ELSEVIER

Antiviral Research 56 (2002) 219–231

Antiviral Research

www.elsevier.com/locate/antiviral

Inhibition of host kinase activity altered by the LMP2A signalosome—a therapeutic target for Epstein–Barr virus latency and associated disease

Lori Cooper, Richard Longnecker *

Department of Microbiology and Immunology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611, USA

Received 5 April 2002; accepted 16 July 2002

Abstract

Epstein–Barr virus (EBV) is a human herpesvirus that establishes a lifelong latent infection in the majority of the human population. The virus resides in a latent state in B lymphocytes and is associated with a variety of cancers in the human host. In normal individuals, latent infection with EBV typically poses no health risk, but upon immunosuppression, either following organ transplantation or HIV infection, malignancies and lymphoproliferative diseases can result. Latent membrane protein 2A (LMP2A) is a virally encoded membrane protein that is expressed in EBV latent infection and in most of the tumors associated with EBV infection. Previous studies have indicated that LMP2A expression alters the activity of the Src family protein tyrosine kinases, the Syk protein tyrosine kinase, the Btk protein tyrosine kinase, and phosphatidylinositol 3-kinase (PI3-kinase). In this study, inhibitors of each of these kinases were tested using an in vitro system dependent on LMP2A expression for B cell colony formation in IL-7 containing methylcellulose media. Of the inhibitors tested, only piceatannol, a Syk tyrosine kinase inhibitor, demonstrated a specific effect on LMP2A expressing cells and not control cells. These studies provide a basis for targeting LMP2A function in EBV latency and may allow for the identification of novel therapeutics for the treatment or eradication of EBV latent infections and associated proliferative disorders.

Keywords: Epstein–Barr virus (EBV); Syk tyrosine kinase; Phosphatidylinositol 3-kinase

1. Introduction

Epstein–Barr virus (EBV) is one of eight herpesviruses that infect and establish latent infections in the human population (for review, Longnecker, 1998; Rickinson and Kieff, 1996). In most infected humans, latent infection poses no risk, but in some individuals, EBV latent infection

* Corresponding author. Tel.: +1-312-503-0467; fax: +1-312-503-1339

E-mail address: r-longnecker@northwestern.edu (R. Longnecker).

0166-3542/02/$ - see front matter
PII: S0166-3542(02)00110-9

is an important co-factor for the development of cancers such as nasopharyngneal carcinoma (NPC), Burkitt's lymphoma (BL), and Hodgkin's Disease (HD) (for review, Longnecker, 1998; Rickinson and Kieff, 1996). Some of the most serious consequences of EBV latent infection arise in individuals with immune dysfunction as a result of immune suppression for organ transplantation or HIV infection (for review, Cohen, 2000; Longnecker, 1998; Rickinson and Kieff, 1996). In these individuals, EBV latent infection can result in severe life-threatening lymphoproliferative disease making the development of therapeutics against EBV latent infection of vital importance.

EBV has a large double-stranded genome that encodes for over 75 viral proteins important for lytic viral replication and the subsequent production of infectious virions (for review, Kieff, 1996; Longnecker, 1998). In contrast to lytic infection, EBV, like all herpesviruses, expresses few if any viral genes in latent infection (for review, Kieff, 1996; Longnecker, 1998). Studies analyzing gene expression in normal individuals harboring EBV latent infections demonstrate three distinct patterns of latent gene expression that depend on the differentiation stage and location of the latently infected B cell. In the peripheral blood, EBV is restricted to memory B cells that are resting and express very few viral genes (Babcock et al., 1999, 1998; Miyashita et al., 1997). Most well documented is LMP2A, but other studies have indicated that the EBERs and BARTs are also expressed in peripheral B cells harboring EBV (Babcock et al., 2000; Chen et al., 1995, 1999; Qu and Rowe, 1992; Tierney et al., 1994). Naive B cells in tonsils express a repertoire of viral genes similar to what is observed in EBV-infected LCLs grown in tissue culture including the EBNAs, LMP1, and LMP2A (Babcock and Thorley-Lawson, 2000; Babcock et al., 2000; Ikeda et al., 2000; Joseph et al., 2000b; Kurth et al., 2000). Germinal center centroblasts and centrocytes as well as tonsillar memory B cells harboring EBV express a more restricted pattern of latent gene expression including EBNA1, LMP1, and LMP2A (Babcock and Thorley-Lawson, 2000; Babcock et al., 2000; Chen et al., 1999; Ikeda et al., 2000; Joseph et al., 2000a,b; Kurth et al., 2000). This pattern of expression is similar to that observed in EBV positive tumors such as NPC and HD (for review, Longnecker, 1998; Rickinson and Kieff, 1996; Thorley-Lawson, 2001). The universal expression of LMP2A in all latently infected B cells in the human host and in NPC and HD positive for EBV suggests that LMP2A may be a good target for the development of antiviral strategies directed against EBV latent infection and EBV-associated proliferative disorders.

Our previous studies have shown that LMP2A forms a signalosome in B lymphocytes by recruiting and associating with proteins important for normal B cell signal transduction (for review, Merchant et al., 2001; Portis et al., 2002). In B lymphocytes, the LMP2A signalosome performs several functions. By associating with and altering the activity of the Src family protein tyrosine kinases and the Syk protein tyrosine kinase, LMP2A is able to block normal B cell receptor signal transduction, while simultaneously providing a B cell receptor-like signal that promotes B cell development and survival (Burkhardt et al., 1992; Caldwell et al., 1998; Fruehling and Longnecker, 1997; Fruehling et al., 1998; Merchant et al., 2000; Miller et al., 1995, 1994). From our studies, we have proposed several roles for LMP2A in EBV latent infection. First, LMP2A may prevent activation of lytic EBV replication by cell surface mediated signal transduction. This function of LMP2A would be important in preventing lytic replication in latently infected B lymphocytes as they circulate in the peripheral blood, bone marrow, or lymphatic tissue where they might encounter antigens, super antigens, or other ligands which could engage B cell receptors and activate EBV lytic replication. Second, by mimicking normal B cell receptor signals LMP2A may be important in allowing EBV to gain access and persist in certain B cell populations. Both of these functions would insure that EBV latent infection is maintained in infected individuals.

We have characterized LMP2A function using both in vitro and in vivo approaches. These studies have indicated that LMP2A is able to bind and alter the activity of numerous proteins involved in normal B cell receptor signal transduction (Fig. 1). The Src family protein tyrosine kinases, the Syk

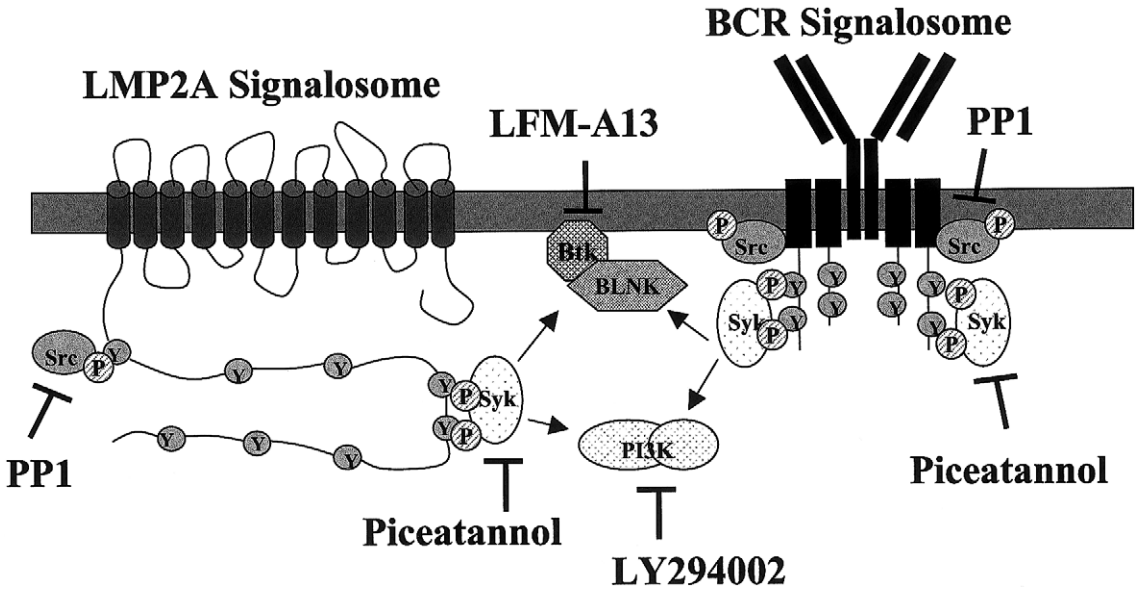

Fig. 1. Host proteins important in LMP2A in vitro and in vivo function. Components of the B cell receptor (BCR) signalosome and the LMP2A signalosome are shown. LMP2A binds the Src family protein tyrosine kinases and the Syk protein tyrosine kinase by SH2-phosphotyrosine interactions. This binding results in the activation of Btk and PI3-kinase. Many of these same signaling molecules are used by the B cell receptor signalosome. In contrast to the B cell receptor that needs antigen binding to stimulate the signal transduction cascade, LMP2A is a constitutive signalosome not requiring exogenous stimulation to signal. Inhibitors that block the relevant kinases are shown. See Table 1 for more information in regard to the specific inhibitors.

protein tyrosine kinase, Bruton's tyrosine kinase (Btk), and phosphatidylinositol 3-kinase (PI3-Kinase) are all activated following B cell receptor signal transduction and we have demonstrated that LMP2A directly targets each of these important cellular kinases (Engels et al., 2001; Fruehling and Longnecker, 1997; Fruehling et al., 1998; Merchant et al., 2000; Merchant and Longnecker, 2001; Swart et al., 2000). In particular, we have shown that the Syk protein tyrosine kinase binds to LMP2A by tandem SH2 domains when LMP2A tyrosine 74 and tyrosine 85 are phosphorylated and this interaction is required for LMP2A function (Fruehling and Longnecker, 1997; Merchant et al., 2000). Like Syk, the Src family tyrosine kinases also bind via an SH2 domain interaction when tyrosine 112 of LMP2A is phosphorylated (Fruehling et al., 1998). Tyrosines 74 and 85 form an immunoreceptor tyrosine-based activation motif (ITAM), whereas tyrosine 112 is contained within a sequence motif that has been shown to bind optimally to the Src family protein tyrosine kinases SH2 domain (Cambier, 1995; Songyang et al., 1993, 1994). We have demonstrated that Btk is critical for the generation of two key aspects of the LMP2A in vivo phenotype (Merchant and Longnecker, 2001). It is required for the survival of B cell receptor negative cells and the up regulation of CD19, an important co-stimulatory cell surface protein in B cell activation. We have also shown that PI3-kinase is activated in LMP2A expressing B cells which results in the phosphorylation of the serine-threonine kinase Akt (Swart et al., 2000). Akt is a multifunctional mediator of PI3-kinase involved in delivering anti-apoptotic signals through various mechanisms. LMP2A mediates this constitutive phosphorylation of Akt via PI3-K and requires the recruitment of the Syk kinase and the Src kinases to mediate this effect. Our identification of host proteins targeted by LMP2A provides the basis for

the testing of specific inhibitors performed in the current study.

To test for the specific effects of known inhibitors on LMP2A function, we utilized transgenic mice we previously constructed in which LMP2A is expressed in B lymphocytes (Caldwell et al., 2000, 1998). Analysis of these mice has allowed for the identification and characterization of an LMP2A growth and survival signal in primary B cells. This important function of LMP2A has not been observed using EBV transformed lymphocytes or in continuous B cell lines in tissue culture making the LMP2A transgenic mice a valuable model system for dissecting LMP2A functions. In particular, we used bone marrow B cells harvested from our LMP2A transgenic line E (TgE) plated in methylcellulose media containing IL-7. Normal precursor B cell growth in IL-7 containing methylcellulose media requires progression beyond the $CD43^+$ stage, a transition dependent upon heavy chain immunoglobulin gene rearrangement and expression (Era et al., 1991; Spanopoulou et al., 1994). Thus, in bone marrow B cells from wild type mice, a high proportion of the resulting cells express a functional B cell receptor as monitored by surface immunoglobulin expression. Those cells that do not express a B cell receptor, express a pre B cell receptor consisting of the expressed rearranged heavy chain gene and the surrogate light chain (Era et al., 1991; Spanopoulou et al., 1994). In TgE LMP2A transgenics, despite the absence of heavy chain immunoglobulin gene rearrangement, precursor bone marrow B cells transit beyond the $CD43^+$ stage and form colonies in the absence of B cell receptor or pre B cell receptor expression (Caldwell et al., 2000, 1998). Normally B cells that do not express a functional B cell receptor or pre B cell receptor rapidly undergo apoptosis (Cheng et al., 1995; Kitamura et al., 1991; Lam et al., 1997; Mombaerts et al., 1992; Shinkai et al., 1992; Turner et al., 1995). These results indicate that LMP2A provides signals that alter B cell development and ensures cell survival in the absence of normal B cell receptor signals.

In this study, multiple inhibitors against specific host proteins involved in LMP2A function were tested for any LMP2A specific effects by comparing apoptosis and colony formation of LMP2A transgenic and wild type bone marrow B cells in the presence or absence of the inhibitors. While LMP2A and wild type control cells were similarly susceptible to apoptosis when treated with each of the inhibitors, the Syk inhibitor piceatannol reduced the proliferation of LMP2A transgenic B cells in IL-7 containing media when compared with littermate control wild type cells.

2. Materials and methods

2.1. Mice

Construction and characterization of EμLMP2A transgenic mice has been previously described (Caldwell et al., 2000, 1998). All mice were housed at the Northwestern University Center for Comparative Medicine in accordance with university animal welfare guidelines.

2.2. Inhibitors

Piceatannol is a naturally derived Syk inhibitor (Geahlen and McLaughlin, 1989) which was purchased from Biomol. The Src family inhibitor PP1 (Hanke et al., 1996) was purchased from Biomol. The Src family inhibitor PP2 (Hanke et al., 1996) and the control inhibitor PP3 (Traxler et al., 1997) were purchased from Calbiochem. LY294002 (Vlahos et al., 1994), a PI3-Kinase inhibitor, was obtained from Cell Signaling Technologies. The Btk inhibitor LFM-A13 (Mahajan et al., 1999) was purchased from Calbiochem. Stock solutions for all of the inhibitors were made with DMSO. A similar amount of DMSO was added to each culture for the untreated controls.

2.3. Isolation of bone marrow cells for growth in IL-7-containing methylcellulose media with inhibitors

Bone marrow cells were flushed from femurs and tibia by using cold sterile 1 × PBS. Samples were then centrifuged at 1500 rpm for 10 min and supernatants were poured off. Red blood cells were lysed in 10 ml of red blood cell lysis buffer (150 mM NH_4Cl, 17 mM Tris base, pH 7.2) for 10

Table 1
Protein tyrosine kinase inhibitors

Inhibitor	Kinase target	Source	IC50[a]	Reference
Piceatannol	Syk protein tyrosine kinase	Biomol	50 μM	(Geahlen and McLaughlin, 1989)
LY294002	Phosphatidylinositol 3-kinase	Cell Signaling Technology	3 μM	(Vlahos et al., 1994)
PP1	Src family protein tyrosine kinases	Biomol	ND	(Hanke et al., 1996)
PP2	Src family protein tyrosine kinases	Calbiochem	ND	(Hanke et al., 1996)
PP3	Control for PP2	Calbiochem	ND	(Traxler et al., 1997)
LFM-A13	Btk protein tyrosine kinase	Calbiochem	ND	(Mahajan et al., 1999)
LFM-A11	Control for LFM-A13	Calbiochem	ND	(Mahajan et al., 1999)

[a] IC50 was experimental calculated by growth in methylcellulose containing IL-7 media. ND, not determined because there was no reduction in colony formation for any of the inhibitor concentrations tested.

min, samples were centrifuged at 1500 rpm for 10 min, supernatants were poured off, and cells resuspended in 10 ml sterile 1 × PBS. Following counting, cells were centrifuged at 1500 rpm for 10 min and resuspended to 1×10^7 per ml in serum-free DMEM (Biowhittaker). Varying amounts of different inhibitors were added to a total of 1×10^6 bone marrow cells and seeded in 3 ml of murine preB Methocult containing IL-7 (Stem Cell Technologies). Inhibitors used and relevant sources are listed in Table 1. All the inhibitors were dissolved in DMSO. The concentrations of each inhibitor used in the various experiments are shown in Table 1. Cells were vortexed briefly to evenly distribute cells and were plated into 60-mm-diameter dishes. Cultures were grown for 7 days at 37 °C in 5% CO_2. Plates were scanned into Adobe Photoshop before being harvested for use in flow cytometry. Colonies were counted from the scanned images. The number of colonies in the untreated samples were approximately 100 for both wild type and TgE bone marrow cells per 1×10^6 plated bone marrow cells. Comparisons of colony formation in treated and untreated controls were analyzed by the Student's *t*-test.

2.4. Flow cytometry

Cell samples were prepared by washing methylcellulose cultures with 10 ml 1 × PBS, centrifuging at 1500 rpm for 10 min, and resuspending in FACS buffer (Caldwell et al., 2000, 1998). Flow cytometry was done as previously described (Caldwell et al., 2000, 1998). All antibodies were purchased from BD Pharmingen. Samples were run on a Becton Dickinson FACScan and data were analyzed using CELLQUEST software.

2.5. Apoptosis of bone marrow cells after inhibitor treatment

Bone marrow cells were collected as described above. Following cell counting, cells were centrifuged at 1500 rpm for 10 min and resuspended at 1×10^7 per ml in Iscove's media (Gibco) containing 30% FBS. Varying amounts of different inhibitors were added to 1×10^6 cells bone marrow cells, in duplicate. To half the bone marrow samples IL-7 (R&D Systems) was added. Cells were grown for 20 and 68 h at 37 °C in 5% CO_2. Cells were centrifuged at 1500 rpm for 10 min and apoptosis was monitored using an Apo-Direct kit from BD Pharmingen.

3. Results

3.1. Colony formation of LMP2A transgenic TgE bone marrow cells is decreased when compared with wild type bone marrow cells in the presence of Piceatannol in IL-7 containing methylcellulose media

Inhibitors for Btk, Syk, PI-3Kinase, and Src family tyrosine kinases were purchased from the commercial sources indicated in Table 1. IC50s for colony formation in IL-7 containing methylcellulose for each kinase inhibitor were determined by

plating wild type bone marrow cells isolated from 6-week-old C57BL/6 mice in the absence of inhibitors and varying doses of each inhibitor. Colonies were scored 7 days after plating. There was no reduction in colony formation for both Src family kinase inhibitors and for the Btk inhibitor even at the highest concentrations tested (data not shown). IC50 values obtained for the Syk inhibitor piceatannol and for the PI3-Kinase inhibitor LY294002 (Table 1) were very similar to the previously published IC50s (Geahlen and McLaughlin, 1989; Oliver et al., 1994; Peters et al., 1996; Vlahos et al., 1994) for each of these inhibitors assuming that both Syk and PI3-Kinase activity are essential for B cell outgrowth in IL-7 containing methylcellulose. To determine if either piceatannol or LY294002 would specifically interfere with the previously reported LMP2A signal that allows B cell receptor negative colonies to form in IL-7 containing methylcellulose media, bone marrow cells from TgE and wild type littermate controls were plated in the presence and absence of each of these inhibitors shown using concentrations bracketing the IC50 as determined for wild type B cells. Following 7 days of incubation at 37 °C, the resultant colonies were counted visually. Shown in Fig. 2A is an example photomicrograph of the resulting colony formation for a DMSO control treated culture. The number of colonies on inhibitor treated plates were normalized using the DMSO control plates for each concentration. Both inhibitors, piceatannol and LY294002, showed a reduction in colony numbers in a dose dependent manner. The results from multiple experiments are shown in Fig. 2B for both inhibitors. As seen in Fig. 2B, piceatannol had a greater effect on colony formation in the TgE bone marrow cells when compared with wild type littermate control bone marrow cells reducing colony formation at all three concentrations tested by more than 50%. In contrast, treatment with the PI3-Kinase inhibitor LY294002 reduced colony formation for each concentration tested similarly for the TgE bone marrow cells and the wild type littermate control cells. These studies suggest that in TgE bone marrow cells, LMP2A is able to promote B cell survival and growth by directly targeting the Syk protein tyrosine kinase when compared with wild type bone marrow B cells expressing either a B cell receptor or a pre B cell receptor. The Src family tyrosine kinase inhibitors PP1 and PP2, and the Btk inhibitor LFM-A13 were also tested despite having no effect on colony formation of wild type bone marrow B cells. As may have been predicted from our earlier results demonstrating an absence of an effect on wild type bone marrow B cell colony formation, these inhibitors also had no effect on colony formation of TgE bone marrow B cells in IL-7 containing methylcellulose at even the highest concentrations tested when compared with wild type littermate controls (data not shown).

3.2. B cell receptor surface expression in wild type and TgE bone marrow cells cultured in IL-7 containing media with and without kinase inhibitors

Our previous studies have shown that there is a near complete absence of B cell receptor expression in TgE bone marrow cells when grown in IL-7 containing methylcellulose (Caldwell et al., 2000, 1998). This is in contrast to wild type bone marrow cells where approximately 40% of the resulting cells express B cell receptors as monitored by the expression of membrane bound immunoglobulin (IgM; Caldwell et al., 2000, 1998). A similar number of colonies result in the wild type and TgE bone marrow cells with the only difference being the loss of B cell receptor or IgM expression (Caldwell et al., 2000, 1998). We have previously shown that LMP2A selectively blocks immunoglobulin heavy chain rearrangement altering normal B cell development and provides a survival signal allowing IgM^- B cells to accumulate in the periphery (Caldwell et al., 2000, 1998). To test if any of the inhibitors block the LMP2A induced growth and survival signal, ability of LMP2A to allow B cell receptor negative cells to form colonies in IL-7 containing methylcellulose media and to confirm that the resulting colonies in the TgE bone marrow B cells were IgM^-, wild type and TgE bone marrow cells following growth in IL-7-containing methylcellulose media with inhibitors were examined by flow cytometry (Fig. 3). Cells were stained with antibodies to the pan-B cell marker CD19 and immunoglobulin M (IgM).

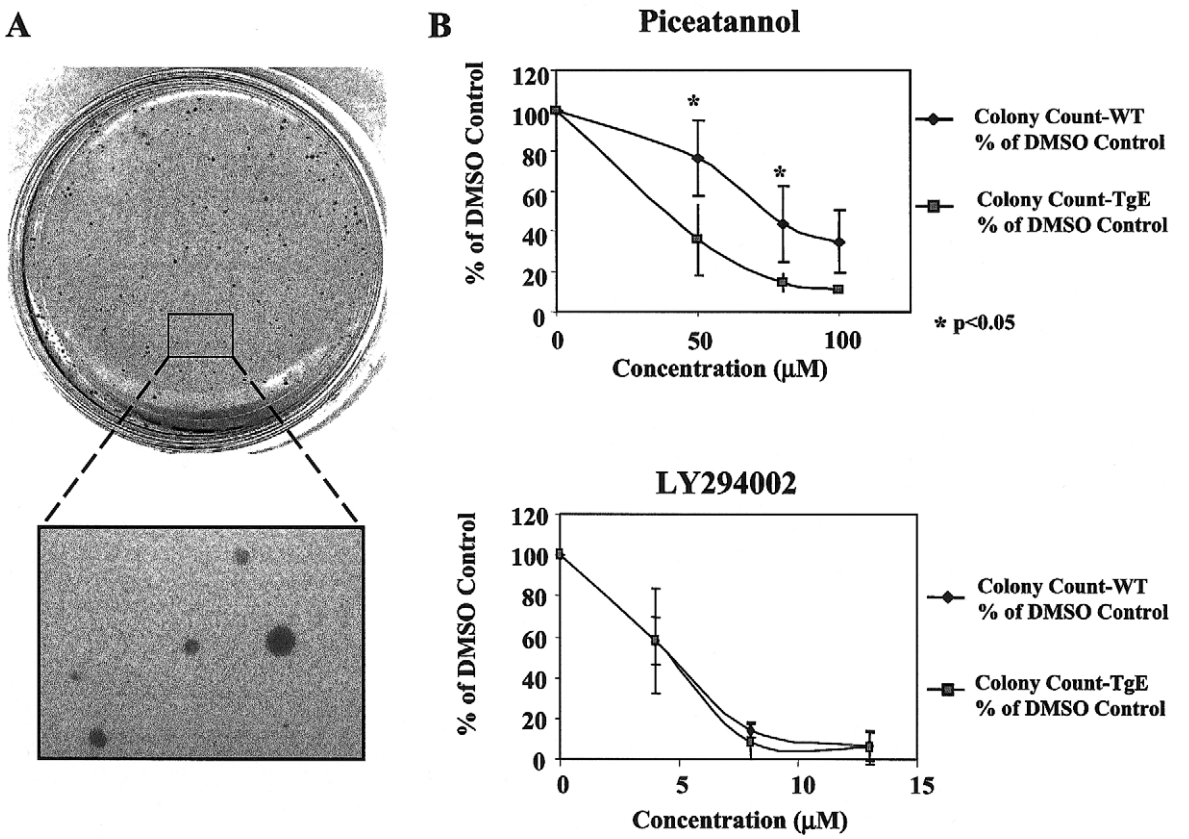

Fig. 2. Growth of wild type and TgE bone marrow cells in IL-7-containing methylcellulose media. (A) Bone marrow B cells were plated in the presence and the absence of piceatannol and LY294002 in IL-7 containing methylcellulose media. After 7 days of incubation macroscopic colonies become visible. The photomicrograph, as an example, is from an untreated control. (B) Macroscopic colonies were counted in control DMSO treated cultures and cultures treated with various dosages of piceatannol and LY294002. There was an approximate 50% reduction in colony number for the LMP2A TgE bone marrow cells when compared with control cells for each dose of piceatannol whereas both wild type and LMP2A TgE bone marrow cells were equally sensitive to LY294002 at all doses tested. Experiments were done multiple times for each data point as follows: Picetannol-0μM, $n = 6$; 50 μM, $n = 4$; 80 μM, $n = 6$; 100 μM, $n = 3$. LY294002-0μM, $n = 6$; 4 μM, $n = 4$; 8 μM, $n = 3$; 13 μM, $n = 5$. Data was analyzed by the Student's t-test.

DMSO treated wild type littermate control mice showed the normal pattern of B cell staining with both $CD19^+$, IgM^- B cells (Fig. 3A, 60%) and $CD19^+$, IgM^+ B cells (Fig. 3A, 40%). In the wild type samples treated with piceatannol and LY294002, there was a small increase in the percentage of $CD19^+$, IgM^- cells when compared with $CD19^+$, IgM^+ cells (Fig. 3A, compare 60 with 67 and 63% for the inhibitor treated cultures). In the DMSO control treated TgE bone marrow B cells there was an almost complete absence of $CD19^+$, IgM^+ cells (Fig. 3A, 2%) with most of the cells being $CD19^+$, IgM^- (Fig. 3, 97%) as has been previously reported (Caldwell et al., 2000, 1998). Both the piceatannol and LY294002 treated TgE bone marrow cells had very similar percentages of $CD19^+$, IgM^+ and $CD19^+$, IgM^- B cells, although the percentage of $CD19^+$, IgM^+ B cells were slightly higher in the LY294002 treated samples (Fig. 3A, 7%) when compared with either the piceatannol or DMSO treated cultures (Fig. 3A, 2% for both). These experiments were performed multiple times and are summarized in Fig. 3B. The small changes in $CD19^+$, IgM^+ cells in both TgE bone marrow B cells and wild type cells were not reproducibly observed and likely repre-

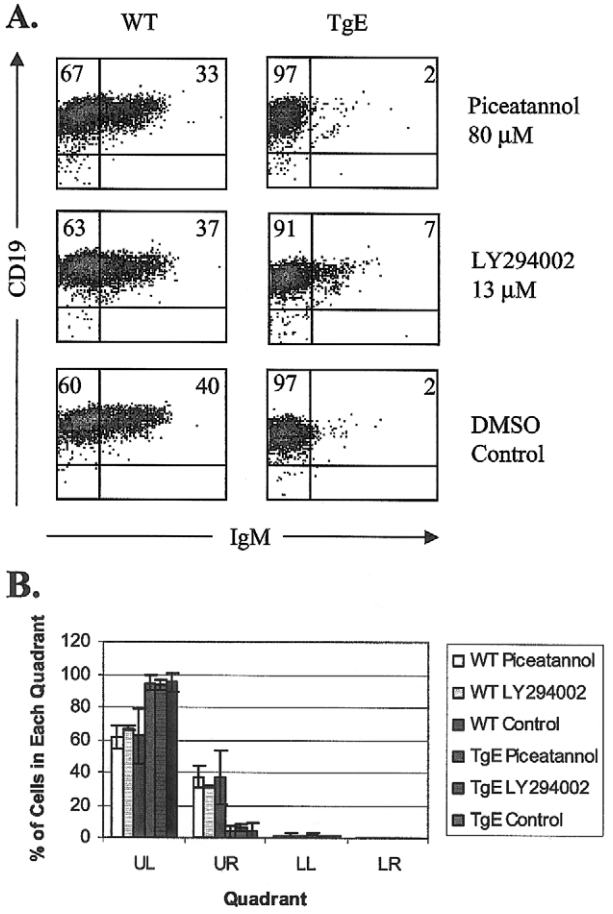

Fig. 3. FACS analysis of wild type littermate controls and TgE bone marrow cells. (A) 10^6 IL-7 cultured bone marrow cells from wild type and TgE mice were analyzed for CD19 and IgM expression by flow cytometry following outgrowth in IL-7-containing methylcellulose media 7 days after plating. Cells were plated in the presence of 80 um piceatannol, 13 uM LY294002, or untreated. The percent of total lymphocytes that are CD19+IgM$^-$ and CD19+IgM$^+$ are indicated. (B) Summary of FACS analysis from multiple experiments (Piceatannol, $n=4$; LY294002, $n=2$; DMSO, $n=4$).

sent experimental variation. These results indicate despite reduced colony formation in the piceatannol treated TgE bone marrow B cells, they are phenotypically similar to the untreated TgE bone marrow B cells in regard to B cell receptor expression.

3.3. Apoptosis is similar in TgE and wild type littermate control B cells when treated with piceatannol

To investigate if apoptosis of the cells treated with the inhibitors may explain the dose dependent decrease in the number of cell colonies in piceatannol-treated bone marrow cultures, the induction of apoptosis was analyzed in bone marrow B cells treated with piceatannol. To ensure that IL-7 was not providing a survival signal for these cells, bone marrow cells were treated in both the presence and absence of IL-7. Cells were treated with 80 mM piceatannol. This concentration chosen for piceatannol gave the greatest difference in colony number in the previous experiments. Bone marrow cells were treated for 20 and 68 h in the presence and absence of inhibitors with and without IL-7. The percentage of B cells undergoing apoptosis was determined by using a Apo-Direct kit (Pharmingen) and gating on B220 positive cells (Fig. 4). Like CD19, B220 is a pan-B cell marker. Piceatannol treatment in the presence of IL-7 for 20 h resulted in 33% apoptotic cells in wild type bone marrow cells and 29% apoptotic cells in the TgE bone marrow cells (Fig. 4A, piceatannol). LY294002 treatment resulted in fewer apoptotic cells when compared with the piceatannol treated cells (Fig. 4A, LY294002). Less apoptosis was detected in the DMSO treated control cells, 11% for wild type and 10% for TgE bone marrow cells (Fig. 4A, DMSO). Almost identical results were obtained in the absence of IL-7 (Fig. 4B). As might have been expected, when the bone marrow cells were treated for 68 rather than 20 h, a greater amount of apoptosis was observed when the cells were treated with piceatannol or LY294002 when compared with the 20 h treatment (Fig. 4C and 4D). But, similar to the results with the 20 h treatment, there appeared to be no difference when the TgE bone marrow cells were compared with wild type littermate control bone marrow cells treated with piceatannol or LY294002 (Fig. 4C and 4D), although there was an increase in the total numbers of apoptotic cells when compared with the cells treated for 20 h. Interestingly, there was very little increase in apoptosis in the DMSO treated cells when the incubation period was

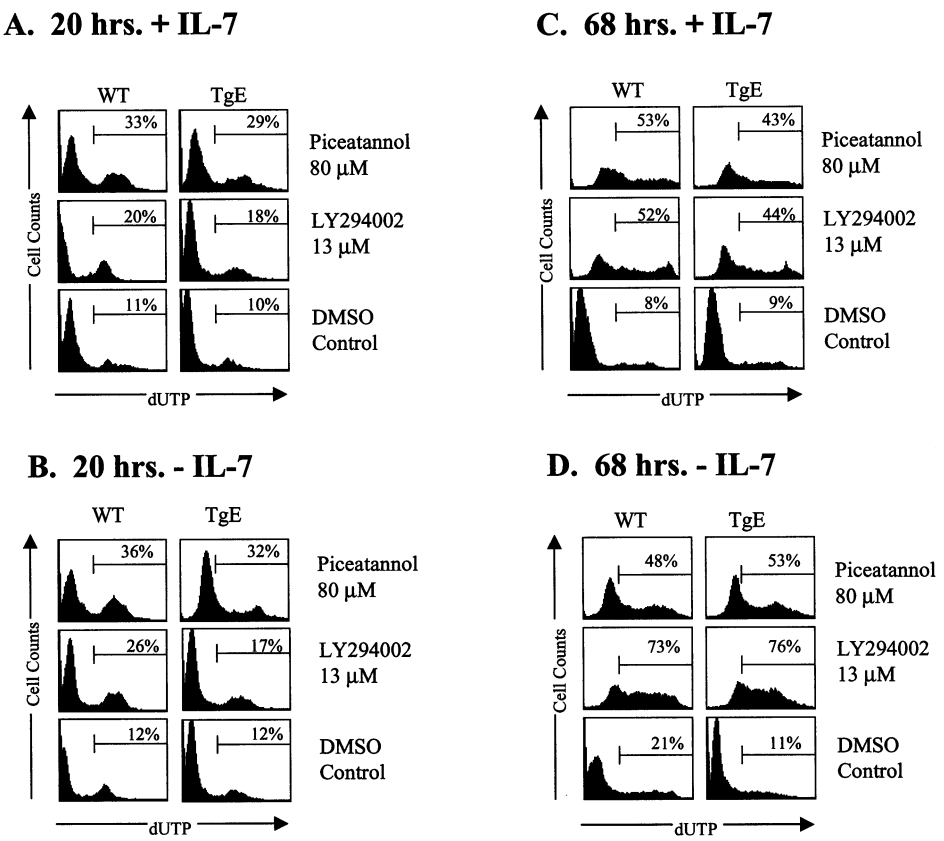

Fig. 4. Apoptosis of bone marrow B cells following treatment with piceatonnal and LY294002. (A–D) Bone marrow cells from WT and TgE mice were cultured in Iscove's media with and without IL-7 and either piceatannol (80 μM), LY294002 (20 μM) or DMSO. Percentage of apoptotic cells is indicated. Only cells positive for B220, a pan B cell marker, are shown. (A) Cells were treated for 20 h at 37 °C in the presence of IL-7. (B) Cells were treated for 20 h at 37 °C in the absence of IL-7. (C) Cells were treated for 68 h at 37 °C in the presence of IL-7. (D) Cells were treated for 68 h at 37 °C in the absence of IL-7.

extended 68 h. The experiments described in Fig. 4 were performed on two separate occasions and the results were similar. These suggest that the absence of colony formation in IL-7 containing methylcellulose in the TgE bone marrow B cells may result from reduced proliferative capacity of the LMP2A expressing TgE bone marrow B cells and not by the induction of apoptosis when the cells are treated with piceatannol.

4. Discussion

There is considerable impetus for the development of novel therapeutics for the treatment of herpesvirus related diseases in humans. Most members of the herpesvirus family can cause debilitating and life threatening diseases in both the immune competent and the immune compromised human host (for review, Roizman et al., 1994). These diseases can result from primary infection, activation of latent virus to lytic replication, and latent infection. Current therapies for treatment of human herpesvirus infections are directed at lytic virus replication and not at latent infections in the human host. In the current manuscript, we have begun experiments to evaluate if kinase inhibitors that inhibit host proteins that are targets of LMP2A may interfere with the normal function of LMP2A in latent infection. These inhibitors, as long as they do not interfere dramatically with normal host cell function, may

offer a therapeutic approach to treat EBV latent infections. The rationale for this approach is based on our underlying hypothesis that LMP2A is essential for latency of EBV in the human host. LMP2A is the only EBV message that has routinely been reported by multiple laboratories to be expressed in peripheral B lymphocytes from normal individuals harboring EBV latent infections (Babcock et al., 1999; Qu and Rowe, 1992; Tierney et al., 1994). In addition, LMP2A is expressed in all EBV associated cancers in the human host with the exception of BL (for review, Rickinson and Kieff, 1996). Inhibitors that target LMP2A function may be beneficial both before the onset of EBV-associated proliferative disorders and even after EBV-associated tumors are identified. For example, treatment of EBV latent infections in individuals at risk for development of EBV-related lymphomas prior to their development may have merit. If the number of B cells harboring the virus can be reduced, the incidence of EBV-related lymphomas may also be reduced. The treatment of EBV associated cancers with inhibitors directed against LMP2A may also have merit. LMP2A may have important roles for tumor progression and tumor survival. Data for such an LMP2A function may be present in both HD and NPC. In HD, Reed–Sternberg cells are characterized as germinal center derived B lineage cells that generally contain mutations resulting in the absence of B cell receptor (BCR) expression (Braeuninger et al., 1997; Kanzler et al., 1996; Marafioti et al., 2000). By mimicking signals derived from a BCR, a function we have previously shown for LMP2A, LMP2A may provide a signal that would maintain these cells in the absence of a BCR. Normally, B cells that lack a BCR are rapidly eliminated by apoptosis (Cheng et al., 1995; Kitamura et al., 1991; Lam et al., 1997; Mombaerts et al., 1992; Shinkai et al., 1992; Turner et al., 1995). LMP2A has been shown to alter normal epithelial cell development (Scholle et al., 2000) indicating a potential role for LMP2A in either NPC tumor development or tumor cell survival.

In the current paper, we tested commercially available kinase inhibitors targeted at host cell proteins we had previously shown to be targets of LMP2A (Fig. 1). We tested the effects of these inhibitors on LMP2A-dependent B cell outgrowth and BCR-dependent outgrowth in IL-7 containing methylcellulose cultures using bone marrow B cells from our LMP2A transgenic mice and normal wild type littermate controls. Three of the inhibitors, PP1, PP2, and LFM-A13, had no effect on outgrowth of B cells in the LMP2A transgenics or the wild type control B cells. PP1 and PP2 are Src family kinase inhibitors and LFM-A13 inhibits Btk. Two other inhibitors, piceatannol and LY294002, decreased the outgrowth of B cells in IL-7 containing methylcellulose and caused an increase in apoptosis. Piceatannol is an inhibitor of Syk. The wild type cells were less sensitive to piceatannol in the B cell outgrowth assays whereas both inhibitors behaved similar in regard to inducing apoptosis in LMP2A transgenic or wild type littermate control bone marrow cells. LY294002 is a PI3-kinase inhibitor. Wild type and LMP2A transgenic bone marrow B cells showed no differences in proliferation or apoptosis when treated with LY294002 indicating that LY294002 had no specific effects on LMP2A transgenic bone marrow B cells.

The results with the Syk inhibitor piceatannol are not surprising based on our previous studies indicating a central role of the Syk kinase in LMP2A function. Syk autophosphorylation and kinase activity is increased in LMP2A expressing cell lines and the interaction of LMP2A with Syk is essential for the ability of LMP2A to block B cell signal transduction and promote B cell development and survival both in transgenic mice and IL-7 containing methylcellulose cultures (Fruehling and Longnecker, 1997; Merchant et al., 2000; Miller et al., 1995). We were surprised that none of the additional inhibitors were able to block LMP2A function as we have also shown that these kinases are also important for LMP2A function. This may be a result of the poor specificity of the inhibitors for the kinase in question, the relatively high concentrations required for kinase inhibition, or poor availability of the inhibitor in our IL-7 containing methylcellulose containing cultures. Finally, LMP2A may not increase the activity of these kinases when compared with the activity of these kinases required for normal B cell outgrowth

and colony formation in IL7 containing methylcellulose.

Much has been learned recently in regard to the function of proteins expressed during EBV latent infections. To date none of these viral proteins contain enzymatic activity, but rather bind and alter the function of normal cellular proteins some with enzymatic activity. Often these interactions activate an entire signal transduction cascade much like what is observed for LMP2A (Fig. 1). In lieu of designing specific inhibitors that target the interactions of cellular proteins with LMP2A, we have used our knowledge of LMP2A function and have begun testing previously described drugs that inhibit specific host proteins that are targeted by LMP2A. We have provided data suggesting that targeting at least one cellular kinase may block the activation of the Syk kinase by LMP2A and block the resulting proliferation of LMP2A expressing B cells. We intend on continuing these studies as new kinase inhibitors become available and plan on extending these results of the current studies by in vivo testing of the Syk inhibitor piceatannol in our LMP2A transgenic mice.

Acknowledgements

R. Longnecker is supported by Public Health Service grants CA62234, CA73507, and CA93444 from the National Cancer Institute and DE13127 from the National Institute of Dental and Craniofacial Research. R. Longnecker is a Stohlman Scholar of the Leukemia and Lymphoma Society of America. We would like to thank members of the Longnecker and Spear Laboratories for their help in these studies. In addition, we thank Toni Portis, Patrick Dennis, and Becca Katzman for kindly reading the manuscript prior to submission.

References

Babcock, G.J., Thorley-Lawson, D.A., 2000. Tonsillar memory B cells, latently infected with Epstein–Barr virus, express the restricted pattern of latent genes previously found only in Epstein–Barr virus-associated tumors. Proc. Natl. Acad. Sci. USA 97 (22), 12250–12255.

Babcock, G.J., Decker, L.L., Volk, M., Thorley-Lawson, D.A., 1998. EBV persistence in memory B cells in vivo. Immunity 9, 395–404.

Babcock, G.J., Decker, L.L., Freeman, R.B., Thorley-Lawson, D.A., 1999. Epstein–Barr virus-infected resting memory B cells, not proliferating lymphoblasts, accumulate in the peripheral blood of immunosuppressed patients. J. Exp. Med. 190 (4), 567–576.

Babcock, J.G., Hochberg, D., Thorley-Lawson, A.D., 2000. The expression pattern of Epstein–Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell. Immunity 13 (4), 497–506.

Braeuninger, A., Kuppers, R., Strickler, J.G., Wacker, H.H., Rajewsky, K., Hansmann, M.L., 1997. Hodgkin and Reed–Sternberg cells in lymphocyte predominant Hodgkin disease represent clonal populations of germinal center-derived tumor B cells. Proc. Natl. Acad. Sci. USA 94 (17), 9337–9342.

Burkhardt, A.L., Bolen, J.B., Kieff, E., Longnecker, R., 1992. An Epstein–Barr virus transformation-associated membrane protein interacts with src family tyrosine kinases. J. Virol. 66 (8), 5161–5167.

Caldwell, R.G., Wilson, J.B., Anderson, S.J., Longnecker, R., 1998. Epstein–Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. Immunity 9, 405–411.

Caldwell, R.G., Brown, R.C., Longnecker, R., 2000. Epstein–Barr virus LMP2A-induced B-cell survival in two unique classes of EuLMP2A transgenic mice. J. Virol. 74, 1101–1113.

Cambier, J.C., 1995. New nomenclature for the Reth motif (or ARH1/TAM/ARAM/YXXL). Immunol. Today 16, 110.

Chen, F., Zou, J.Z., di Renzo, L., 1995. A subpopulation of normal B cells latently infected with Epstein–Barr virus resembles Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP1. J. Virol. 69, 3752–3758.

Chen, H., Smith, P., Ambinder, R.F., Hayward, S.D., 1999. Expression of Epstein–Barr virus BamHI-A rightward transcripts in latently infected B cells from peripheral blood. Blood 93 (9), 3026–3032.

Cheng, A.M., Rowley, B., Pao, W., Hayday, A., Bolen, J.B., Pawson, T., 1995. Syk tyrosine kinase required for mouse viability and B-cell development. Nature 378, 303–306.

Cohen, J.I., 2000. Epstein–Barr virus infection. New Engl. J. Med. 343 (7), 481–492.

Engels, N., Merchant, M., Pappu, R., Chan, A.C., Longnecker, R., Wienands, J., 2001. Epstein–Barr virus latent membrane protein 2A (LMP2A) employs the slp- 65 signaling module. J. Exp. Med. 194 (3), 255–264.

Era, T., Ogawa, M., Nishikawa, S., Okamoto, M., Honjo, T., Akagi, K., Miyazaki, J., Yamamura, K., 1991. Differentiation of growth signal requirement of B lymphocyte precursor is directed by expression of immunoglobulin. EMBO J. 10 (2), 337–342.

Fruehling, S., Longnecker, R., 1997. The immunoreceptor tyrosine-based activation motif of Epstein–Barr virus

LMP2A is essential for blocking BCR-mediated signal transduction. Virology 235, 241–251.

Fruehling, S., Swart, R., Dolwick, K.M., Kremmer, E., Longnecker, R., 1998. Tyrosine 112 of latent membrane protein 2A is essential for protein tyrosine kinase loading and regulation of Epstein–Barr virus latency. J. Virol. 72, 7796–7806.

Geahlen, R.L., McLaughlin, J.L., 1989. Piceatannol (3,4,3′,5′-tetrahydroxy-trans-stilbene) is a naturally occurring protein-tyrosine kinase inhibitor. Biochem. Biophys. Res. Commun. 165 (1), 241–245.

Hanke, J.H., Gardner, J.P., Dow, R.L., Changelian, P.S., Brissette, W.H., Weringer, E.J., Pollok, B.A., Connelly, P.A., 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J. Biol. Chem. 271 (2), 695–701.

Ikeda, T., Kobayashi, R., Horiuchi, M., Nagata, Y., Hasegawa, M., Mizuno, F., Hirai, K., 2000. Detection of lymphocytes productively infected with Epstein–Barr virus in non-neoplastic tonsils. J. Gen. Virol. 81 Pt 5, 1211–1216.

Joseph, A.M., Babcock, G.J., Thorley-Lawson, D.A., 2000a. Cells expressing the Epstein–Barr virus growth program are present in and restricted to the naive B-cell subset of healthy tonsils. J. Virol. 74 (21), 9964–9971.

Joseph, A.M., Babcock, G.J., Thorley-Lawson, D.A., 2000b. EBV persistence involves strict selection of latently infected B cells. J. Immunol. 165 (6), 2975–2981.

Kanzler, H., Kuppers, R., Hansmann, M.L., Rajewsky, K., 1996. Hodgkin and Reed–Sternberg cells in Hodgkin's disease represent the outgrowth of a dominant tumor clone derived from (crippled) germinal center B cells. J. Exp. Med. 184 (4), 1495–1505.

Kieff, E., 1996. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Epstein–Barr Virus and Its Replication. Fundamental Virology. Lippincott-Raven, pp. 1109–1162.

Kitamura, D., Roes, J., Kuhn, R., Rajewsky, K., 1991. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. Nature 350, 423–426.

Kurth, J., Spieker, T., Wustrow, J., Strickler, G.J., Hansmann, L.M., Rajewsky, K., Kuppers, R., 2000. EBV-infected B cells in infectious mononucleosis: viral strategies for spreading in the B cell compartment and establishing latency. Immunity 13 (4), 485–495.

Lam, K., Kuhn, R., Rajewsky, K., 1997. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell 90, 1073–1083.

Longnecker, R., 1998. Molecular biology of Epstein–Barr virus. In: McCaance, D. (Ed.), Human Tumor Viruses. American Society for Microbiology, pp. 133–172.

Mahajan, S., Ghosh, S., Sudbeck, E.A., Zheng, Y., Downs, S., Hupke, M., Uckun, F.M., 1999. Rational design and synthesis of a novel anti-leukemic agent targeting Bruton's tyrosine kinase (BTK), LFM-A13 [alpha-cyano-beta-hydroxy-beta- methyl-*N*-(2,5-dibromophenyl)propenamide]. J. Biol. Chem. 274 (14), 9587–9599.

Marafioti, T., Hummel, M., Foss, H.D., Laumen, H., Korbjuhn, P., Anagnostopoulos, I., Lammert, H., Demel, G., Theil, J., Wirth, T., Stein, H., 2000. Hodgkin and Reed–Sternberg cells represent an expansion of a single clone originating from a germinal center B-cell with functional immunoglobulin gene rearrangements but defective immunoglobulin transcription. Blood 95 (4), 1443–1450.

Merchant, M., Longnecker, R., 2001. LMP2A survival and developmental signals are transmitted through Btk-dependent and Btk-independent pathways. Virology 291, 46–54.

Merchant, M., Caldwell, R.G., Longnecker, R., 2000. The LMP2A ITAM is essential for providing B cells with development and survival signals in vivo. J. Virol. 74 (19), 9115–9124.

Merchant, M., Swart, R., Katzman, R.B., Ikeda, M., Dykstra, M.L., Pierce, S.K., Longnecker, R., 2001. The function of LMP2A for the development and activation of B cells. Int. Rev. Immunol. 20, 131–161.

Miller, C.L., Lee, J.H., Kieff, E., Longnecker, R., 1994. An integral membrane protein (LMP2) blocks reactivation of Epstein–Barr virus from latency following surface immunoglobulin crosslinking. Proc. Natl. Acad. Sci. USA 91 (2), 772–776.

Miller, C.L., Burkhardt, A.L., Lee, J.H., Stealey, B., Longnecker, R., Bolen, J.B., Kieff, E., 1995. Integral membrane protein 2 of Epstein–Barr virus regulates reactivation from latency through dominant negative effects on protein-tyrosine kinases. Immunity 2, 155–166.

Miyashita, E.M., Yang, B., Babcock, G.J., Thorley-Lawson, D.A., 1997. Identification of the site of Epstein–Barr virus persistence in vivo as a resting B cell. J. Virol. 71 (7), 4882–4891.

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., Papaioannou, V.E., 1992. RAG-1-deficient mice have no mature B and T lymphocytes. Cell 68, 869–877.

Oliver, J.M., Burg, D.L., Wilson, B.S., McLaughlin, J.L., Geahlen, R.L., 1994. Inhibition of mast cell Fc epsilon R1-mediated signaling and effector function by the Syk-selective inhibitor, piceatannol. J. Biol. Chem. 269 (47), 29697–29703.

Peters, J.D., Furlong, M.T., Asai, D.J., Harrison, M.L., Geahlen, R.L., 1996. Syk, activated by cross-linking the B-cell antigen receptor, localizes to the cytosol where it interacts with and phosphorylates alpha-tubulin on tyrosine. J. Biol. Chem. 271 (9), 4755–4762.

Portis, T., Cooper, L., Dennis, P., Longnecker, R., 2002. The LMP2A signalosome-a therapeutic target for Epstein–Barr virus latency and associated disease. Front Biosci. 7, D414–D426.

Qu, L., Rowe, D., 1992. Epstein–Barr virus latent gene expression in uncultured peripheral blood lymphocytes. J. Virol. 66 (6), 3715–3724.

Rickinson, A.B., Kieff, E., 1996. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Epstein–Barr virus, Fields Virology. Lippincott-Raven Publishers, Philadelphia, PA, pp. 2397–2446.

Roizman, B., Whitley, R.J., Lopez, C., 1994. The Human Herpesviruses. Raven Press, New York.

Scholle, F., Bendt, K.M., Raab-Traub, N., 2000. Epstein–Barr virus LMP2A transforms epithelial cells, inhibits cell differentiation, and activates Akt. J. Virol. 74, 10681–10689.

Shinkai, Y., Rathbun, G., Lam, K., Oltz, E.M., Stewart, V., Mendelsohn, M., Charon, J., Datta, M., Young, F., Stall, A.M., Alt, F.W., 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell 68, 855–867.

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., et al., 1993. SH2 domains recognize specific phosphopeptide sequences. Cell 72 (5), 767–778.

Songyang, Z., Shoelson, S.E., McGlade, J., Olivier, P., Pawson, T., Bustelo, X.R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., et al., 1994. Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. Mol. Cell. Biol. 14 (4), 2777–2785.

Spanopoulou, E., Roman, C.A.J., Corcoran, L.M., Schlissel, M.S., Silver, D.P., Nemazee, D., Nussenzweig, M.C., Shinton, S.A., Hardy, R.R., Baltimore, D., 1994. Functional immunogloubulin transgenes guide ordered B-cell differentiation in Rag-1-deficient mice. Genes Dev. 8, 1030–1042.

Swart, R., Ruf, I.K., Sample, J., Longnecker, R., 2000. Latent membrane protein 2A-mediated effects on the Phosphatidylinositol 3-kinase/Akt Pathway. J. Virol. 74, 10838–10845.

Thorley-Lawson, A.D., 2001. Epstein–Barr virus: exploiting the immune system. Nat. Rev. Immunol. 1, 75–82.

Tierney, R.J., Steven, N., Young, L.S., Rickinson, A.B., 1994. Epstein–Barr virus latency in blood mononuclear cells: analysis of viral gene transcription during primary infection and in the carrier state. J. Virol. 68 (11), 7374–7385.

Traxler, P., Bold, G., Frei, J., Lang, M., Lydon, N., Mett, H., Buchdunger, E., Meyer, T., Mueller, M., Furet, P., 1997. Use of a pharmacophore model for the design of EGF-R tyrosine kinase inhibitors: 4-(phenylamino)pyrazolo[3,4-ɒ]pyrimidines. J. Med. Chem. 40 (22), 3601–3616.

Turner, M., Mee, P.J., Costello, P.S., Williams, O., Price, A.A., Duddy, L.P., Furlong, M.T., Geahlen, R.L., Tybulewicz, V.L., 1995. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature 378 (6554), 298–302.

Vlahos, C.J., Matter, W.F., Hui, K.Y., Brown, R.F., 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J. Biol. Chem. 269 (7), 5241–5248.