that LCLs could be categorized as permissive or non-permissive for lytic reactivation, and permissivity did not appear to be correlated with a particular cell surface phenotype. We considered that, although an LCL's permissivity could not be predicted by its surface markers or morphology, it might be predicted by the LCL's distinct patterns of gene expression. The transcription factor XBP1 is important in terminal differentiation of B cells into antibody-secreting plasma cells (PCs) and was recently found to induce the EBV Z promoter to produce the lytic switch gene BZLF1/ZEBRA. We hypothesized that higher levels of XBP1 and other PC factors would correlate with higher rates of spontaneous lytic reactivation. We also expected to find other patterns of cellular gene expression which could explain the heterogeneity between LCLs in their lytic permissivity. Differences in LCLs from patients who differ in the prognosis of their EBV-related disease might reflect the biology of their EBV-immortalized B cells *in situ*.

Several genes were identified, by microarrays and RT-PCR, as differentially expressed between permissive and non-permissive LCLs. Although PC factors did not correlate with permissivity, a group of factors (*EBF1*, *PAX5*, *TCF3*, *ETS1*) that inhibit PC differentiation did correlate with non-permissivity. One group found at higher levels in permissive LCLs was genes associated with the unfolded protein response (UPR), a multi-functional pathway that responds to and prepares for ER stress by enabling the cell to fold proteins at a higher rate. Although XBP1 is a UPR gene in addition to being a PC factor, we observed mostly upstream ER-located chaperones overexpressed in permissive LCLs, rather than UPR-related transcription factors.

UPR chaperone Grp94 was overexpressed in permissive LCLs at the protein level as well, and this overexpression was consistent throughout the cell lines rather than concentrated in the cells experiencing lytic reactivation. Treatment with the ER stress-inducing drug thapsigargin was used to investigate the effect of the UPR on permissive and non-permissive LCLs. The UPR reliably induced lytic reactivation in both groups, but this did not necessarily lead to lytic DNA replication. UPR induction was detectable earlier (at 12 hours, rather than 24 hours) in early-passage LCLs than in late-passage LCLs. Also, LCLs that were not lytically induced by the classical inducers TPA and butyrate, possibly because they were late-passage, were inducible by thapsigargin.

We demonstrate here that in Latency III-expressing B cell lines, the unfolded protein response is one of the factors that contribute to lytic reactivation, and that this effect may be independent of the connection between the UPR and the splicing of XBP1 to its active form. These results will later be accompanied by analysis of the degree to which the UPR is induced after ER stress in this same group of LCLs which differ in

passage number and lytic permissivity. This further characterization of UPR activity must be followed by studies into the other cellular pathways whose influence on lytic reactivation was revealed by our gene expression analysis.

5.2 RESULTS

5.2.1 Comparison of gene expression between permissive and non-permissive LCLs by microarray.

From the HapMap project it is clear that LCLs differ in their expression of a wide range of cellular proteins, as a result of polymorphisms [222], so we used LCLs as a system to detect connections between cellular gene expression and EBV activity in latently infected cells. We selected two permissive LCLs (LCL33 and LCL76) and two non-permissive LCLs (LCL21 and LCL38), maintained them in separate ACV^+ and ACV^- conditions for two months, and isolated DNA and RNA two days after passage at the same density. cDNA was hybridized to Illumina HumanRef-8 v2 BeadChips containing probes for 20589 human mRNA sequences. For every probe, we first compared the average intensity of the 4 LCLs grown with ACV to the average intensity of the 4 LCLs grown without ACV **(Figure 14A)**, and found no significant effects on any cellular genes. We then compared the average intensity of the permissive samples (LCL33 ACV^+ and ACV^-, LCL76 ACV^+ and ACV^-) to the average intensity of the non-permissive samples (LCL21 ACV^+ and ACV^-, LCL38 ACV^+ and ACV^-), and found a much greater degree of variation **(Figure 14B)**, although the R^2 value of 0.993 indicates how similar the phenotypes of the cells in this study are.

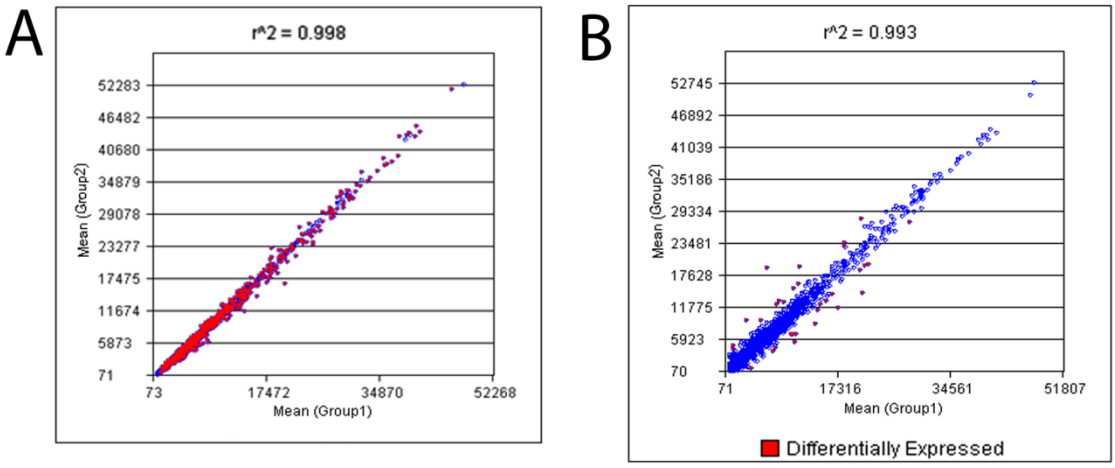

Figure 14. Data quality analysis of microarray results. (A) For all 20589 probes, 4 LCLs maintained with acyclovir were compared to the same 4 LCLs maintained without acyclovir. No genes show significant differential expression between the two populations. **(B)** For all probes, 2 permissive LCLs, grown with and without ACV, were compared to 2 non-permissive LCLs grown with and without ACV. Red points represent probes with J5 > 32.50.

We used efficiency analysis of several transformations of the data to identify the process that would generate the most repeatable list of genes with significant absolute differences in expression between permissive and non-permissive LCLs. These transformations included log (base 10 and base 2), square root, subtraction of global minimum, and removal of lowest 5% of values. Online caGEDA software [197] indicated that the most consistent determination of significance came from data that was untransformed and had been defined as significant using the J5 formula **(Figure 2)**.

Table 4. Illumina probes differentially expressed between permissive and non-permissive LCLs, as determined by J5 score of absolute difference.

All transcripts with J5 score >32.50							
Unigene transcript ID	**J5***	**Symbol**	**Synonyms**	**Unigene transcript ID**	**J5***	**Symbol**	**Synonyms**
Hs.9754	-123.52	**ATF5**		Hs.436446	81.04	**ARMET**	
Hs.523012	-69.63	**DDIT4**	REDD1	Hs.192374	69.33	**HSP90B1**	Grp94, gp96
Hs.654444	-61.07	**S100A4**	MTS1, Metastasin	Hs.514107	59.05	**CCL3**	MIP-1α
Hs.546259	-53.02	**H3F3A**	H3 histone 3A	Hs.303116	56.89	**SDF2L1**	
Hs.125867	-43.06	**EVL**		Hs.512304	44.01	**CCL3L3**	MIP-1α
Hs.369438	-40.63	**ETS1**		Hs.702021	41.19	**HSPA8**	Hsc70
Hs.375957	-40.45	**ITGB2**	Integrin β2, LFA-1, CD18	Hs.70327	40.23	**CRIP1**	
Hs.75256	-40.31	**RGS1**		Hs.534322	39.75	**HLA-DRB1**	HLA II DR-β1
Hs.419240	-38.72	**SLC2A3**	GLUT3	Hs.434937	39.47	**PPIB**	Cyclophilin B
Hs.107740	-38.72	**KLF2**	L-KLF	Hs.26663	38.94	**HERC5**	CEBP1,
unknown	-37.12	unknown	unknown	Hs.317192	37.63	**DNAJB11**	ERdj3,
Hs.415067	-34.06	**CORO1A**	p57/ Coronin-1	Hs.1908	37.29	**SRGN**	Serglycin
Hs.514581	-33.67	**ACTG1**	γ-actin	Hs.434081	37.07	**PSME2**	PA28β, REGβ
Hs.568346	-33.13	**CT45-4**		Hs.642990	36.50	**STAT1**	
				Hs.534322	36.19	**HLA-DRB1**	HLA II DR-β1
*** negative value: overexpressed in non-permissive LCLs**				Hs.458485	36.08	**ISG15**	IFI15, G1P2
				Hs.108957	33.84	**RPS27L**	
*** positive value: overexpressed in permissive LCLs**				Hs.702021	33.17	**HSPA8**	Hsc70
				Hs.517307	32.84	**MX1**	MxA, IFI78
				Hs.374191	32.51	**LEPREL1**	P3H2 prolyl hydroxylase

By this measure, the most repeatable threshold for significance was J5=32.5, and 34 transcripts representing 31 genes were found to be above this threshold. 18 were overexpressed in permissive LCLs, and 13 overexpressed in non-permissive LCLs **(Table 4)**. The supplementary material to our most recent publication [195] contains lists of all the transcripts whose overexpression in either non-permissive or permissive LCLs led to a J5 score above 10.0. We also quantified the difference between permissive and non-permissive LCLs by measuring the fold difference, or ratio between the averages of the groups, without removing outliers. Our recent publication also contains lists of the

transcripts that were overexpressed by a fold difference above 1.75 in non-permissive or permissive LCLs. After analyzing these results, we singled out genes which scored highly by at least one measure of significance for further study.

5.2.2 Differentially expressed sets of genes in permissive and non-permissive LCLs.

In light of the small sample size used with this microarray, we used statistical analysis software not just to identify individual genes, but also to seek out patterns of overexpression in entire groups of genes. These groups of genes included “gene ontologies” associated with intracellular pathways, interaction between cells, or larger-scale biological processes, curated by the Gene Ontology Consortium [223]. These groups of genes also included “gene sets” which were derived mostly from the results of other microarray experiments, partially from a database of known sequence motifs in each gene’s promoter, and partially from the consensus pathways compiled by GenMAPP, KEGG, and BioCarta [224].

DAVID, employing a canonical database of gene ontologies and other groups of genes such as KEGG pathways [225], was used to analyze lists of genes significantly overexpressed in permissive or non-permissive LCLs, and identify gene ontologies that contained these genes. We used four different definitions of significance (J5 > 10.0; J5 > 7.0; fold difference > 1.75; fold difference > 1.5) to create these lists, to see whether the genes identified by one method would also be identified by others. All gene ontologies that contained at least one significant gene, and were associated with

immunology or transcription factor activity, were collected. Similar ontologies (e.g. “transcription”, “transcription factor activity”, and “regulation of transcription”) were combined, and all such ontologies are listed in **Table 5**. The small number of differentially expressed genes identified from these categories is expected from a comparison of cells with such similar phenotypes.

GSEA (Gene Set Enrichment Analysis) was used to analyze the results as a whole, ranking all genes by significance of their difference between the two groups of LCLs, and then identifying gene sets that were overrepresented at either extreme of the ranking [224]. Curated gene sets associated with non-permissive LCLs included genes underexpressed in AIDS-associated primary effusion lymphoma [226]; downregulated by cell cycle suppressors p21 and p53 [227]; overexpressed in CD10+ hematopoietic progenitor cells with B cell potential [228]; and upregulated in multiple myeloma cell lines after activation by N-ras or IL-6 [229]. Curated gene sets associated with permissive LCLs included genes upregulated by p53, IFN-α or IFN-γ [230-232]; overexpressed in all types of plasma cells *in vivo* [233]; overexpressed in peripheral blood B cells of lupus patients [234]; and involved in the oxidative phosphorylation pathway.

Motif-based gene sets associated with non-permissive LCLs included those with promoter sequences predicted to be activated or repressed by transcription factors PAX8, PAX6, NF-IL3, FOXO1, EGR1/2/3, E2F1, CUTL1/CDP, and several microRNAs including the oncogenic miR-155, -221, and -222, and the anti-inflammatory miR-346 [235-237]. Motif-based gene sets associated with permissive LCLs were those with motifs for ATF-6, XBP-1, HTF-1, and STAT5A, and microRNAs miR-338 and miR-

518A/E/F. Although dozens of both motif-based and curated gene sets were enriched at a false discovery rate (FDR) < 0.25 in non-permissive LCLs, only seven motif-based gene sets were enriched at FDR < 0.50 in permissive LCLs. The most enriched gene sets detected by GSEA are shown in **Table 6**. The normalized enrichment score and false discovery rate used to define enrichment of gene sets are described in Subramanian *et al.* [224].

Table 5. Relevant gene ontologies containing genes overexpressed in permissive or non-permissive LCLs.

Gene ontologies	Genes associated with the ontology
Immune system processes	
lymphocyte differentiation	**AICDA , CBFB ,** CD74 , **CD79A , EGR1 , HDAC9 , IL4 ,** IL15 , **KLF6 , TCF3**
humoral immune response	BST2 , **CCR6 ,** EBI3 , **LY86 ,** PDCD1 , **ST6GAL1** , STAP1
innate immune response	**COLEC12 ,** LAG3 , **TUBB**
immune system development	**AICDA , BCL11A , CBFB ,** CD74 , **CD79A , EGR1 , ETS1 ,** HCLS1 , **HDAC9 , ID2 ,** IL15 , IL27RA , **IL4 , KLF6 ,** LMO2 , **LRMP ,** MAL , MB , **MYH9 , PRL ,** RPS19 , **RUNX1 , SWAP70 , TCF3 ,** TIMP1 , **WNT3A**
BCR signaling pathway	**CD79A, CD79B ,** NFATC1 , **PRKCA ,** SHC1
lymphocyte activation / proliferation: negative regulation	LAG3 , **TNFRSF13B**
lymphocyte activation / proliferation: positive regulation	**CD81 ,** CDKN1A , EBI3 , IFNG , IL15 , **IL4 ,** TNFSF13B
lymphocyte activation / proliferation: unspecified	**AICDA , BANK1 ,** BST2 , **CBFB , CD7 ,** CD74 , **CD79A , CXCR4 , EGR1 , HDAC9 ,** IL27RA , **KLF6 ,** PAG1 , **SWAP70** , **TCF3 ,** TNFRSF14 , **WAS**
antibody production / lymphocyte mediated immunity	CD74 , EBI3 , IFNG , **IL4 ,** IL27RA , LAG3 , LY9 , **SWAP70 ,** TNFSF13B , **TUBB**
immune response: negative regulation	IL27RA
immune response: positive regulation	**CD79A , CD79B ,** IL15 , IL27RA , TNFSF13B
immune response: unspecified	ADA, AIM2 , C1QBP , **EBI2 , EDG6 ,** FAIM3 , FCN1 , HLA-C, HLA-DMB , HPA-DPA1 , **HLA-DRA1 ,** HLA-DRB5 , **HLA-E , ICOS , IFI27 , IFI30 ,** IFI35 , IFI44 , IGJ , IL1R2 , **INPP5D ,** ISG15 , **LCP2 , LILRA2 ,** MIST , MNDA , OAS1 , **OAS1 ,** OAS2 , PSME1 , PSME2 , **RGS1 ,** TAP1 , TNFRSF17 , TRIM22
inflammatory response	**AIF1 , ALOX5AP ,** ANXA1 , CCL1 , CCL3 , CCL3L3 , CCL4L2 , **CCR7 , CXCR4 , HDAC9 , IL1A , ITGB2 , LY86 , MIF ,** NCR3 , OLR1 , **PLA2G4C ,** PRDX5 , **PRKCA , PTAFR ,** SERPINA1
cell adhesion	**ADAM15 , AEBP1 , AMICA1 , ARHGDIB ,** CCL4L2 , CD33 , **CD9 , CD96 , CD99 , CDH17 ,** CDH2 , CEACAM1 , **CLDN10 , COL24A1 ,** COL8A1 , CTNNA2 , **CYFIP2 , DCBLD2 ,** DSC2 , **EDG1 ,** EPDR1 , FEZ1 , **FLOT2 , FLRT3 ,** GMDS , GPNMB , **GPR56 ,** IL32 , **ITGA9 , ITGB1 , ITGB2 ,** JAM2 , **JUP , KAL1 , LAMA5 , LAMB1 , LGALS3BP ,** LY9 , **MYBPC2 , MYBPC2 , MYBPH , NINJ2 ,** NPNT , **NRCAM ,** NRP1 , OLR1 , **PFN1 ,** PKP2 , **PLEKHC1 , PTEN ,** RND3 , **SCARB1 , SSPN ,** TGFBI , TINAG , **TLN2 ,** TPBG , VANGL2
cytokine activity	CCL1 , CCL3 , CCL3L3 , CCL4L2 , **CKLF , CMTM3** , **CMTM7 ,** CD70 , EBI3 , GDF15 , IFNG , IL15 , **IL1A ,** IL32 , **IL4 , MIF , PRL , PTEN ,** TNFSF4 , TNFSF13B , TXLNA

Table 5 (contd.)

Gene ontologies	Genes associated with the ontology
Transcription factor activity	
regulation of transcription: negative	**BHLHB2 , DNMT1 , EGR1 , HDAC9 , ID2 ,** ID3 , **JARID1B , KLF12 ,** LEF1 , **LRRFIP1 , NFKB1 , NRIP1 ,** PFDN5 , PPARG , PSMC5 , **RBM9 , SMARCA4 , SUZ12 ,** TFCP2L1 , **TH1L , TRIB3 ,** TRIM22 , TRPS1
regulation of transcription: positive	**ARNT , CAMKK2 , CBFB ,** CHURC1 , EDF1 , **EDG1 , EGR1 ,** FHL2 , **HNRPD ,** IFNG , **IL4 , KLF2 , KLF6 ,** LEF1 , MAGED1 , **MYB , NCOA3 , NEUROD1 , NEUROG2 ,** NFATC1 , **NFKB1 , NRIP1 ,** NUP62 , **PDLIM1 ,** PPARG , PRRX1 , PSMC5, **RUNX1 , SERTAD2 , SMARCC1 , TCF3 , TEAD4 , UBB**
regulation of transcription: unspecified	**AEBP1 , ATF5 , BCL11A , BHLHB5 ,** C14orf156 , CALR , **CAMKK2 , CARHSP1 , CBFB ,** CBX6 , **CDCA7 , CEBPD ,** CREB3L2 , **CRY1 , E2F2 ,** EGR2 , EMX1 , EOMES , **ETS1 ,** FOXA3 , GFI1 , GTF2E2 , HCLS1 , **HDAC1 , HES4 , HES6 ,** HMBOX1 , **HMGB2 , HMX2 , HNRPD , IL4 ,** IRF5 , JUND , LDB2 , LITAF , **LZTS1 , MCM2 , MCM7 ,** MNDA , MORF4L1 , **MYT1 , NFIB , NR2F6 , OLIG1 ,** PBX3 , **PFN1 ,** POLR2A , POLR2F , PTRH2 , **PTTG1 , RAB26 , RUNX1 ,** RUNX3 , SND1 , **SOX18 , SOX4 ,** SP140 , **SSRP1 ,** STAT1 , STAT4 , TCEAL4, **THRSP , TSC22D3 , TXNIP , UHRF1 , YWHAZ ,** ZBTB20 , ZFHX4 , ZNF165 , **ZNF358 , ZNF395 , ZNF462**
regulation of gene expression: unspecified	EBI3 , **EIF1 ,** EIF5A , **GPX1 , H2AFY2 , IGF2BP2 ,** IGF2BP3 , LAG3
transcription cofactor activity: coactivator	**ARNT , CBFB ,** EDF1 , FHL2 , MAGED1 , **NCOA3 , NRIP1 , PDLIM1 ,** PRRX1 , **SERTAD2 , SMARCA4 , SMARCC1**
transcription cofactor activity: corepressor	**ATF5 , HDAC9 ,** ID3 , **KLF12 , NRIP1 ,** PFDN5 , **RBM9 ,** TFCP2L1 , **TRIB3** , TRIM22
transcription cofactor activity: unspecified	**HES6 ,** LDB2 , PSMC5 , SND1

We ranked all 20,589 transcripts in the microarray for the significance of their difference between permissive and non-permissive LCLs, ranking them by both J5 score and mean fold difference, and made a list of all transcripts with a fold difference > 1.5 or a J5 score > 7.0. This generated 533 transcripts overexpressed in non-permissive LCLs (15.0% of them detected by both J5 and fold change), and 551 transcripts overexpressed in permissive LCLs (10.3% of them detected by both J5 and fold change). NIAID's DAVID online software was used to generate a list of all gene ontology terms associated with any genes in the lists; all terms relevant to immunology or transcription factor activity were saved in a spreadsheet. The gene categories in the left column of this table resulted from combining closely related GOterms into larger categories.

Bold: overexpressed in non-permissive LCLs.

Plain: overexpressed in permissive LCLs.

Underlined: significant by both J5 score and fold difference.

Table 6. Gene Sets enriched in permissive or non-permissive LCLs.

Enriched in permissive LCLs					
	Curated gene set name	**Description**	**Size**	**NES**	**FDR**
1	TARTE_PC	Overexpressed in plasmablast and plasma cell subsets	75	2.66	0.000
2	IFNA_HCMV_6HRS_UP	Induced in human fibroblasts 6hr after IFN-α treatment	48	2.12	0.002
3	MOOTHA_VOXPHOS	Involved in oxidative phosphorylation	80	1.99	0.023
4	P53GENES_ALL	Transactivated by wild-type p53 in a yeast system	17	1.93	0.049
5	DAC_PANC50_UP	Induced by demethylation in pancreatic cancer cell lines	43	1.92	0.040
6	TAKEDA_NUP8_HOXA9_8D_UP	Induced by oncoprotein NUP98-HOXA9 in CD34+ cells	134	1.90	0.045
7	DAC_IFN_BLADDER_UP	Induced by IFN and by demethylation in bladder cancer cell lines	17	1.90	0.043
8	HSA00190_OXIDATIVE_PHOSPHORYLATION	oxidative phosphorylation (KEGG pathway)	112	1.90	0.039
9	DER_IFNA_UP	Induced in HT1080 fibroblasts 6hr after IFN-α treatment	63	1.85	0.064
10	ELECTRON_TRANSPORT_CHAIN	Involved in oxidative phosphorylation	98	1.82	0.085
11	HSA01510_NEURODEGENERATIVE_DISEASES	neurodegenerative diseases (KEGG pathway)	36	1.79	0.105
12	SANA_IFNG_ENDOTHELIAL_UP	Induced by IFN-γ in primary endothelial cells	72	1.78	0.114
13	RESISTANCE_XENOGRAFTS_UP	Overexpressed in extremely chemotherapy-resistant xenografts	27	1.77	0.116
14	CMV_8HRS_UP	Upregulated by early HCMV infection of human fibroblasts	29	1.77	0.111
15	OXIDATIVE_PHOSPHORYLATION	oxidative phosphorylation (GenMAPP pathway)	60	1.76	0.115
16	BENNETT_SLE_UP	Overexpressed in PBMCs of children with active SLE	28	1.76	0.108
17	CMV_HCMV_TIMECOURSE_ 24HRS_DN	Downregulated by late HCMV infection of human fibroblasts	38	1.76	0.102
18	CMV_ALL_UP	Upregulated by either early or late HCMV infection of fibroblasts	89	1.75	0.098
19	MMS_HUMAN_LYMPH_HIGH_ 24HRS_UP	Upregulated by the mutagen MMS in human TK6 LCLs	18	1.75	0.097
20	AD12_32HRS_DN	Downregulated by late adenovirus infection of HeLa cells	15	1.73	0.118
21	PORPHYRIN_AND_CHLOROPHYLL_METABOLISM	porphyrin and chlorophyll metabolism (GenMAPP pathway)	19	1.72	0.122
22	HSA03010_RIBOSOME	ribosome (KEGG pathway)	65	1.72	0.122
23	ALCALAY_AML_NPMC_UP	Overexpressed in AML cases with cytoplasmic NPM localization	131	1.67	0.191
24	LEE_TCELLS5_UP	Overexpressed in intrathymic T progenitors, compared to all later stages of T cell development	18	1.66	0.188
25	SCHUMACHER_MYC_UP	Induced by Myc-driven proliferation of human B cell line P493-6	53	1.66	0.193
26	ARAPPATHWAY	ADP-ribosylation factor / vesicular trafficking (BioCarta pathway)	20	1.65	0.192
27	UVB_NHEK1_C1	Upregulated by UV-B light in normal keratinocytes	47	1.65	0.185
28	AGED_MOUSE_CORTEX_DN	Downregulated in cerebral cortex of aged (22 mo) mice (vs. 2 mo)	45	1.64	0.191
29	HSA05110_CHOLERA_INFECTION	*Vibrio cholerae* infection (KEGG pathway)	40	1.64	0.197
30	AD12_24HRS_DN	Downregulated by late adenovirus infection of HeLa cells	18	1.62	0.215
	Motif gene set name	**Description**	**Size**	**NES**	**FDR**
1	V$ATF6_01	Targets of **ATF6** [TGACGTGG]	102	1.72	0.146
2	V$HTF_01	Targets of **HTF1** [NNWWWWNGMCACGTCATYNYWNNN]	58	1.65	0.163
3	GGAANCGGAANY_UNKNOWN	motif GGAANCGGAANY [no known TF]	90	1.58	0.212
4	AGCGCTT,MIR-518F,MIR-518E,MIR-518A	Targets of microRNA **518A, 518E, 518F** [AGCGCTT]	18	1.57	0.180
5	V$STAT5A_04	Targets of **STAT5A** [NNNTTCYN]	171	1.51	0.259
6	ATGCTGG,MIR-338	Targets of microRNA **338** [ATGCTGG]	114	1.45	0.384
7	V$XBP1_01	Targets of **XBP1** [NNGNTGACGTGKNNNWT]	115	1.43	0.416
Enriched in non-permissive LCLs					
	Curated gene set name	**Description**	**Size**	**NES**	**FDR**
1	CROONQUIST_IL6_STARVE_UP	Upregulated by IL6 in a multiple myeloma cell line (vs. IL6-starved)	33	2.22	0.002
2	DOX_RESIST_GASTRIC_UP	Overexpressed in chemoresistant gastric cancer cell lines	39	2.21	0.002
3	IDX_TSA_UP_CLUSTER3	Upregulated 8hr into adipocyte differentiation of 3T3-L1 cells	84	2.19	0.001
4	ZHAN_MM_CD138_PR_VS_REST	Overexpressed in PR (proliferation) subset of MM (vs. other cases)	40	2.18	0.001
5	LE_MYELIN_UP	Overexpressed in damaged nerves or EGR2Lo/Lo nerves	87	2.17	0.001
6	LEE_TCELLS3_UP	Overexpressed in intrathymic T progenitors and double-positive thymocytes, compared to all later stages of T cell development	95	2.14	0.001
7	P21_P53_ANY_DN	Downregulated by p21, in a p53-dependent fashion, in OvCa cells	43	2.13	0.001
8	CROONQUIST_IL6_RAS_DN	Upregulated by N-ras in a MM cell line (vs. IL6-exposed)	23	2.12	0.001

Table 6 (contd.)

9	ADIP_DIFF_CLUSTER5	Upregulated 24hr into adipocyte differentiation of 3T3-L1 cells	36	2.12	0.001
10	ZHAN_MM_CD138_CD1_VS_REST	Overexpressed in mild CD-1 subset of MM (vs. other cases)	40	2.08	0.002
11	GREENBAUM_E2A_UP	Overexpressed in E2A-deficient pre-B cells	32	2.08	0.002
12	P21_P53_MIDDLE_DN	Downregulated by p21, in a p53-dependent fashion, in OvCa cells	22	2.06	0.002
13	LIAN_MYELOID_DIFF_TF	Transcription factors upregulated during neutrophil differentiation	33	2.01	0.004
14	LI_FETAL_VS_WT_KIDNEY_DN	Overexpressed in Wilms' tumor (vs. normal fetal kidney)	151	2.01	0.005
15	SERUM_FIBROBLAST_CELLCYCLE	Cell cycle genes upregulated in fibroblasts by serum exposure	118	1.99	0.006
16	KLEIN_PEL_DN	Underexpressed in PEL cases (vs. other NHLs and normal B cells)	56	1.95	0.012
17	HYPOXIA_REG_UP	Induced by hypoxia in renal tubular epithelial cells	36	1.93	0.015
18	UVB_NHEK1_DN	Downregulated by UV-B light in normal keratinocytes	242	1.91	0.020
19	HUMAN_TISSUE_THYMUS	Expressed specifically in human thymus	16	1.90	0.022
20	BREAST_DUCTAL_CARCINOMA_GENES	Overexpressed in high-tumor-grade breast ductal carcinoma	18	1.89	0.025
21	ZHAN_MM_MOLECULAR_CLASSI_UP	Overexpressed in mild CD-1 subset of MM (vs. CD-2 subset)	51	1.88	0.024
22	UVC_HIGH_D2_DN	Downregulated by UV-C light in normal keratinocytes	33	1.88	0.024
23	SHEPARD_BMYB_MORPHOLINO_DN	Downregulated by knockdown of B-*myb* in zebrafish	176	1.87	0.029
24	HDACI_COLON_SUL16HRS_UP	Upregulated by the NSAID sulindac in Caco-2 cells	37	1.86	0.029
25	HADDAD_HSC_CD10_UP	Overexpressed in hematopoietic stem cells with B cell potential (vs. T/NK cell potential)	254	1.86	0.030
26	IRITANI_ADPROX_UP	Lymphatic endothelium-specific genes induced by Prox-1	23	1.85	0.030
27	VERNELL_PRB_CLSTR1	Upregulated by E2F, downregulated by pRB and p16 in U2OS cells	60	1.85	0.030
28	DNA_REPLICATION_REACTOME	DNA replication reactome (GenMAPP pathway)	44	1.84	0.032
29	AGED_MOUSE_RETINA_ANY_UP	Upregulated in retina of aged (16 mo) mice (vs. 3 mo)	21	1.84	0.033
30	ETSPATHWAY	ETS / macrophage differentiation (BioCarta pathway)	18	1.83	0.033
	Motif gene set name	**Description**	**Size**	**NES**	**FDR**
1	GGCACTT,MIR-519E	Targets of microRNA **519E** [GGCACTT]	122	1.85	0.064
2	GTATTAT,MIR-369-3P	Targets of microRNA **369-3p** [GTATTAT]	208	1.84	0.035
3	GCGCCTT,MIR-525,MIR-524	Targets of microRNA **525, 524** [GCGCCTT]	15	1.80	0.046
4	GGCAGAC,MIR-346	Targets of microRNA **346** [GGCAGAC]	41	1.76	0.061
5	GCCNNNWTAAR_UNKNOWN	motif GCCNNNWTAAR [no known TF]	125	1.74	0.057
6	KTGGYRSGAA_UNKNOWN	motif KTGGYRSGAA [no known TF]	64	1.74	0.049
7	GTGTGAG,MIR-342	Targets of microRNA **342** [GTGTGAG]	66	1.74	0.043
8	AGCATTA,MIR-155	Targets of microRNA **155** [AGCATTA]	132	1.71	0.053
9	GTACTGT,MIR-101	Targets of microRNA **101** [GTACTGT]	253	1.68	0.067
10	V$CDPCR3_01	Targets of **CUTL1 / CDP** [CACCRATANNTATBG]	46	1.68	0.061
11	V$POU6F1_01	Targets of **POU6F1** [GCATAAWTTAT]	206	1.67	0.059
12	ATGTAGC,MIR-221,MIR-222	Targets of microRNA **221, 222** [ATGTAGC]	138	1.65	0.073
13	V$EGR3_01	Targets of **EGR3** [NTGCGTGGGCGK]	65	1.64	0.076
14	AAAGGAT,MIR-501	Targets of microRNA **501** [AAAGGAT]	126	1.61	0.096
15	V$FOXO4_02	Targets of **MTTL7 / FOXO4** [NNGTTGTTTACNTN]	225	1.60	0.100
16	V$PAX8_01	Targets of **PAX8** [NNNTNNNGNGTGANN]	29	1.60	0.094
17	V$PAX6_01	Targets of **PAX6** [NNNNTTCACGCWTGANTKNNN]	85	1.59	0.100
18	SMTTTTGT_UNKNOWN	motif SMTTTTGT [no known TF]	355	1.59	0.099
19	GAGACTG,MIR-452	Targets of microRNA **452** [GAGACTG]	93	1.58	0.096
20	TCTGGAC,MIR-198	Targets of microRNA **198** [TCTGGAC]	84	1.58	0.093
21	TTAYRTAA_V$E4BP4_01	Targets of **NFIL3 / E4BP4** [TTAYRTAA]	216	1.58	0.092
22	ATGCAGT,MIR-217	Targets of microRNA **217** [ATGCAGT]	111	1.58	0.089
23	V$CDP_02	Targets of **CUTL1 / CDP** [NWNATCGATTANYNN]	96	1.56	0.099
24	V$FREAC2_01	Targets of **FOXF2 / FREAC2** [NNANNGTAAACAANNN]	226	1.55	0.109
25	SGCGSSAAA_V$E2F1DP2_01	Targets of **E2F1:DP2 heterodimer** [SGCGSSAAA]	144	1.55	0.108
26	TTTGTAG,MIR-520D	Targets of microRNA **520D** [TTTGTAG]	328	1.52	0.134
27	V$EVI1_01	Targets of **EVI1** [WGAYAAGATAAGATAA]	16	1.52	0.138
28	CAGTATT,MIR-200B,MIR-200C,MIR-429	Targets of microRNA **200B, 200C, 429** [CAGTATT]	460	1.51	0.138
29	V$E2F1_Q6	Targets of **E2F1** [TTTSGCGS]	195	1.51	0.137
30	V$OSF2_Q6	Targets of **RUNX2 / OSF2** [ACCACANM]	223	1.51	0.138

NES = Enrichment score normalized to gene set size. **FDR** = Estimated false discovery rate.

5.2.3 Genes overexpressed in permissive or non-permissive LCLs, detected by quantitative RT-PCR.

To further explore hypotheses suggested by the microarray results, we subjected a larger group of LCLs (seven permissive and seven non-permissive) to an RT-PCR assay for cellular gene expression. We investigated 92 genes, which were identified by strongly differential expression in the microarray, by relevance to B cell or EBV biology, or both. Seven of the selected genes (*BCL11A, BCL6, CD40LG, CT45-4, HLA-DRB3, HLAD-DRB5, U2B7*) were not detected in the majority of samples. For the remaining 85 genes, we used the $2^{-\Delta\Delta Ct}$ method to quantify relative gene expression, normalizing the results to an endogenous control and then comparing that value to the value for a reference sample, LCL17. Each measurement was normalized twice, with either *GAPDH* or *B2M* as the endogenous control gene, and these two normalized values were averaged together. The mean for seven permissive LCLs (mean EBV DNA/cell 42.9, s.d. 23.6) was compared to the mean for seven non-permissive LCLs (mean EBV DNA/cell 1029.1, s.d. 520.6), and all 85 genes (plus two more endogenous controls, *HPRT1* and *ACTB*) were characterized for significance of the difference between permissive and non-permissive LCLs. All cellular genes measured by RT-PCR were ranked both by *p*-value of the difference derived from a two-sample t-test (ranked in **Table 7**) and by relative fold difference between the two populations of LCLs (indicated by colored background in **Table 7**).

Table 7. Genes investigated with RT-PCR array, ranked by significance of the difference between populations of LCLs.

Gene symbol	p-value * (low>high)	p-value * (high>low)	Synonyms
CD40	**0.00003**		
EVL	**0.00112**		
EBF1	***0.00182***		***EBF***
PTPRK	***0.00466***		
LRMP	**0.00481**		Jaw1
ID2	***0.00522***		
ETS1	**0.00559**		
CD79B	**0.00874**		Ig-beta
TNFSF13B	**0.01126**		BAFF / BLyS
TSC22D3	**0.01355**		**GILZ / Glucocorticoid-induced leucine zipper**
CD99	**0.02085**		
PAX5	**0.02562**		BSAP
CD69	**0.03061**		
ITGB2	**0.03495**		CD18 / Integrin Beta2
RAC2	**0.03500**		
EBI2	**0.03834**		**IL27B**
TCF3	**0.03856**		E2A
NFATC1	**0.04748**		
RGS1	**0.05508**		
BHLHB2	**0.05551**		Dec1
CCR7	**0.05738**		**CD197**
HERC5	**0.05866**		CEB1/Cyclin E Binding protein
EGR1	***0.07464***		***Zif-268***
GNA15	**0.07588**		**Galpha15**
KLF2	**0.07731**		**LKLF**
EDG6	**0.09377**		S1P4
CRIP1	**0.09469**		
ATF1	**0.10263**		
TNFRSF13C	**0.10518**		BAFFR / BR3 / CD268
CD38	***0.11367***		
CD79A	**0.11696**		Ig-alpha / Mb-1
STAT1	**0.11783**		
STAP1	**0.11961**		BRDG1
INPP5D	**0.13569**		SHIP1
CD19	**0.13617**		
ATF2	**0.13691**		
CD48	**0.13960**		
MYB	**0.15182**		
CXCR4	**0.15510**		CD184
HLA-DMB	**0.18788**		
IFI27	***0.19137***		
EBI3	**0.19279**		
S100A4	**0.21019**		Mts1 / Metastasin
ATF4	**0.22466**		

Table 7 (contd.)

IL2RB	**0.24372**		CD122
CCL3	**0.24501**		Mip-1alpha
SLC2A3	**0.25367**		GLUT3
IRF5	**0.25371**		
GFI1	**0.29951**		Znf-163
CORO1A	**0.30293**		p57 / coronin-1
CD70	**0.30440**		CD27L / TNFSF7
ATF6	**0.31090**		
ATF5	**0.32549**		
SRGN	**0.33410**		PRG1 / Serglycin
XBP1	**0.33587**		
PRDM1	**0.34917**		Blimp-1
TIMP1	**0.38669**		
TNFRSF14	**0.39945**		HVEM
CCL4	**0.43494**		Mip-1beta
ID3	**0.43625**		
FAIM3	**0.44830**		
HPRT1	**0.45451**		
DDIT4	**0.46010**		REDD1 / RTP801
TNFRSF8	**0.47262**		CD30
JUND	**0.47416**		
ACTB		**0.48297**	
IRF4		**0.40400**	MUM-1
TP53		**0.37892**	p53
BST2		**0.36339**	
TAP1		**0.32637**	
IL15		**0.21073**	
EGR2		**0.17723**	
TNFRSF17		**0.15706**	BCMA / CD269
RAB31		**0.15491**	**Rab22b**
CDKN1A		**0.10813**	p21
HSPA8		**0.10770**	
SDC1		**0.10088**	**Syndecan / CD138**
FKBP11		**0.09225**	
CD27		**0.08568**	
PPIB		**0.06984**	cyclophilin B
LEPREL1		***0.06700***	
ARMET		**0.04494**	
CALR		**0.01762**	Calreticulin
SDF2L1		**0.01597**	
CCR10		**0.01493**	
DNAJB11		**0.01158**	
HSP90B1		**0.00930**	**Grp94**

*** from a 2-sample t test; n=7 in each group.**

Genes with orange background also had fold change > 3.00.

Genes with yellow background also had fold change > 2.00.

Table 8 shows all the genes whose differential expression was significant at a level of $p < 0.05$, or a fold difference greater than twofold. Although the three PC-associated transcription factors investigated (*XBP1*; *IRF4*/MUM1; *PRDM1*/Blimp-1) were not overexpressed in permissive LCLs, we did detect a difference in expression of *SDC1*, encoding the plasma cell surface marker Syndecan-1/CD138; and *CCR10*, encoding a chemokine receptor (the CCL27/CCL28 receptor) that is upregulated on PCs and EBV-immortalized B cell lines, but not EBV^+ BL lines [238, 239]. The difference in *CCR10* expression was small but consistent, while the high fold difference in *SDC1* expression was the result of two data points **(Figure 15A)**. Interestingly, although permissive LCLs did not show transcription factor expression indicative of increased plasma cell differentiation, non-permissive LCLs overexpressed *EBF1*, *PAX5*, *TCF3*/E2A, and *ETS1*, which are all components of a TF network that maintains the proliferating B cell phenotype and inhibits the XBP-1/Blimp-1 network **(Figure 15B)**.

The three genes most overexpressed in non-permissive LCLs were *EBF1*, *ID2*, and *PTPRK*. *ID2* encodes a non-DNA-binding transcription factor thought to inhibit E2A and Pax5 activity and in turn be downregulated by EBF-1 and Pax5 [240]. *PTPRK* encodes a receptor tyrosine phosphatase that regulates activity of proteins including Src and EGFR, and mediates anti-proliferative and pro-migratory effects of TGF-β [241-243]. *ID2* is overexpressed in most cases of HD, particularly EBV+ cases, while *PTPRK* seems to be specifically downregulated by EBNA1 in EBV+ HD [243, 244]. **Table 8** shows that these were the only genes that were both overexpressed as determined by significance of $p < 0.01$, and overexpressed by fold difference greater than threefold, in non-permissive LCLs. Subsequent RT-PCR analysis of four permissive and four non-

permissive LCLs for *EBF1*, *ID2*, and *PTPRK* expression at six timepoints further established this pattern (data not shown). A Western blot showed that EBF1 was also overexpressed in non-permissive LCLs at the protein level (data not shown).

Table 8. Genes found by RT-PCR array to be most differentially expressed between permissive (high-copy) and non-permissive (low-copy) LCLs.

	p-value *	fold change		p-value *	fold change
p-value <0.05 (low vs. high)			**p-value <0.05 (high vs. low)**		
CD40	**<0.001**	1.76	DNAJB11	**0.012**	1.59
EVL	**0.001**	1.84	CCR10	**0.015**	1.49
LRMP (Jaw-1)	**0.005**	1.98	SDF2L1	**0.016**	1.77
ETS1	**0.006**	1.38	CALR	**0.018**	1.52
CD79B	**0.009**	1.54	ARMET	**0.045**	1.49
TNFSF13B (BAFF)	**0.011**	1.68	**Fold difference > 2.0 (high / low)**		
CD99	**0.021**	1.38	LEPREL1	0.067	**4.53**
PAX5 (BSAP)	**0.026**	1.42	SDC1 (Syndecan)	0.101	**2.85**
ITGB (Integrin β2)	**0.035**	1.47	RAB31 (Rab22b)	0.155	**2.11**
RAC2	**0.035**	1.23	CD27	0.086	**2.02**
TCF3 (E2A)	**0.039**	1.31	**Both**		
NFATC1	**0.048**	1.67	**HSP90B1 (Grp94)**	**0.009**	**2.16**
Fold difference > 2.0 (low / high)					
IFI27	0.191	**15.46**			
EGR1 (Zif-268)	0.075	**3.98**	*** from a 2-sample t-test; n=7 in each population**		
CD38	0.114	**3.38**			
KLF2	0.077	**2.11**			
CCR7 (EBI1)	0.057	**2.10**			
GNA15 (Gα15)	0.076	**2.09**			
Both					
EBF1	**0.002**	**3.25**			
PTPRK	**0.005**	**3.42**			
ID2	**0.005**	**4.23**			
TSC22D3 (GILZ)	**0.014**	**2.20**			
CD69	**0.031**	**2.63**			
EBI2	**0.038**	**2.03**			

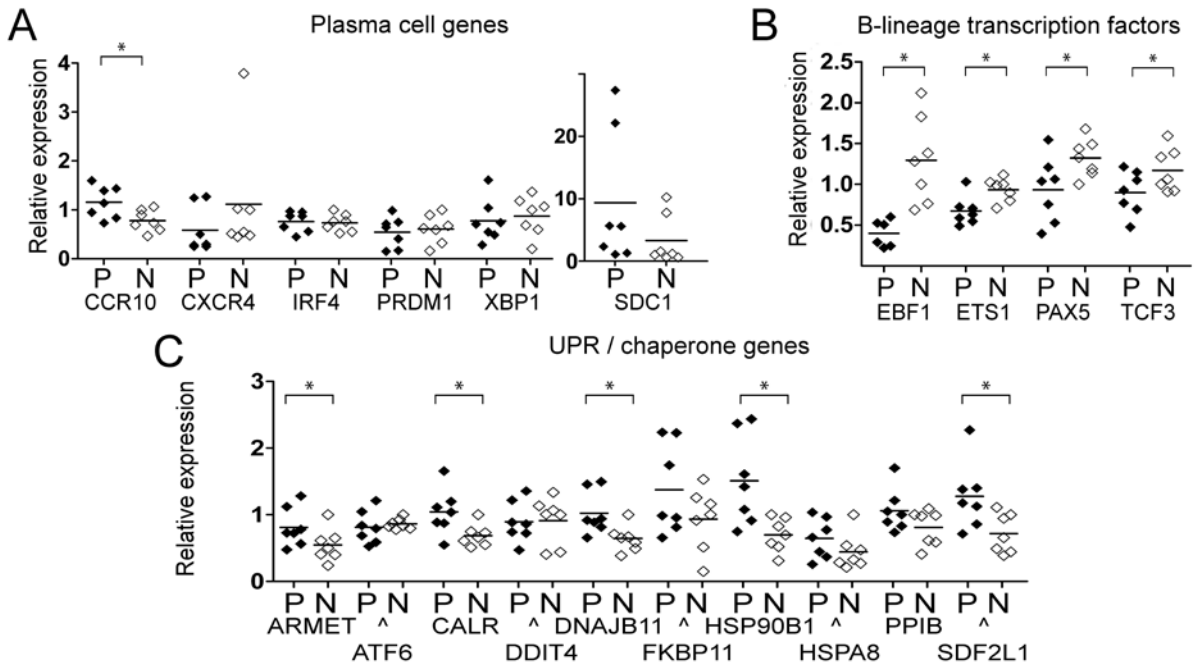

Figure 15. Differential expression of genes from three pathways of interest. Permissive LCLs (n=7) and non-permissive LCLs (n=7) were measured for expression of 90 cellular mRNAs, using RT-PCR and normalizing to both *B2M* and *GAPDH*. **(A)** Relative expression of plasma cell genes. **(B)** Relative expression of transcription factors that maintain B-cell lineage. **(C)** Relative expression of UPR / ER chaperone genes. Horizontal bars show the means of the permissive (filled) and non-permissive (open) groups, and asterisks indicate $p < 0.05$ from Student's *t* test.

Five of the ten genes significantly overexpressed in permissive LCLs **(Table 8, right column)** are associated with ER stress or the unfolded protein response (UPR). The UPR is a sequence of events induced by accumulation of unfolded proteins in the ER, leading to rapid breakdown of these proteins followed by enhanced new protein synthesis and sometimes autophagy. UPR genes suggested by the microarray results and then measured using RT-PCR included *ARMET*, *ATF6*, *CALR*, *DDIT4*, *DNAJB11*, *FKBP11*, *HSP90B1*, *HSPA8*, *PPIB*, and *SDF2L1*, encoding ER proteins, and the

downstream transcription factors *XBP1* and *ATF4*. Eight of the ten ER genes were overexpressed in permissive LCLs **(Figure 15C)**.

XBP1 and *ATF4* expression did not differ between the two groups of LCLs, but were positively associated with viral LMP1 expression **(Figure 16A,B)**. *XBP1* and *ATF4* were more strongly correlated with each other despite not being correlated with the pattern of other UPR genes **(Figure 16C)**. This suggests that the way cell lines differ in constitutive expression of UPR chaperones is independent of the way they differ in induction of downstream UPR transcription factors. The chaperone and cochaperone proteins could be described as "housekeeping proteins" because of their essential role in the metabolism of nearly all cells, but are not expected to be as uniformly expressed across cell lines and conditions as some housekeeping proteins that are used as endogenous controls.

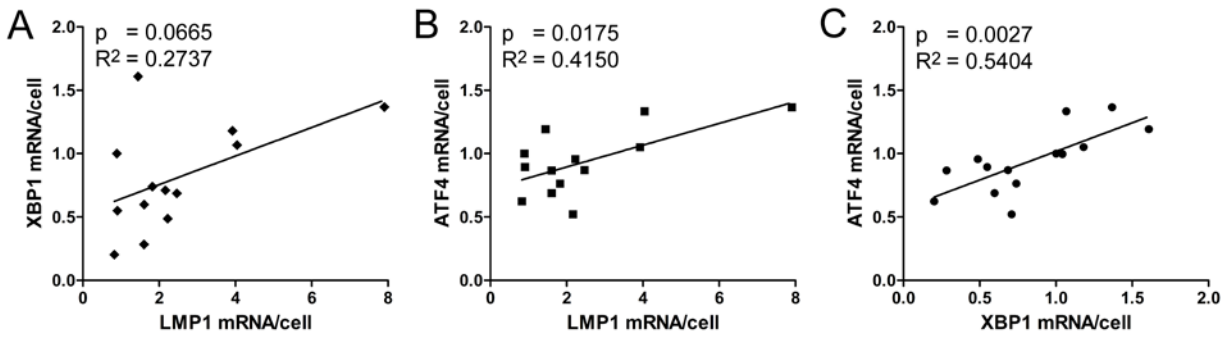

Figure 16. Correlations between downstream UPR transcription factors and LMP1. 19 LCLs were measured using the RT-PCR array for cellular genes, and also using primers designed by our lab for latent viral genes. **(A,B)** The association between LMP1 and UPR factors ATF and XBP1 is closer than the association between any of these three genes and EBV lytic reactivation. **(C)** ATF4 and XBP1 are significantly correlated in their expression.

5.2.4 Permissivity of LCLs for spontaneous lytic reactivation is not associated with enhanced splicing of XBP-1 to its active form.

One branch of the UPR leads to upregulation of XBP-1 mRNA, and another leads to splicing of XBP-1 mRNA to produce its active XBP-1s isoform. Although lytic permissivity did not correlate with XBP-1 expression, we considered that it might correlate with its splicing. To look for such a relationship, we determined the relative quantities of spliced and unspliced transcripts by SYBR Green PCR followed by dissociation curve analysis to distinguish the two isoforms. No relationship was detected **(Figure 17A)**, suggesting that EBV reactivation does not induce the UPR despite being found at higher levels in cell lines with more propensity for UPR activity.

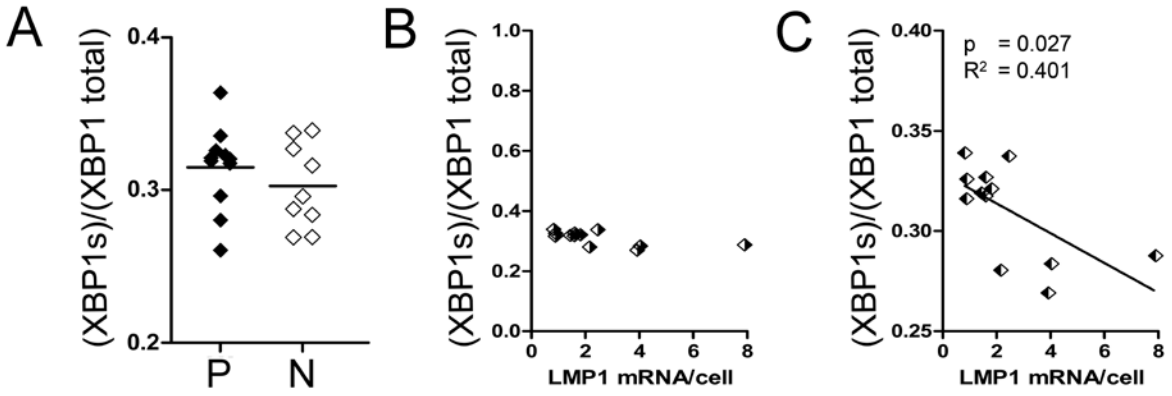

Figure 17. Splicing of XBP1 is not correlated to EBV activity in LCLs. (A) Permissive LCLs (n=11) and non-permissive LCLs (n=9) were measured for XBP1 splicing by SYBR Green RT-PCR followed by a melt curve used to detect the relative amounts of XBP1s and XBP1u mRNA. Horizontal bars show the means of the permissive (filled) and non-permissive (open) groups, and asterisks indicate $p < 0.05$ from Student's *t* test. **(B, C)** XBP-1 splicing was plotted against expression of the UPR-regulating viral signaling gene LMP1.

We also considered that XBP1 splicing might correlate with LMP1 expression, as LMP1 appears to correlate with expression of XBP1 and ATF4. The relationship is a negative correlation that can be seen with a very focused y-axis **(Figure 17C)**. However, the distinction between (XBP1s)/(XBP1 total) ratios of 0.25 and 0.35 **(Figure 17B)** is probably physiologically negligible, as other studies find vastly greater differences in XBP1 splicing under different conditions of cell stress [245-248]. The distinction between permissive and non-permissive LCLs may lie instead in their propensity for UPR induction rather than their background level of UPR activity.

5.2.5 The upstream UPR protein Grp94 is more highly expressed in permissive LCLs.

Grp94 (Gp96, *HSP90B1*) is a member of the glucose-regulated protein (GRP) family of chaperone proteins that are induced by ER stress. Grp94 is an essential chaperone for the folding of multiple TLRs, integrins, and IGF-II, though it is not important for plasma cell function [249, 250]. Grp94 is expressed at abundant levels in low-stress conditions, explaining the extremely broad cell tropism of the VSV virus which depends on Grp94 for cell entry [251]. Cells that consistently produce high levels of GRP proteins (Grp94, Grp78/BiP, calreticulin) are better prepared to resist ER stress-induced apoptosis [252].

We considered that a high level of Grp94 could indicate a cell line that can more easily accommodate the ER stress associated with lytic virus production, thus containing many lytic genomes instead of undergoing apoptosis soon after the lytic viral proteins begin to be expressed. However, since the permissive cell lines are more likely

to undergo the initial step of ZEBRA induction, it is more likely that lytic reactivation is downstream of some UPR pathway. This may indicate a minority of cells undergoing both acute UPR activity and lytic EBV replication, or it may indicate that all cells in that particular LCL have a high basal level of UPR activity and only a few trip over into ZEBRA induction. First we needed to extend the RT-PCR data by measuring the protein level of Grp94 in LCLs. A Western blot stained with polyclonal antibodies against Grp94 is shown in the upper panel of **Figure 18A**, with staining of the same blot for GAPDH in the lower panel. Quantification of the bands on two Western blots, followed by normalizing each Grp94 band to its corresponding GAPDH band, indicates that the permissive LCLs express about twice as much Grp94 as non-permissive LCLs **(Figure 18B)**. Two of the eight LCLs in these experiments were not among those used in the earlier RNA experiments.

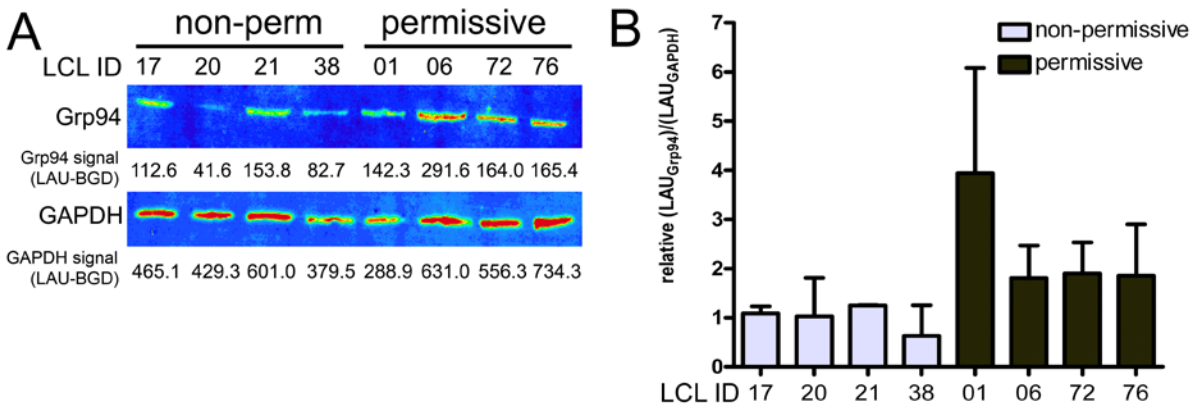

Figure 18. Grp94, the protein encoded by UPR gene *HSP90B1*, is overexpressed in permissive LCLs. (A) LCLs were pelleted while proliferating, lysed and measured by Western blot. Image Gauge software was used to quantify each band, by measuring LAU (arbitrary intensity units) of a box containing the band, and subtracting background (BGD) LAU of adjacent boxes from the same lane. **(B)** Average of two Western blots, of samples taken on different days. For each blot, (LAU-BGD) values for Grp94 were divided by (LAU-BGD) for GAPDH, and the mean Grp94/GAPDH ratio for the four non-permissive LCLs was set as 1. In an unpaired student's *t* test comparing permissive and non-permissive groups, $p < 0.01$.

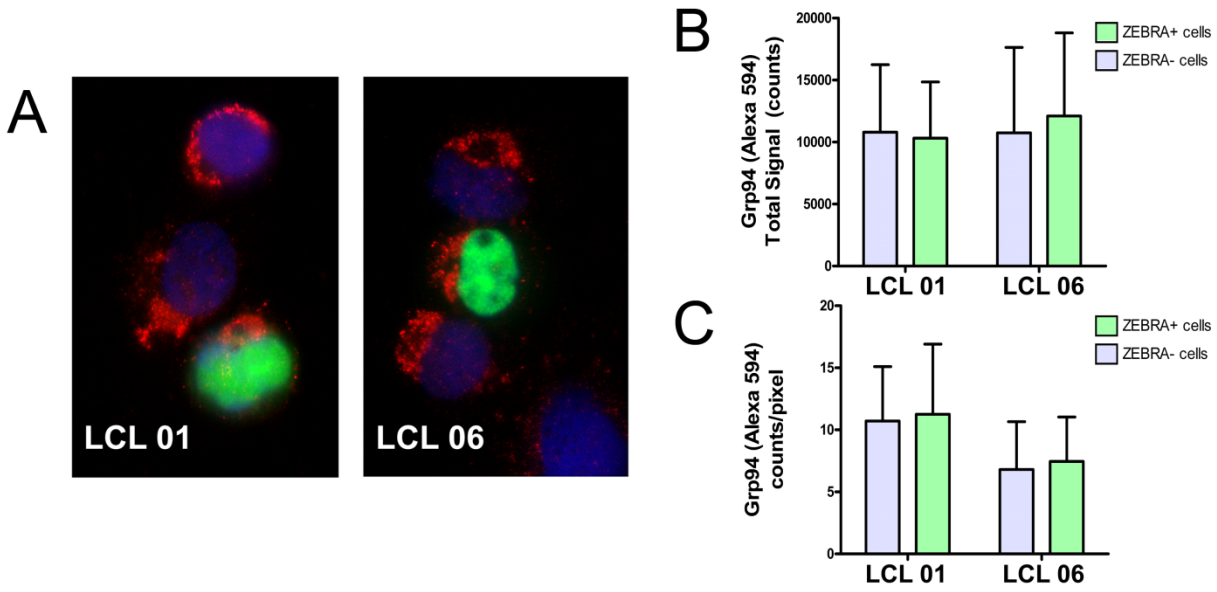

Figure 19. Grp94 overexpression in permissive LCLs is not concentrated in cells producing the lytic switch protein ZEBRA. (A) Permissive LCL 01 and 06 were spun onto slides and visualized using Nuance filters and software. ZEBRA+ (green) cells do not contain proportionately more Grp94 (red). **(B)** Cells were measured for total Grp94 (Alexa 594) signal intensity in ZEBRA+ (n=15 in LCL 01, n=24 in LCL 06) and ZEBRA- (n=91 in LCL 01, n=205 in LCL 06) cells. **(C)** Cells were measured for the ratio of Grp94 (Alexa 594) signal (counts) to cell area (pixels). For all graphs, bars represent mean ± SD.

5.2.6 Grp94 overexpression in permissive LCLs is not concentrated in cells with lytic EBV reactivation.

Next, we addressed the question of whether enhanced UPR activity in permissive LCLs is downstream of EBV reactivation, by looking at Grp94 expression and lytic gene expression in individual cells. If EBV reactivation in permissive LCLs leads to two- or three-fold upregulation of UPR genes in these LCLs, it would be because the small minority of cells with lytic EBV experience massive UPR upregulation. **Figure 19A** shows examples of two LCLs stained for Grp94 (red) and

ZEBRA (green), with the blue nuclear stain DAPI. The ZEBRA+ cells do not appear to be any brighter for Grp94 than ZEBRA-negative cells.

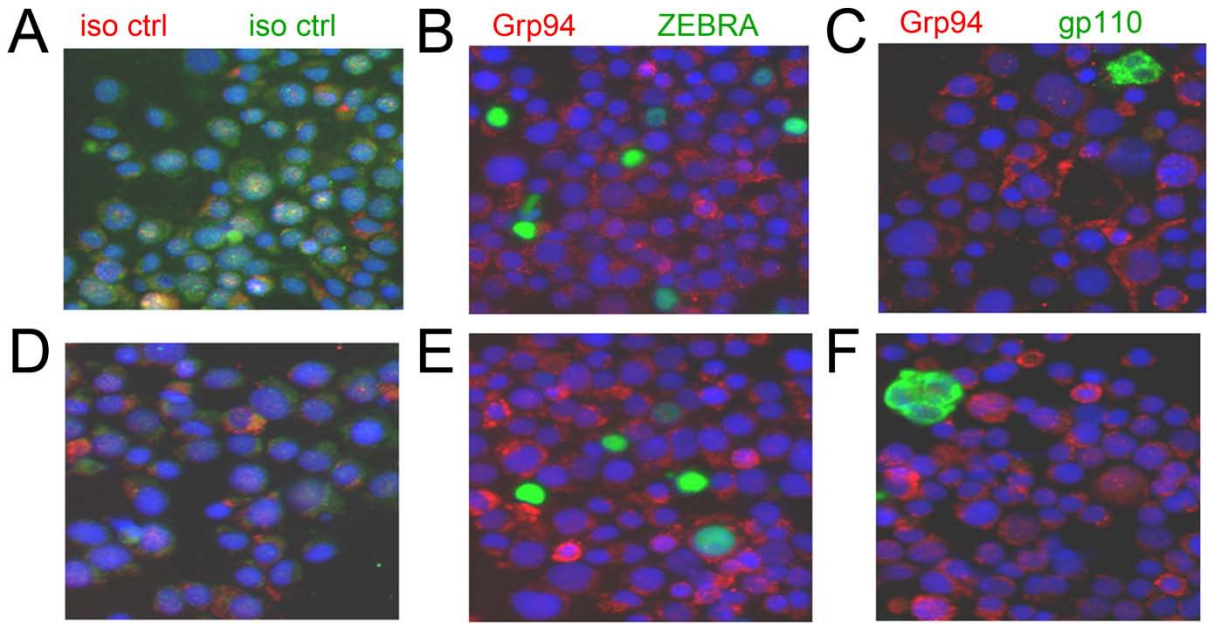

Figure 20. More examples of Grp94 and lytic viral protein expression in LCLs. Permissive LCL76 was spun down onto slides at a higher cell density than used in Fig. 19. Slides were stained with DAPI and normal mouse and rabbit IgG **(A,D)**; mouse α-ZEBRA and rabbit α-Grp94 **(B,E)**; or mouse α-gp110 and rabbit α-Grp94 **(C,F)** – followed by secondary staining with Alexa 488-conjugated α-mouse and Alexa 647-conjugated α-rabbit. Nuance filters and software were used to define the spectra for A488, A647, and DAPI, and unmix the light data collected into these three colors. Cells vary in brightness for Grp94 and can be clearly defined as positive or negative for ZEBRA and gp110.

Several microscope fields were imaged for these proteins, and Nuance software was used to define cells as “areas of interest”. Cells defined as AOIs by both red and green fluorescent intensity were considered ZEBRA+ cells, and cells defined as AOIs by only red fluorescent intensity were considered ZEBRA-negative cells. The range of Grp94 intensity per pixel was very similar between ZEBRA+ and ZEBRA-negative cells (Figure 19B). Also, the difference between LCL01 and LCL06 in Grp94 intensity per

pixel mirrors the difference between LCL01 and LCL06 in Grp94 content per cell in **Figure 18B**. **Figure 20** shows another permissive LCL, LCL76, stained for Grp94 (red) and either ZEBRA (green) (**Figure 20B, E**) or gp110 (green) (**Figure 20C, F**).

5.2.7 The calcium pump inhibitor Thapsigargin induces lytic reactivation.

After observing evidence of greater propensity for UPR activity in permissive LCLs, we looked for effects of artificially inducing the UPR in these cells. Thapsigargin is an inhibitor of Ca^{2+} ATPases in intracellular membranes, and leads to calcium stores in the ER being emptied into the cytoplasm, thus making protein folding less efficient and causing ER stress [253]. It leads to unfolded protein response activation in LCLs, accompanied by increased XBP-1 splicing, which seems to be counteracted by EBV EBNA3C [133, 254]. We found in pilot experiments that, as might be expected, thapsigargin induces lytic reactivation in most LCLs as well as Latency I-expressing Raji cells. We then looked for distinctions between permissive and non-permissive LCLs. We hypothesized that the increased levels of proteins like Grp94 in permissive LCLs would mean they respond more quickly to thapsigargin. In addition, the drug might confer a permissive, high-EBV-copy phenotype on non-permissive LCLs.

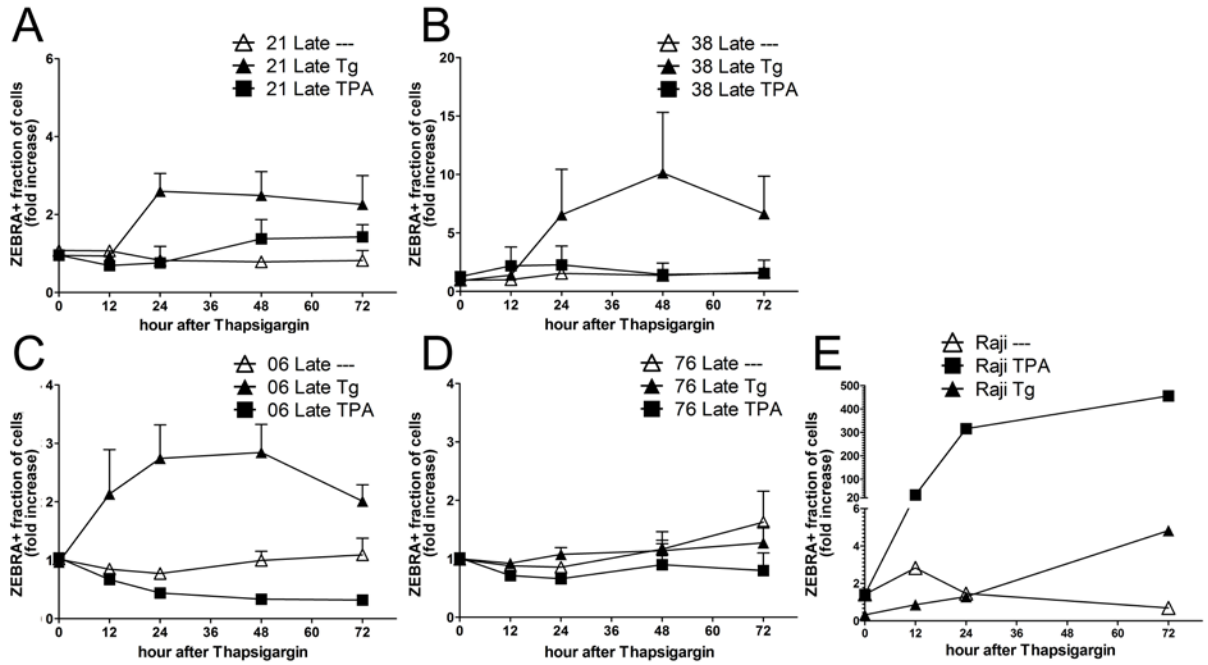

Figure 21. Thapsigargin enhances lytic EBV reactivation in LCLs. Late-passage LCLs (between 5.5 and 8 mo in culture) were grown at high passage in three drug conditions: thapsigargin (500 nM); TPA (2 nM) and sodium butyrate (500 µM); or negative control (0.1% DMSO). At the indicated timepoints, cells were isolated, fixed, permeabilized and stained with Alexa Fluor 488-labeled anti-ZEBRA antibody. The ZEBRA+ percent of cells was identified by gating as in **Figure 10**. **(A,B)** Tg induction of ZEBRA peaks at 24-48 hr in non-permissive LCL21 and LCL38. **(C)** Tg induction of ZEBRA peaks at 24-48 hr in permissive LCL06. **(D)** Neither Tg nor TPA/NaB causes ZEBRA induction in permissive LCL76. **(E)** The same concentrations of TPA/NaB and Tg were used to induce ZEBRA in the BL cell line Raji.

Several experiments were conducted to analyze the inducing ability of thapsigargin (Tg) in LCLs. 2 µM had the same effect as 500 nM in all four LCLs examined in the first experiment, so in subsequent experiments 500 nM was selected. Both non-permissive LCLs showed an increase in the percentage of cells that trip over into producing lytic switch protein ZEBRA **(Figure 21A, B)**. Permissive LCL76 showed no effect while permissive LCL06 showed a significant increase **(Figure 21C, D)**, which was by far the most significant increase observed in terms of number of cells induced (e.g. an increase from ~2.6% of cells ZEBRA+ to ~6.5% of cells ZEBRA+, compared to

an increase from 1 ZEBRA+ cell out of 1000 to 5 out of 1000). LCL06 also was the only line to show ZEBRA induction as early as the 12-hour timepoint after thapsigargin stimulus.

Interestingly, the well-established lytic EBV induction cocktail of TPA and sodium butyrate (NaB) had little to no effect on all four LCLs. As a positive control to confirm that the mixture was active, we cultured Raji cells in TPA/NaB **(Figure 21E)** and observed that the number of ZEBRA+ cells was increased by more than 300-fold (from 0.07% ZEBRA+ cells to 14.74% ZEBRA+ cells) by the 24-hour timepoint.

The pattern of lytic induction in these experiments was for the ZEBRA+ percent of cells to peak between 24 hours and 48 hours, and to be declining by 72 hours. As the cells were not proliferating in Tg- or TPA/NaB-treated cells, the decrease at 72 hours presumably indicates the progression of apoptosis pathways.

5.2.8 Early-passage LCLs show quicker UPR-driven lytic induction.

Earlier work had suggested that early-passage cell lines like LCLs are more responsive to TPA induction of lytic viral proteins than LCLs in long-term culture. Early in their passage, LCLs were more tumorigenic in SCID mouse models, and produced more IL-6. Early-passage LCLs are expected to be more responsive to environmental stimuli of lytic reactivation both *in vitro* and *in vivo*, but production of lytic DNA and infectious virus may not be enhanced to the same degree as production of lytic proteins [255, 256]. We examined effects of thapsigargin-induced stress on six early-passage LCLs (permissive: LCL01, LCL06, LCL76; non-permissive: LCL17, LCL21, LCL38).

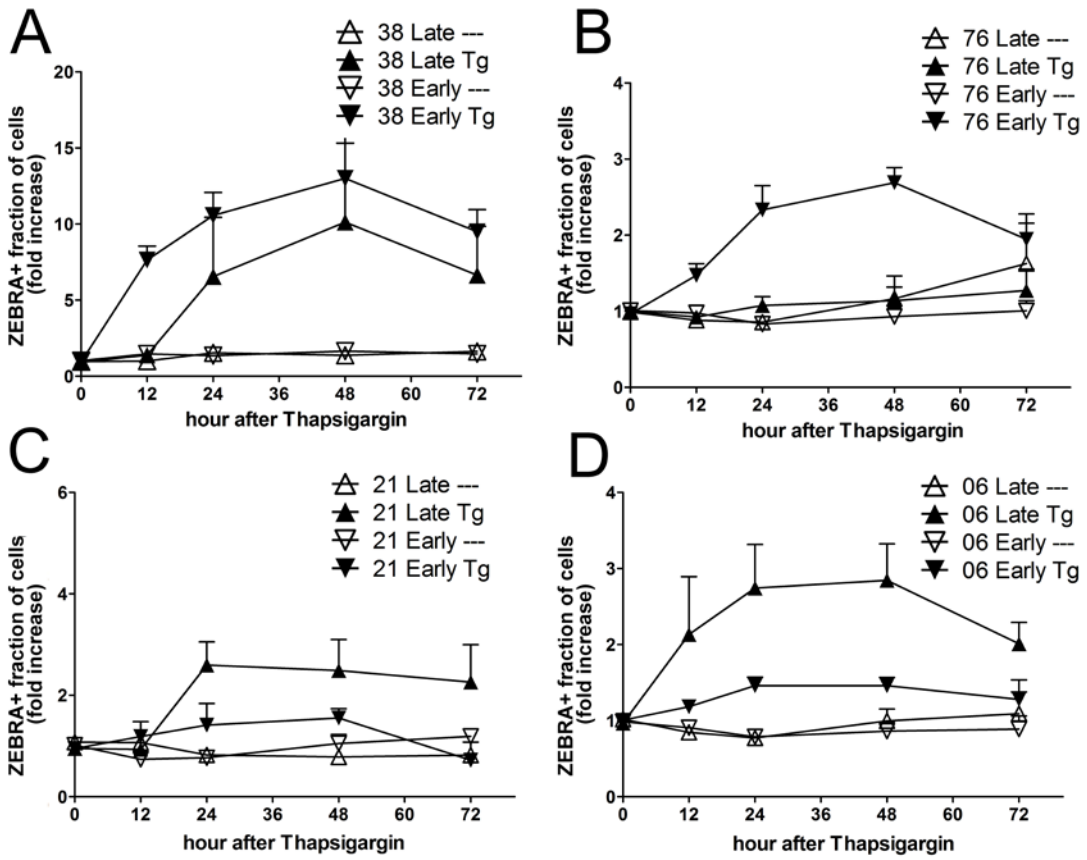

Figure 22. Early-passage LCLs show faster thapsigargin-induced lytic reactivation. Early-passage (between 2.5 and 5.5 mo in culture) and late-passage (between 5.5 and 8 mo) LCLs were grown with or without thapsigargin and stained as described in **Figure 21**. **(A,B)** In LCL38 and LCL76, Tg induction of ZEBRA is detectable at 12hr at early passage only. **(C)** In LCL21, Tg induction of ZEBRA peaks at a higher level at late passage, but is detectable at 12hr at early passage. **(D)** Tg induction of ZEBRA is detectable at 12hr in both early- and late-passage LCL06.

Early-passage LCL38 **(Figure 22B)** and LCL76 **(Figure 22D)** showed more induction of ZEBRA than the late-passage LCLs. This was particularly clear at the 12-hour timepoint, at which there is no detectable induction in the late-passage LCLs.

LCL21 **(Figure 22A)** and LCL06 **(Figure 22C)** show less induction of ZEBRA at early than late passage numbers. However, at 12 hours, early-passage LCL06 increases slightly, to about half of its peak level of ZEBRA induction (119% of the 0-hour baseline, compared to 91% of the baseline with no drug), which was similar to the induction detected at late passage (213% of the 0-hour baseline, compared to 85% with no drug). LCL21 showed an increase to 118% of baseline ZEBRA+ percentage at 12 hours, compared to a decrease to 74% with no drug. This was not a significant difference, but in late-passage LCL21 the values were 93% of baseline with Tg, and 107% with no drug.

Early-passage LCL01, in one experiment, saw the ZEBRA+ fraction of cells increase to 142% of the 0-hour baseline after 12 hours with thapsigargin, and peak at 322% of the baseline at 48 hours. LCL01 with TPA/NaB did not increase after 12 hours, and LCL01 with no drug only increased to 113% of the baseline.

Early-passage LCL17, in two experiments, saw the ZEBRA+ fraction of cells increase to an average of 578% of the 0-hour baseline after 12 hours with thapsigargin, and peak at 18 times the original value at 48 hours. LCL17 with TPA/NaB increased only to 231% of the baseline at 12 hours, and LCL17 with no drug did not increase.

5.2.9 Enhanced ZEBRA induction does not imply enhanced lytic DNA replication.

In experimental settings where full lytic reactivation is induced, either by TPA, BCR cross-linking, TGF-β2, or addition of a hormone that releases a repressor from the Z promoter, it is expected that ZEBRA expression will be greatly enhanced at 24 hours,

along with some production of viral DNA replication machinery, followed by DNA replication detectable at 48 hours [257-260]. Early-passage and late-passage LCLs were cultured with or without thapsigargin, and measured for EBV and cellular DNA at six timepoints **(Figure 23)**.

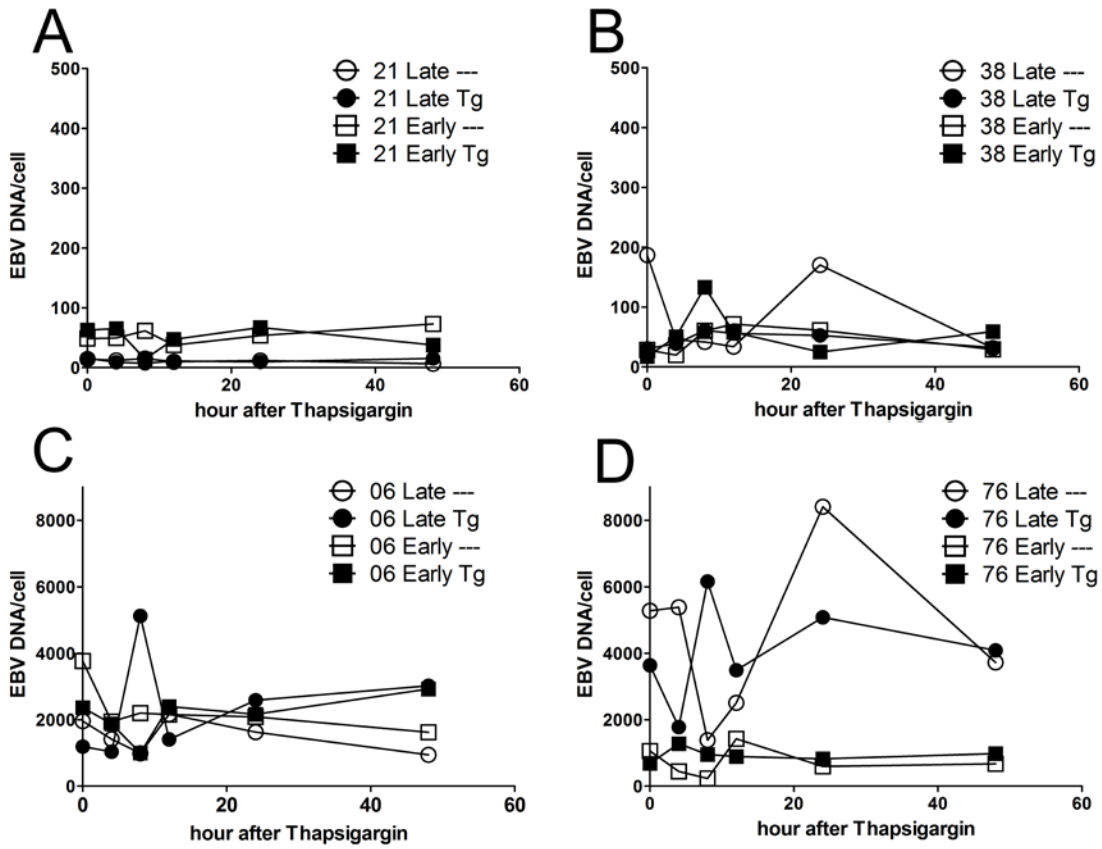

Figure 23. Induction of early lytic events is not accompanied by lytic DNA replication. LCLs were cultured with or without thapsigargin as described in Figure 21. DNA pellets were taken at the indicated timepoints, lysed and measured for cellular (GAPDH) and viral (gp350) DNA using real-time PCR, with two technical replicates for each gp350 measurement.

Addition of thapsigargin did not induce a high-EBV-copy phenotype in non-permissive LCL21 and LCL38. In none of the four LCLs was EBV DNA replication enhanced at 48 hours to the degree that would be expected in cell lines that have been

induced to undergo all events of the lytic virus cycle. The amount of EBV DNA in permissive LCL06 was higher with thapsigargin, at both early and late passage. Early-passage LCL06 reached 2925 EBV copies/cell at 48 hours, compared to 1622 in LCL06 with no drug. Late-passage LCL06 reached 3020 EBV copies/cell at 48 hours, compared to 943 in LCL06 with no drug.

LMP1 has been reported to enhance the ATF4/PERK/CHOP pathway of the UPR, while EBNA3C has been reported to increase phosphorylation and activation of the PERK substrate eIF2α despite blocking upstream UPR steps through interaction with Gadd34 and SUMO [133, 261]. Further investigation of the mRNAs induced by thapsigargin in these LCLs is underway, and will give a better picture of the kinetics of the UPR, its XBP1-dependent and XBP1-independent pathways, and its effect on lytic and latent viral activity in this heterogeneous set of LCLs.

6.0 DISCUSSION

6.1 HETEROGENEITY OF LCLS.

Lymphoblastoid cell lines are useful models for the processes of EBV Latency III and for EBV-infected proliferating B cells in disease, particularly PTLD. They are used as living genetic repositories and as HLA-matched immunological targets. By profiling a large panel of LCLs we have investigated the extent to which latently infected B cells vary in permissivity for EBV reactivation. Spontaneous reactivation in the cultured LCLs was characterized by viral DNA replication and the production of early and late lytic proteins. Depending on the cell line, between 0.1% and 8% of cells in an LCL were producing the lytic switch protein ZEBRA and, of these producers, about 20% made late structural proteins. The detection of glycoprotein B by flow cytometry and IFA shows that in permissive LCLs the entire process of lytic reactivation is upregulated, rather than an abortive expression of early lytic proteins and EBV DNA replication.

LCLs could be classified into two groups. One group was composed of “permissive” LCLs with high and fluctuating amounts of EBV DNA/cell (> 200 copies/cell), lower levels of latent gene expression, and high frequency of lytic gene positive cells. The other group consisted of “non-permissive” LCLs that had low amounts of EBV DNA/cell (8 – 200 copies/cell), variable levels of latent gene

expression, and a low frequency of lytic gene positivity. Just as permissive cell lines vary within a wider range for their production of lytic genes, non-permissive cell lines vary within a wider range for their production of latent genes. Statistical analysis suggested a weak negative relationship between the abundance of latent viral genes in an LCL and its permissivity for lytic reactivation.

The only latent gene for which there was a positive correlation was LMP2A. Although LMP2A may maintain latency by blocking cross-linked BCR from inducing lytic reactivation [262], there is evidence that LMP2A downregulates Pax-5 and EBF-1 expression, enhances differentiation into plasma cells, and induces Z promoter activity, all factors associated with increased lytic reactivation [63, 263, 264]. For every latent transcript, except LMP2A, the mean expression level in permissive LCLs was lower than that in non-permissive LCLs, although this difference was only statistically significant for the EBNA3 genes. These findings do not support models in which EBV latency proteins restrict the induction of the lytic cycle in proliferating cells. Indeed, of all the latent genes measured, LMP1 had the highest level of expression and by far the least difference in expression between permissive and non-permissive LCLs, making it implausible that the variation in LMP1 expression accounts for permissivity towards EBV reactivation. All LCLs were immortalized with the same strain of EBV (B95-8), which minimizes the possibility that permissivity related to virus strain variation. Lytic permissivity did not correlate with the amount of time spent in culture, and culturing LCLs with an inhibitor of lytic replication did not alter their permissivity. All of the above analyses suggest that permissivity for spontaneous lytic reactivation was not influenced by viral factors, with the possible exception of LMP2A.

It was clear from longitudinal analyses of LCLs that the difference in lytic permissivity was a stable property of an LCL. It was reasonable to suspect that lytic permissivity could be due to properties that were intrinsic to the founder B cell. Gross phenotypic properties such as doubling time, clumping, or the morphology and size of cells were not associated with lytic permissivity. Cellular factors, whose expression is expected to be a stable characteristic of a cell line, were investigated. Initially we analyzed expression of a range of B cell-surface markers that define ontological status and observed that only in permissive LCLs did more than 30% of the cells stain positive for B220, a marker for naïve primary B cells. Although this was intriguing, there was not an accompanying low level of CD27 and IgD on permissive LCLs, which would be expected for a naïve cell lineage. We also investigated whether permissive LCLs contained a higher percentage of cells with the $CD27^{hi}$ $CD38^{hi}$ phenotype characteristic of *in vitro* plasmacytoid differentiation, but this was not the case.

6.2 HOST GENE EFFECTS ON LYTIC PERMISSIVITY

6.2.1 Gene set enrichment analysis.

If intrinsic cellular factor(s) were responsible, the variation would need to be subtle. Genetic polymorphisms can lead to varying expression levels of genes in otherwise comparable cells from different individuals. Genetic analyses have connected single nucleotide polymorphisms (SNPs) in the *IFNG, TGFB1*, *IL10* and *IL1A* genes with

EBV-associated PTLD-like disease [265-267]. EBV reactivation after transplantation has been correlated with an *IFNG* genotype that produces low basal levels of IFN-γ [268]. SNPs that lead to a high basal level of *IL-10* expression [269], and SNPs in *HLA-A* [270], have been associated with susceptibility to EBV^+ HD. To learn more about the patterns of cellular gene expression that may influence LCLs' lytic permissivity, we first conducted an RNA microarray.

First we analyzed our data with respect to these earlier studies on genetic effects on lytic reactivation. Gene set analysis of our microarray suggested that genes upregulated by IFN-γ and IFN-α were overexpressed as a whole in non-permissive LCLs. However, *IFNG* itself was overexpressed by 2-fold in permissive LCLs, along with other cytokine genes including *IL15*, *GDF15* (MIC-1), *TNFSF4* (OX40L), *TNFSF13B* (BAFF), two MIP-1α chemokines (*CCL3*, *CCL3L3*) and one MIP-1β chemokine (*CCL4L2*). Non-permissive LCLs showed overexpression of pro-inflammatory cytokines *IL4*, *IL1A*, *MIF*, *PRL* (prolactin), and three other cytokines, *CKLF*, *CMTM3*, and *CMTM7*. Most of these genes were low in absolute amount of transcripts produced, and the results were less robust than for other genes discussed below.

Also overexpressed in non-permissive LCLs were potential target genes of EGR-family transcription factors – which is surprising, since EGR1 (the primary B-cell member of this family) is both induced by ZEBRA and able to induce Rta [164, 271]. Interestingly, among the top microRNAs whose targets were overexpressed in non-permissive LCLs were the oncogenic miRNAs 155 and 221/222. miR-155's targets include PU.1, CEBP/β, BACH1, SOCS1, AID, and SHIP1, and it is upregulated by TGF-β signaling [272-275]. This microRNA is linked to Latency III, Hodgkin's disease, PTLD,

and DLBCL and AML leukemias, but is known to not be associated with Latency I lymphomas [235, 276]. miR-155 orthologs are encoded by oncogenic herpesviruses HHV-8 and MDV-1, and miR-155 is induced by EBV LMP1 [277, 278]. miR-221/222 enhances the progression of several malignancies by inhibiting the cell cycle regulator $p27^{Kip1}$ and is upregulated by MDV-1 in lymphoma cells [237, 279].

Plasma cell-associated genes were expressed differentially in the microarray. Significantly, of the curated gene sets gleaned from earlier publications, the one most overexpressed in permissive LCLs was a group of 80 genes overexpressed in plasma cell and plasmablast subsets and underexpressed in peripheral blood and tonsillar B cells [233]. A few of these genes were individually overexpressed in permissive LCLs, including *HYOU1*, *TNFRSF17*, *PRG1*, *DDOST*, *PPIB*, *HSPA5*, *ARMET*, and *HSP90B1*, the last five of which are associated with the unfolded protein response. Also, when examining sets of genes with shared promoter motifs, we found that among the few overexpressed in permissive LCLs were the sets of genes whose promoters contained recognition sites for XBP-1, ATF-6, and HTF-1. HTF-1 is a rat homologue of XBP-1, and ATF-6 induces the expression of XBP-1 [280].

Just as permissive LCLs might have more expression of PC genes, non-permissive LCLs might have gene expression profiles that support stable proliferation rather than terminal differentiation. Non-permissive LCLs did overexpress a set of genes downregulated by the tumor suppressor genes p21 and p53, and permissive LCLs overexpressed a set of genes upregulated by p53. The gene for p21 (*CDKN1A*) was seen in later experiments to be overexpressed in non-permissive LCLs, though this was not statistically significant (1.3-fold enrichment; p=0.108). With regard to PC

differentiation, non-permissive LCLs overexpressed a gene set associated with a “proliferation” subgroup of multiple myeloma (MM) that predicts a bad prognosis compared to other MM cases [281], and gene sets upregulated in MM cells activated by the oncogene N-ras or exposed to the pro-proliferative cytokine IL-6. Also overexpressed in non-permissive LCLs was a set of genes downregulated in primary effusion lymphoma, identified in a study that concluded that PEL may be derived from plasmablasts [226].

6.2.2 Genes identified by quantitative RNA PCR.

When we compared LCLs grown with and without acyclovir there were negligible differences in gene expression, suggesting that neither the drug treatment nor late lytic viral events influenced the results of the microarray. Genes identified by this microarray study as potentially involved in lytic permissivity, and other genes of interest, were selected for further study using RT-PCR. Many gene expression studies have been done on the effects of EBV infection of B cells, but this is the first to characterize a variety of genetically distinct LCLs, all immortalized in the same way with the same strain of virus.

Thus far, the list of genes known to induce or repress lytic EBV replication is short. In physiological situations, the R promoter mainly responds to ZEBRA [198]. The Z promoter has been extensively characterized for binding sites, including motifs activated by XBP-1 and the ZEBRA protein it encodes [166], and repressed by signaling from CD40 as well as EBV LMP1 [160, 162]. We saw that *CD40* expression correlated negatively with lytic permissivity, although LMP1 expression did not. *CD40* was the

most differentially expressed gene in the RT-PCR comparison of 14 permissive and non-permissive LCLs (**Table 7**). Because *CD40* emerged as significant in this objective, large-scale comparison, we are encouraged that functional significance might attach to other genes detected in the same study.

In general, as shown in **Table 8** (a subset of the larger RT-PCR results shown in **Table 7**), more genes were significantly overexpressed in non-permissive LCLs than in permissive LCLs. In our microarray results the lists of genes overexpressed in non-permissive LCLs also contained more transcription factors, a trend that exists for activators, repressors, coactivators and corepressors **(Table 5)**. This suggests that some EBV-transformed B cell lines repress lytic reactivation more tightly because of a milieu of factors that counteract the cells' tendency to experience a positive feedback loop of ZEBRA expression and trip over into lytic reactivation.

Signaling from CD40 or LMP1 leads to the activation of transcription factors that repress the Z promoter. We detected a number of genes whose expression was greater in the non-permissive LCLs, but none are known to be upregulated by CD40 or LMP1. One study found that Pax-5 and EBF-1 *activity* were enhanced by CD40 signaling, but CD40 did not affect the *levels* of these factors [282]. Several genes known to be upregulated by lytic replication of EBV or other herpesviruses were nonetheless associated with the non-permissive phenotype in our LCLs, further suggesting that our findings did not reflect downstream effects of EBV reactivation. For example, *EGR1*/Zif-268 is directly induced by ZEBRA and directly induces BRLF1 [164, 271], while *ID2* is upregulated by lytic replication of EBV, HHV-8, CMV, and HSV [283-286]. However, we found both these factors overexpressed in LCLs with *less* lytic permissivity. Many genes

we investigated by RT-PCR had been detected in earlier studies to be influenced by EBV Latency III proteins, but were not regulated in either direction in our study (e.g. *JUND*, *EBI3*, *TIMP1*, *TNFRSF14*). This might be expected, since every cell line was producing the full complement of Latency III proteins, and EBNA2 and LMP1 expression were similar across both permissive and non-permissive LCLs.

Most significant was the group of transcription factors which maintain B cell lineage from the pre-B cell phase to the point of terminal differentiation into PCs. During the transition to a plasmablastic phenotype this network of factors, including EBF-1, Pax-5, and E2A, gives way to a network of factors that had been repressed, led by Blimp-1, which suppresses Pax-5 and thus removes restraints on XBP-1 production [287]. The interaction of EBV replication with this network is unclear. Pax5 activates the W promoter (but not the C promoter) used for expression of EBNAs during early establishment of latency, which probably contributes to the B-cell specificity of latent EBV [288]. ZEBRA interacts with Pax5, and seems to repress its transactivation activity of Wp, but also stabilizes Pax5, increasing protein (but not mRNA) levels of Pax5, thus leading to increased induction of B cell proteins like CD19 and CD79A/Ig-α [170, 289].

Although lytically permissive LCLs did not contain more *PRDM1*/Blimp-1, *IRF4*, unspliced *XBP1*, or spliced *XBP1* mRNA, the major members of the opposite network were all overexpressed in non-permissive LCLs. *EBF1*, *PAX5*, and *TCF3*/E2A were among the 18 genes with significantly enhanced ($p < 0.05$) expression in non-permissive LCLs, and *EBF1* had the most significant *p*-value of any gene with a > 3-fold difference between the means of the two populations. In addition, *ETS1*, which encodes

a transcription factor that upregulates EBF-1 and Pax-5 by repressing Blimp-1 activity [290], was one of eight genes overexpressed to $p < 0.01$ in this comparison.

Taking our results as a whole, we suggest that each line of Latency III-transformed B cells has a characteristic frequency of EBV lytic reactivation which is attributable to the intrinsic levels of a few key cellular proteins that influence gene expression. We further suggest that in addition to the known effects of XBP-1 on ZEBRA expression, the XBP-1-containing pathway may be relevant in a different way. When LCLs produce high amounts of factor(s) that *repress* the activity of XBP-1 and Blimp-1, they also repress the level of spontaneous lytic reactivation. From this study the best single candidate for such a controlling transcription factor is EBF-1.

6.3 CONTRIBUTIONS OF THE UNFOLDED PROTEIN RESPONSE

Aside from transcription factors relating to B cell differentiation, the major group of genes expressed differently between the two groups of LCLs was the unfolded protein response genes. The UPR is activated when B cells become antibody-secreting PCs and during some productive virus infections. A terminal UPR leading to apoptosis involves production of CHOP/DDIT3, and activation of the JNK MAP kinase, both of which lead to inhibition of anti-apoptotic Bcl2 proteins and activation of Bim, which moves to the mitochondria and encourages release of cytochrome C. A mild or adaptive UPR is a homeostatic process that involves mainly the production of ER chaperones and growth of the ER [291]. BCR crosslinking and LPS are among the stimuli that induce adaptive ER responses in mature naïve or memory B cells. BCR stimulus with

co-stimulatory signals leads to PC differentiation of memory B cells, but without co-stimulatory signals it induces an adaptive UPR. In immature B cells, BCR alone induces rapid apoptosis, but with additional co-stimulation immature B cells have more of an adaptive UPR [292].

There is no evidence that UPR activation is induced by herpesvirus reactivation [245]. Some herpesviruses inhibit the UPR during productive infection to create an environment that sustains lytic replication, or induce it as part of an immune evasion strategy [293]. EBV LMP1 induces the PERK/ATF-4 pathway of the UPR, which leads initially to further upregulation of LMP1, and then at higher levels to LMP1 degradation by autophagy [261]. This biphasic pattern probably titrates LMP1 expression to ensure an appropriate level of constitutive signaling to sustain the proliferation of EBV-transformed cells.

Expression of two transcription factors downstream of UPR activation, *ATF4* and *XBP1*, correlated positively with LMP1 expression in our study, suggesting that LCLs vary in the degree to which they undergo the LMP1/PERK/ATF-4 feedback cycle. Importantly, this variation was not associated with the level of lytic permissivity. We also investigated ten UPR proteins that localize in the ER, and eight were overexpressed in permissive LCLs, including five of the ten most significantly overexpressed (*HSP90B1*, *DNAJB11*, *SDF2L1*, *CALR*, *ARMET*). These ER proteins did not correlate with LMP1, and did not include any of the stress response proteins recently found to be induced by EBNA3A [294].

We cannot rule out the possibility that this association of UPR gene overexpression with lytic permissivity actually reflects the induction of the UPR by EBV

reactivation. However, the same association was found in LCLs cultured with ACV, and **Figure 19** and **Figure 20** show that UPR proteins are expressed at a consistently high level in permissive LCLs, not concentrated in the minority of cells undergoing lytic reactivation. Also, the induction of ZEBRA protein in all early-passage LCLs cultured with thapsigargin indicates that in addition to causing XBP-1 splicing, UPR induction causes lytic reactivation.

These data suggest that UPR activity is upstream rather than downstream of lytic reactivation, and that permissive and non-permissive LCLs may differ in their propensity for UPR activity. Epigenetic changes, including methylation and deacetylation of Rp and Zp, are expected to cause LCLs to show tighter EBV latency in response to agents like TPA [30]. Early-passage LCLs produce more factors like IL-6 and VEGF that encourage proliferation (and vascularization of tumors) [255, 256]. It has been hypothesized that from the point of view of oncogenesis, it makes sense for cells to show at least the early events of lytic reactivation if it leads to these growth factors being produced, while later on in Latency III-driven proliferation the cells that produce these factors are outcompeted. However, the study which showed this for IL-6 used LCLs from only two donors and also suggested that early-passage LCLs produced more BZLF1 and more late viral proteins, but did not have enhanced lytic DNA replication [256]. In our experience, non-permissive LCLs may become even more tightly latent over time, as measured by ZEBRA+ percent of cells, but permissive LCLs do not become non-permissive, as measured by either lytic protein production or lytic DNA replication.

There is a consensus established that early-passage LCLs are more responsive to inducers of lytic reactivation [30]. We did not seek to repeat these results for TPA,

although we did find that all four late-passage LCLs showed no induction of ZEBRA by TPA and sodium butyrate. After establishing that thapsigargin (Tg) could enhance the rate of lytic reactivation in both Latency I and Latency III cell lines, we investigated whether there was a similar effect of passage number on induction by thapsigargin. Five of six early-passage LCLs had some enhanced ZEBRA production after 12 hours of culture with Tg, while one line (LCL21) showed only minimal enhancement. Meanwhile, only one of four late-passage LCLs had detectable ZEBRA induction at 12 hours.

Given this result, we thought a similar distinction might exist between LCLs that already show signs of being permissive or non-permissive based on expression of cellular factors. In the last part of Aim Two our study did not find distinctions on this count. Non-permissive LCLs had a greater fold increase in the fraction of cells undergoing the switch to lytic EBV, but this still did not bring them to as high a ZEBRA+ fraction of cells as permissive LCL06 or LCL76. Although permissive LCL76 clearly went from Tg-responsive to Tg-unresponsive after months in culture, permissive LCL06 did not.

Our results do suggest that Tg-induced reactivation is more slowly induced in late-passage LCLs. This may result from the same sort of epigenetic alteration described above. As a side effect of faster cell division, certain promoters including Zp and Rp that are less necessary for maintaining the cell line can be temporarily silenced. Latent viral promoters may also be affected – perhaps LMP1, which reinforces UPR induction, is less highly expressed in late-passage LCLs. Previous studies suggest that the change in LMP1 expression over time in culture varies from LCL to LCL [256, 295]. EBNA3C, which restricts UPR induction, is expressed from latent Wp (which is

gradually methylated and silenced over time in culture) and Cp (which takes the place of Wp), so it should be stably expressed over months in favorable culture conditions.

Finally, when measuring the late lytic cycle by looking for enhanced EBV DNA replication, the only LCL which showed any sign of Tg-induced lytic replication was permissive LCL06. Most lytic stimuli (e.g. TPA, BCR ligation) lead to both early and late events in lytic reactivation, even if these stimuli are toxic or inhibit cell proliferation. The standard hypothesis would be that lytic induction by Tg occurs through XBP-1s binding to Zp, but this does not explain the observed lack of lytic DNA replication. Other UPR factors induced by Tg may block the later events of lytic reactivation, like inhibitors of ZEBRA function [30]. This may be one way that EBV undergoes an abortive lytic cycle without causing the cell death that accompanies the full lytic cycle. EBV abortive lytic cycle in B cells is not as well-characterized as abortive lytic EBV or KSHV in epithelial cells [296, 297], but has been suggested as important for immune evasion and growth factor secretion early in the development of EBV+ tumors [256].

Characterization of the Tg-induced UPR in LCLs is underway, which will give clues to how early-passage and late-passage LCLs differ in the speed of UPR induction and EBV reactivation, as well as how permissive and non-permissive LCLs differ in not just basal levels of UPR gene expression, but induction of acute UPR responses upon ER stress. Inducing cells to overexpress individual UPR factors, without actually inducing the UPR through ER stress, might help in identifying the reason why LCLs' basal level of ER-localized UPR factors correlates with their permissivity for lytic reactivation. LCLs are very similar in their viral latency profile, as seen in this study, but surprisingly heterogeneous in their lytic viral activity. As such, they present an intriguing

model for why EBV-host cell interaction differs so much among individuals experiencing virus-driven B cell activation either during the natural course of infection or as part of an EBV-associated malignancy.

6.4 PUBLIC HEALTH SIGNIFICANCE

Epstein-Barr virus is ubiquitous in the human population worldwide, and is associated with an increasing number of cancers and autoimmune diseases. For most of these disorders the link to EBV, either initiating or sustaining, is unclear, so more information is needed on risk factors that cause EBV to have varying effects in different people. Most of the relevant earlier studies that we have built on here are investigations of the way EBV immortalizes human B cells *in vitro*. The cell lines used here resemble the B cells initially infected and activated by the virus in tonsils or other lymphoid organs, and the uncontrolled expression of the full set of viral latent genes is like that found in cases of post-transplant lymphoproliferative disease (PTLD). No transgenic or mutant cells or virus strains were involved, as we were hoping to minimize the artificiality of the system.

We and our collaborators have become increasingly aware that in EBV-seropositive immunocompromised patients, it is unclear what factors influence the development of PTLD. This leads to a need for regular surveillance of EBV levels in circulating leukocytes as a harbinger of neoplasia, although it is not clear what this increased EBV titer represents (possibly an increased number of “bystander cells” infected by an increased number of lytic virions, or possibly EBV-infected cells released

by a subclinical neoplasia). Most of the cell lines used in this study were derived from transplant candidates at different degrees of risk for PTLD, and it will be possible to follow this study up by connecting data on the cell lines with data on the subsequent progression of disease in the patients from whom they were generated.

By using a diverse set of cell lines (each derived from a different donor) we have learned new details about the genetic factors that influence EBV-cellular interaction. The fact that these cell lines all have similar levels of Latency III gene expression indicates that there is one default way in which B cells are immortalized by EBV. However, they vary significantly in permissivity for spontaneous lytic reactivation, indicating that the rate of EBV reactivation is not particularly relevant to B cell immortalization. This diversity is likely found *in vivo* as well, as a property which influences long-term viral persistence as well as transmission by the production of free virus in the tonsils. the effects of different levels of lytic reactivation in different cases of EBV-driven lymphoproliferation are unknown. We have shown that cellular pathways other than plasma cell differentiation – in particular, the unfolded protein response pathway – can influence EBV lytic reactivation. We also have data suggesting the importance of B cell lineage-sustaining transcription factors in repressing lytic reactivation. Although using drugs to repress or induce lytic EBV reactivation has been ineffective in treating cancers, knowledge about genetic factors that predispose people's B cells to more spontaneous reactivation will help us learn about the etiology of chronic EBV-associated diseases.

7.0 FUTURE DIRECTIONS

This study establishes a new way of categorizing lymphoblastoid cell lines, and suggests several sources for the diversity these cell lines show in their tendency to lytically reactivate. Our characterization of lytic permissivity as a phenotype of LCLs is only one of the ways that this diverse group of cell lines could be categorized into phenotypic groups. Earlier studies have categorized LCLs by surface marker phenotype, production of growth factors, production of secreted antibodies, growth rate, chromosomal abnormalities, and ability to be lytically induced by stimuli like TPA or BCR cross-linking. We found a lack of evidence for connections between antibody isotype or surface marker phenotype and lytic permissivity, but have not looked at the production of factors such as IL-6, IL-10, IL-21, and TNF-α. Pilot experiments on the production of virus particles by LCLs suggested that the amount of EBV particles in the supernatant was proportional to the amount of EBV DNA / cell. This could be extended to look into what type of LCLs produce more infectious EBV, since the correlation we have shown between early lytic protein (ZEBRA) and late structural lytic protein (gp110) production in LCLs does not necessarily imply that highly permissive LCLs release more infectious virus.

The next obvious step to take in following up Aim Two is underway. We are analyzing samples of RNA from the same LCLs measured in **Figures 21-23** to learn

more about the unfolded protein response taking place in response to thapsigargin stimulus. These samples should be measured for BZLF1 and BMLF1 expression, for at least LMP1 and EBNA3C (the two latent genes known to influence the UPR), and for several UPR genes for which we have optimized primers. BiP/Grp78 (encoded by *HSPA5*) and ARMET are two proteins which, like Grp94, are XBP1-independent chaperones that are found in the adaptive UPR and contribute to homeostasis [298, 299]. BiP in particular is an abundant protein which blocks oxidative stress. CHOP (*DDIT3*) is also XBP1-independent but causes oxidative stress and is part of the terminal UPR associated with intense ER stress [298, 300]. EDEM1 is an XBP1-dependent protein important for the ERAD pathway for disposal of misfolded proteins that links the UPR to autophagy; the level of EDEM1 expression is closely indicative of XBP1s activity [292]. ERdj4 (*DNAJB9*) and $p58^{IPK}$ (*DNAJC3*) are other XBP1-dependent proteins that serve as co-chaperones in the ER, though $p58^{IPK}$ also oddly seems to counteract the PERK branch of the UPR by being an eIF2α inhibitor [301, 302].

We intend to look at the different rates at which the adaptive UPR genes, the pro-apoptotic CHOP gene, and the genes indicative of XBP1s activity, are induced in permissive and non-permissive LCLs at either early or late passage. Later, it would be interesting to look at how different LCLs differ in UPR response to BCR cross-linking, since that stimulus should be more physiological and less acutely stressful. We suspect that heterogeneity between LCLs in propensity for adaptive UPR induction may confer heterogeneity in their permissivity for lytic EBV induction, through XBP1s or some other mechanism. LCLs are not easily transfected with DNA or RNA, and are probably most

easily infected via lentiviral constructs, which could be used to introduce some individual UPR factor to see its effect on lytic EBV without inducing the UPR at all.

The identification of certain genes as candidates for restricting lytic reactivation (particularly *EBF1*, *ID2*, and *PTPRK*) also suggests future avenues of research. We have created lentiviral vectors for transducing cells with siRNA against these three genes, and are trying to create lentiviral vectors with expression constructs for EBF1 and ID2 (PTPRK being more likely toxic if overexpressed [243]). The effects of overexpressing these genes in a permissive LCL should be easily determined if a reliable expression construct can be produced. Also, we do not know whether the diversity among LCLs in expression of these host genes of interest is the result of genetic diversity, or of epigenetic factors developed during the establishment of cell lines. Another factor we have not yet considered is the variation within LCLs from the same individual – since each LCL in this study was derived from a different donor to try to maximize the heterogeneity of what we thought might be a quite similar group of cell lines.

Finally, it would be feasible to extend this study to different populations of LCLs which have also been characterized for certain properties. We could correlate lytic permissivity of LCLs with their secretion of cytokines or other growth factors, or their ability to differentiate into PCs *in vitro*. A subset of the LCLs were derived from blood from pediatric transplant recipients, and those could be stratified by the donors' risk for PTLD and/or the morbidity of their EBV infections before describing their LCLs' lytic permissivity and expression of EBV latent genes, although it might be more relevant to use spontaneous LCLs generated *ex vivo* from the patient's own cells and EBV.

The GENEVAR extension of the HapMap project has done gene expression profiling on LCLs from 270 individuals who had already been characterized for over 2 million SNPs, finding about 1,500 instances of a gene's genotype influencing its expression or the expression of another gene [222, 303]. These results are only indirectly relevant to most scientists, because they are data from each individual's EBV-immortalized B cells instead of primary tissue or some other type of tissue, but they are directly relevant to those interested in the interaction of EBV with its natural host. A subpopulation of these LCLs, whose expression levels as well as haploptyes for almost all the genes investigated in our study have already been characterized, could be investigated for EBV latent gene expression, lytic gene expression, number of latent episomes per cell, lytic permissivity, and response to stimuli that induce lytic reactivation.

LCLs are a unique system for the study of pathogen-host interactions, thanks to the transforming capability of EBV. This study constitutes one step in the process of finding physiological variations between the host cells from different individuals, which may contribute to the heterogeneity of EBV reactivation in different virus carriers, particularly those at risk for immunosuppression-associated lymphomas. Because of the ease of creating and maintaining LCLs, our laboratory, or any other investigators intrigued by the potential to identify cellular pathways that influence lytic reactivation, have many resources at hand to look for such interactions.

APPENDIX

ABBREVIATIONS USED IN THIS DOCUMENT

AID	Activation-induced cytidine deaminase
AIDS	Acquired immune deficiency syndrome
AILD	Angioimmunoblastic lymphadenopathy with dysproteinemia
AIM	Acute infectious mononucleosis
ALL	Acute lymphoblastic leukemia
AOI	Area of interest
APC	Allophycocyanin
ATF	cyclic-AMP-dependent transcription factor
B2M	β-2 microglobulin
BAFF	B cell-activating factor of the TNF family
BART	BamHI A region rightward transcript
BCL	B cell lymphoma
BL	Burkitt's lymphoma
BSA	Bovine serum albumin
CAEBV	Chronic active EBV infection
CBP	CREB-binding protein
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CLL	Chronic lymphocytic leukemia
Cp	BamHI C-region promoter

CSD	Cytoplasmic signaling domain
CSF	Colony-stimulating factor
Ct	Cycle of threshold
CTAR	C-terminal activator region
CTL	Cytotoxic T lymphocyte
DAPI	4’,6-diamidino-2-phenylindole
dATP	Deoxyadenoside triphosphate
DAVID	Database for Annotation, Visualization, and Integrated Discovery
dCTP	Deoxycytidine triphosphate
DDIT	DNA damage-inducible transcript
DEPC	Diethylpyrocarbonate
dGTP	Deoxyguanosine triphosphate
DLBCL	Diffuse large B-cell leukemia
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dTTP	Deoxythymidine triphosphate
EA	Early lytic antigen
EBER	Epstein-Barr virus-encoded small RNA
EBF	Early B-cell factor
EBNA	Epstein-Barr virus nuclear antigen
EBNA-LP	EBNA leader protein
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetra-acetic acid
EGFR	Epidermal growth factor receptor
eIF	Eukaryotic translation initiation factor
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
ERK	Extracellular signal-regulated protein kinase
FACS	Fluorecence-activated cell sorting
FBS	Fetal bovine serum
FDC	Follicular dendritic cell

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Germinal center(s)
GO	Gene ontology
GEDA	Gene Expression Data Analyzer
gp	Glycoprotein
GPCL	Genomics and Proteomics Core Laboratories
GRP	Glucose-regulated protein
GSEA	Gene Set Enrichment Analysis
GSK	Glycogen synthase kinase
HAT	Histone acetyltransferase
HD	Hodgkin’s disease
HDAC	Histone deacetylase
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRS cells	Hodgkin/Reed-Sternberg cells
HSC	HSP cognate
HSP	Heat-shock protein
HUGO	Human Genome Organization
ID	Inhibitor of DNA binding
IFA	Immunofluorescence assay
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
IM	infectious mononucleosis
IR	Internal repeat
IRF	Interferon response factor
ISG	Interferon-stimulated gene
JAK	Janus kinase
JNK	c-Jun N-terminal protein kinase

KEGG	Kyoto Encyclopedia of Genes and Genomes
KSHV	Kaposi’s sarcoma herpesvirus
LAU	Luminescence arbitrary units
LAU-BGD	LAU minus background
LCL	Lymphoblastoid cell line
LCV	Lymphocryptovirus
LMP	Latent membrane protein
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MDV	Marek’s disease virus
MES	3-(N-morpholine)ethanesulfonic acid
MFI	Median fluorescent intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
miR	microRNA
MM	Multiple myeloma
MOPS	3-(N-morpholine)propanesulfonic acid
mRNA	Messenger RNA
NaB	Sodium butyrate
NK	Natural killer
NPC	Nasopharyngeal carcinoma
OHL	Oral hairy leukoplakia
PAGE	Polyacrylamide gel electrophoresis
PBL	Plasmablastic lymphoma
PBS	Phosphate-buffered saline
PC	Plasma cell
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEL	Primary effusion lymphoma
PERK	Pancreatic ER kinase
PFA	Paraformaldehyde

PI3K	Phosphatidylinositol 3-OH kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PML	Promyelocytic leukemia
PMSF	Phenylmethanesulfonyl fluoride
PTLD	Post-transplant lymphoproliferative disorder
PTPR	Protein tyrosine phosphatase, receptor-type
PVDF	Polyvinylidine fluoride
Qp	BamHI Q-region promoter
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RTA	BRLF1-encoded transactivator
RT-PCR	Reverse transcription + PCR
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SHM	Somatic hypermutation
SNP	Single-nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TCF	Transcription factor
TCL	T cell lymphoma
TF	Transcription factor
Tg	Thapsigargin
Tm	Melting temperature
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TNFSF	TNF superfamily
TNFRSF	TNF receptor superfamily
TPA	12-O-tetradecanoylphorbol-13-acetate
TR	Terminal repeat
TLR	Toll-like receptor

TRAF	TNFR-associated factor
VCA	Viral capsid antigen
VSV	Vesicular stomatitis virus
Wp	BamHI W-region promoter
XBP	X-box binding protein
ZEBRA	BZLF1-encoded broadly reactive activator
Zp	BamHI Z-region promoter

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