The Src family tyrosine kinase, Lyn, negatively regulates Akt activation in LMP2A-expressing B lymphocytes

by

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B.Sc., University of Victoria, 2005

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biology

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Abstract

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The Epstein-Barr virus (EBV) protein, Latent Membrane Protein 2A (LMP2A), is critical for maintaining viral latency and provides pro-survival and pro-migratory signals to EBV-positive B and epithelial cell malignancies. The N-terminus of LMP2A contains several protein-protein interaction motifs involved in the recruitment of cellular signalling proteins and it is through the recruitment of these proteins that LMP2A is able to initiate signalling. In B lymphocytes, LMP2A's ability to initiate signalling was originally proposed to proceed via a two step mechanism. Firstly, recruitment of the Lyn tyrosine kinase to the tyrosine phosphorylated YEEA site in LMP2A allows for tyrosine phosphorylation of the LMP2A immunoreceptor tyrosine-based activation motif (ITAM). This, in turn, facilitates the recruitment and activation of Syk tyrosine kinase which then initiates downstream signalling events. However, recent findings suggest this model may not be correct and argue that Syk recruitment to LMP2A is independent of the YEEA site. Therefore, we undertook a series of experiments to better understand the role of the YEEA motif and Lyn in the initiation of LMP2A signalling in B lymphocytes. We found that the YEEA site was not absolutely required for tyrosine phosphorylation of the

LMP2A ITAM, or for LMP2A to activate Syk. Using siRNA to silence Lyn expression in LCLs, we found that reducing Lyn expression inhibited the ability of LMP2A to promote Syk tyrosine phosphorylation. In contrast, DG75 B cells or Lyn-deficient DT40 B cells transiently expressing higher levels of LMP2A did not require Lyn for LMP2A-mediated Syk phosphorylation. Furthermore, Lyn was not required for LMP2A-mediated Akt activation in DG75 B cells, but rather Akt activation was significantly enhanced in LMP2A-expressing cells where Lyn was reduced by siRNA. We propose that Lyn negatively regulates LMP2A-mediated Akt activation by phosphorylating Syk on Y323, which serves to recruit the c-Cbl E3 ubiquitin ligase to Syk and targets Syk for ubiquitin-mediated degradation. In sum, this work provides novel insight into how LMP2A uses Lyn to initiate and titre signalling in B cells and brings to light an unappreciated role for Lyn as a negative regulator of LMP2A-mediated Akt activation.

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List of Abbreviations

AIDS	acquired immunodeficiency syndrome
AP-1	Activator Protein-1
BART	Bam A rightward transcript
BCR	B-cell receptor
BL	Burkitt's Lymphoma
BLC	B lymphocyte chemoattractant
BLNK	B cell linker protein
BSA	bovine serum albumin
BTK	Bruton's tyrosine kinase
CIP	calf intestinal phosphatase
CR2	complement receptor 2
CTL	cytotoxic T cell
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GAG	glycosaminoglycan
GC	germinal centre
GSK3β	glycogen synthase kinase 3β
GST	glutathione-S-transferase
HA	hemagglutinin
HBS	HEPES-buffered saline
HCMV	human cytomegalovirus
HD	Hodgkin's disease
HDAC	histone deacetylase
HHV	human herpes virus
HRP	horseradish peroxidase
HRS	Hodgkin Reed-Sternberg
HSV	herpes simplex virus
ICAM-1	intercellular cell adhesion molecule 1
IFN	interferon
IFR	interferon regulatory factor
Ig	immunoglobulin
IGF-1	insulin-like growth factor 1
IL	interleukin
IM	infectious mononucleosis

ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
JAK	Janus Kinase
JNK	c-Jun N-terminal kinase
KSHV	Kaposi's sarcoma-associated herpesvirus
LCL	lymphoblastoid cell line
LMP	latent membrane protein
LMP2A	latent membrane protein 2A
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MHC II	major histocompatibility complex class II
mL	millilitre
mTOR	mammalian target of rapamycin
NK	natural killer cell
NPC	nasopharyngeal carcinoma
oriP	origin of replication
PARP	poly(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PKR	RNA activated protein kinase
PP1	4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
PP3	4-amino-7-phenylpyrazol[3,4-d]pyrimidine
pRb	retinoblastoma protein
RBP-Jκ	recombination signal-binding protein Jκ
RNA	ribonucleic acid
SAP	SLAM-associated protein
SCID	severe combined immunodeficiency
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel eletrophoresis
SFK	src family kinase
SH2	src homology 2
SH3	src homology 3
siRNA	small interfering ribonucleic acid
STAT	signal transducers and activators of transcription
TBS	Tris-buffered saline
TBST	Tris-buffered saline + 0.05% tween
TCF	T cell factor
TCR	T cell receptor
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAF	tumour necrosis factor receptor-associated factor
μg	microgram
UGDH	UDP-glucose dehydrogenase
μM	micromolar
VSV	Varicella-Zoster virus
XLP	X-linked lymphoproliferative syndrome

Chapter 1: Introduction

1.1 Herpesviruses: An Overview

Herpesviruses are a large, diverse family of double-stranded DNA viruses that infect a wide range of hosts from fungi and frogs to horses and humans (1, 2). Although they have a wide host range, all herpesviruses share a common virion structure, a linear double-stranded DNA genome densely packed within an icosahedral capsid (1-3). This capsid is surrounded by a layer of proteins, called the tegument, and enveloped in a lipid bilayer embedded with glycoproteins.

All herpesviruses infect their hosts for life (1). They establish latency in cells where the virus lies dormant until reactivated by some environmental cue (1). During latency, the viral genome is maintained in a covalently closed, circular form and a small subset of viral genes are expressed (2). Productive infection, including both primary infection and viral reactivation, is often associated with host disease (3).

The herpesvirus family has been divided into three subgroups (α, β, or γ) based on host species, host-cell range, and length of reproductive cycle (1, 3-6). In these three subgroups there are eight viruses able to infect humans, many of which are associated with disease in the host (2). These diseases range from non-life threatening illnesses such as oral herpes to more serious illnesses such as cancer (2, 4). α-herpesviruses are neurotrophic viruses, meaning they establish latency within sensory neurons (1, 2, 4, 5). This subgroup includes herpes simplex viruses (HSV) 1 and 2, the causative agents of oral and genital herpes respectively. This group also includes the Varicella-zoster virus (VZV) which causes chicken pox upon primary infection and shingles upon reactivation.

The predominant cellular site of latency for the β-herpesviruses is not well defined and infection with this viral subtype is not generally associated with serious disease in the host (1, 2, 4, 5). This subgroup includes human cytomegalovirus (HCMV) and human herpesviruses (HHV) 6 and 7.

γ-herpesviruses are a group of lymphotrophic viruses (1, 2, 4, 5). They are able to infect and establish latency in both B and T lymphocytes, but on occasion may infect epithelial cells as well (1). γ-herpesviruses cause transient lymphoproliferative disease in the host upon primary infection and their latent infection is associated with several human malignancies (1, 2, 4). Due to their ability to infect and cause serious disease in humans, γ-herpesviruses are an important subject of research. Improving our knowledge of the molecular biology behind their latent infection may prove beneficial to understanding their association with certain human maladies, particularly cancer. Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) are both γ-herpesviruses linked to human cancers. More specifically, EBV, which causes infectious mononucleosis (IM) upon primary infection, has been associated with a number of lymphoid and epithelial malignancies including nasopharyngeal carcinoma (NPC), Burkitt's lymphoma (BL) and Hodgkin's disease (HD) (see Section 1.2.4 for more details) (1).

1.2 Epstein-Barr Virus

1.2.1 Discovery

Epstein-Barr virus (EBV) was discovered as a result of the work by British surgeon, Dr. Denis Burkitt. While working in Uganda, Burkitt discovered a childhood

lymphoma which presented with tumours in the jaw. This previously uncharacterized lymphoma is now known as Burkitt's Lymphoma (BL) (5). The distribution of BL, which Burkitt reported to be dependent on climatic factors such as temperature and rainfall, intrigued British pathologist Dr. Anthony Epstein (6). Epstein suggested to Burkitt that an oncogenic virus may be playing a role in this disease. Burkitt began sending Epstein BL biopsies and, in 1964, the Epstein-Barr virus was discovered by electron microscopy within a cultured BL cell line by Epstein and Yvonne Barr making it the first human tumour virus to be identified (7). A short time later, EBV was determined to be the causative agent of infectious mononucleosis (IM), a connection that was made quite by accident when an EBV sero-negative laboratory technician contracted IM and subsequently tested positive for EBV (8, 9). EBV is now known to be a ubiquitous virus infecting approximately 90% of the world's population (10, 11).

1.2.2 Primary infection and persistence

Although 90% of the worlds population is latently infected with EBV, not all experience the symptoms of IM as infection during infancy or early childhood is often asymptomatic (12). EBV is primarily transmitted through the saliva, but may also be passed through blood or tissue transplantation. EBV entering through the saliva, crosses the epithelial cell barrier of the tonsils before infecting the underlying, naïve B lymphocytes (13). EBV enters B cells by binding of the viral glycoprotein gp350/220 to CD21 on the B cell surface which is followed by vesicular internalization of the virus (14, 15). Major histocompatibility class II (MHC II) molecules on the surface of B cells serve as co-receptors for EBV (16). CD21, also known as complement receptor 2 (CR2),

is expressed primarily on B cells and follicular dendritic cells but also, to a lesser extent, on thymocytes and mature T cells (17, 18). In addition to B and T cells, EBV also infects epithelial cells, but how it gains access to this CD21-negative cell type is not yet well understood. It has been speculated that EBV virions remain on the surface of B cells and from there are efficiently transferred to epithelial cells (19). Alternatively, the expression of another cell surface marker able to bind EBV on the surface of epithelial cells may mediate infection of this cell type (20).

Like all herpesviruses the life cycle of EBV consists of two stages of infection. The lytic stage is associated with primary infection and reactivation. Lytic infection is characterized by the production of new infectious virus particles and the release of these virions results in host-cell lysis. During the second stage of infection, known as latency, the virus enters a quiescent state and no new viral particles are produced. The latent stage of EBV infection persists for life in the body's memory B cell compartment (21-23). During latency only a small subset of viral genes are expressed from the EBV genome including six EBV nuclear antigens (EBNAs), three latent membrane proteins (LMPs), two EBV non-polyadenylated RNAs (EBERs), and BamA rightward transcripts (BARTs) (24). The differential expression of these genes is believed to be important for the development and differentiation of EBV-infected naïve B cells into resting memory B cells (Table 1.1). The pattern of latency gene expression also differs for many EBV-associated diseases (Table 1.2).

How persistent EBV infection is established specifically in the body's memory B cell compartment is still not well understood. Based on *in vitro* studies of EBV infection, and some *in vivo* data, a model of EBV infection has been constructed. It is believed,

Table 1.1 EBV Latency types and associated gene expression patterns (25)

Latency Type	EBV Gene Expression
Latency I	EBNA1, EBERs
Latency II	EBNA1, LMP1, LMP2A/2B, EBERs
Latency III	all latency genes
Lytic	all lytic genes

Table 1.2 EBV latency and associated diseases

EBV-associated Disease	Latency
Nasopharyngeal Carcinoma	II
Gastric Carcinoma	I/II
NK/T cell Lymphomas	II
Infectious Mononucleosis	III
X-linked lymphoproliferative disease	III
Immunoblastic Lymphomas	III
Burkitt's Lymphoma	I
Hodgkin's Disease	II

that when EBV infects a naïve tonsillar B cell all the latency genes are expressed causing the cell to become activated and proliferate as though it were responding to antigen (13, 25). This pattern of viral gene expression, which is referred to as Latency III or the growth program, drives the EBV-infected B cell to differentiate into an active blast, migrate to the follicle and form a germinal centre (GC) (Figure 1.1) (Table 1.1). B cells infected with EBV *in vitro* exhibit this pattern of viral gene expression and will proliferate continuously forming a lymphoblastoid cell line (LCL) (8). *In vivo*, however, the gene expression pattern of these EBV-infected blasts is thought to become more restricted such that only three of the latent proteins, EBNA1, LMP1 and LMP2A, are expressed (25, 26) (Figure 1.1) (Table 1.1). This pattern of gene expression is referred to as Latency II, or the default program. In the GC, an EBV-infected B cell undergoes somatic hypermutation and isotype switching, just as a normal antigen-stimulated B cell would, before differentiating into a memory B cell (27). Latently infected resting memory B cells are then thought to leave the follicle and enter peripheral circulation where they are maintained as normal memory B cells (28) (Figure 1.1). These cells express only EBNA1, a viral gene expression pattern which is referred to Latency I or the Latency Program (25, 26) (Table 1.1). Since these cells are few in number and express very little viral genetic information, they remain safe from immunosurveillance and are nonpathogenic. Instead of EBV driving B cell differentiation, it is also possible that latent EBV infection is found in the memory B cell compartment simply due to the ability of EBV-latent genes to promote long-lived B cells through their pro-survival and anti-apoptotic functions (20).

Persistent EBV infection is characterized by stable numbers of latently-infected

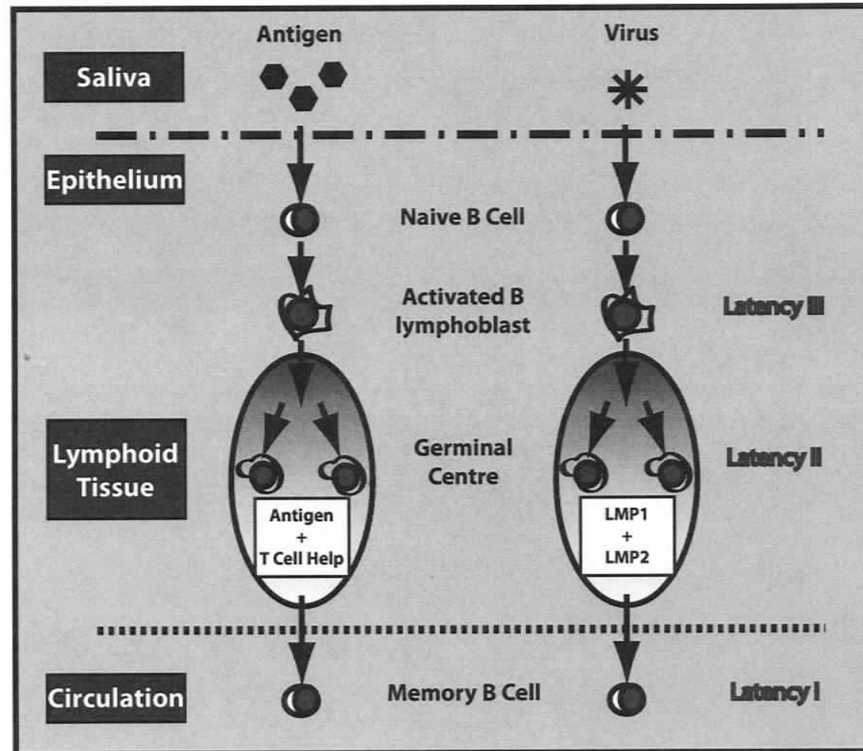

Figure 1.1 – EBV uses normal B-cell biology to establish latent infection in memory B cells. The response of a normal naïve B cell to antigen, resulting in the production of an antigen-specific memory B cell, is diagrammed on the left. The parallel series of steps induced by EBV infection in the process of establishing latency in memory B cells is diagrammed on the right. The latency gene expression patterns are shown in grey on the far right (see Table 1.1 for more details). Adapted from (25).

memory B cells in the blood and by the periodic shedding of infectious virus in the saliva (29). How EBV latency is maintained in this small compartment of resting cells with a finite lifespan is a subject of some debate. It has been suggested that the epithelial cells of the nasopharynx serve as a reservoir for lytic infection, continuously producing virus capable of infecting naïve B cells, thereby replenishing the latently-infected memory B cell pool (reviewed in (24)). It has also been suggested that the source of infectious virus is the B cells themselves, that periodic initiation of lytic replication occurs when a latently-infected memory cell receives signals that cause it to differentiate into an antibody-secreting plasma cell (Figure 1.2) (30). Because antibody-secreting plasma cells migrate into the mucosal epithelium, such a cell would be perfectly placed to release virions onto the musocal surface, which in the case of the tonsils, would be the saliva (24) (Figure 1.2). The number of EBV-positive memory B cells is prevented from becoming too large through a combination of cytotoxic T cell (CTL) removal, cell lysis during viral replication and normal cell death (24).

1.2.3 EBV-latent gene products and their function

Latent gene expression patterns appear to be central to EBV biology as varying types of latency are observed in EBV-positive tumours (Table 1.2). For this reason, it is important to understand the regulation of EBV-latent gene expression and the function of the latency proteins. The linear, double-stranded DNA genome of EBV consists of about 172 kb (31). Approximately sixteen hours after infection and internalization of the virus, the genome circularizes forming an episome that translocates to the nucleus of the host cell (32) (Figure 1.3). Once in the nucleus, the tightly regulated expression of latency

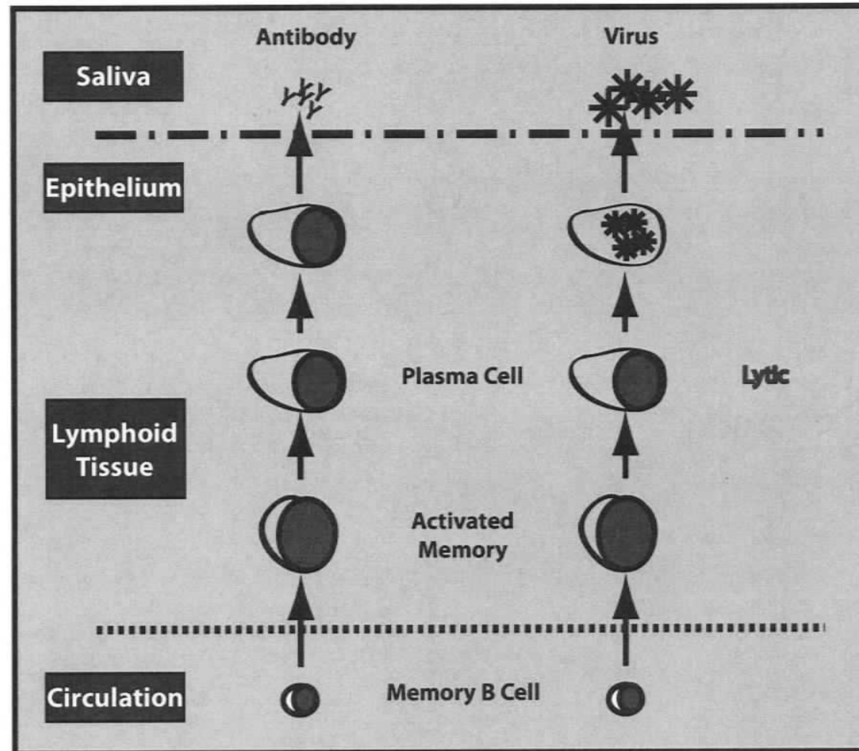

Figure 1.2 – A model for how EBV uses normal B-cell biology to replicate, producing new virus particles that are shed into the saliva. The pathway by which antigen-specific memory cells become activated and differentiate into antibody secreting plasma cells is diagrammed on the left. The parallel pathway by which memory B cells latently infected with EBV become activated, resulting in the shedding of infectious virus particles in the saliva, is diagrammed on the right. Adapted from (25).

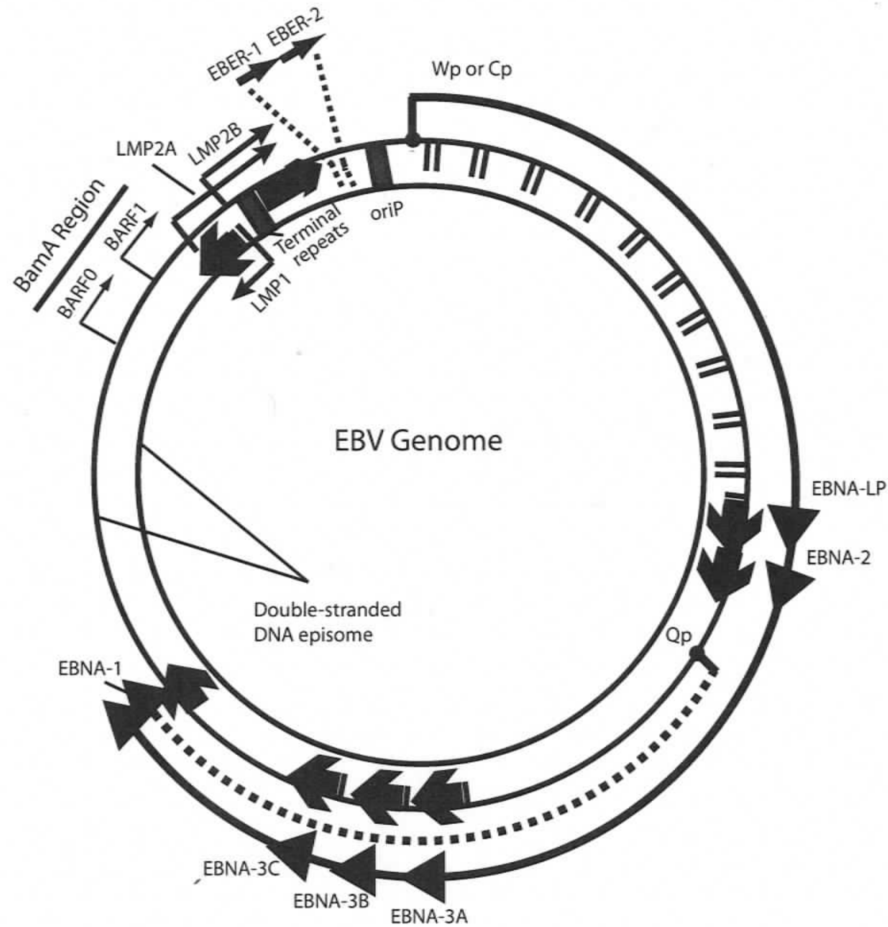

Figure 1.3 - The EBV Genome. This diagram shows the location and transcription of the EBV latent genes on the double stranded DNA episome. The large, solid arrows represent the coding exons for each of the latent proteins and the direction in which they are transcribed. The latent proteins include the six EBNAs (EBNA-1, -2, -3A, -3B, -3C and EBNA-LP) and the three latent membrane proteins (LMP-1, -2A, and -2B). The long solid outer line represents EBV gene transcription during latency III where all EBNAs are transcribed from the Wp or Cp promoter. The EBNAs are encoded by individual mRNAs generated by differential splicing of the same long primary transcript. The shorter dotted inner line represents the EBNA-1 transcript originating from the Qp promoter during latency I and latency II. The location of the non-polyadenylated RNAs (EBER-1 and EBER-2) and the BamA region transcripts (BARF0 and BARF1) are also diagrammed here. Adapted from (107).

genes from the EBV genome begins. The products of EBV-latent gene expression and their functions are discussed below.

1.2.3.1 The EBNAs

There are six EBV nuclear antigens (EBNAs), EBNA-1, -2, -3A, -3B, -3C and -LP, that are expressed from the EBV genome during latency (33). The EBNA proteins function as transcriptional regulators and regulate the transcription of both cellular and viral genes. The EBNA proteins vary in their oncogenic potential and function, as the genes regulated by each EBNA protein differ. EBNA-1 is a 641 amino acid DNA binding protein required for replication and maintenance of the EBV genome (34). EBNA-1 links the episome to chromosomes so the viral genome is replicated in dividing cells as though it were part of the cellular genome (34, 35). EBNA-1 binds the origin of replication (OriP) within the viral episome via its C-terminal DNA binding and dimerization domain (Figure 1.3) (36). Subsequent to EBNA1 binding, all other replication steps are carried out by host cell enzymes during S phase in parallel with the replication of the cellular genome (33). EBNA-1 also negatively regulates its own expression (37) and enhances the transcription of other EBV latency genes, such as *LMP1* (38). EBNA-1 contains a glycine-alanine repeat region that inhibits its proteasomal degradation and the subsequent presentation of EBNA-1 epitopes by MHC class I molecules (39, 40). Inhibiting the presentation of antigenic peptide represents a novel mechanism for bypassing CTL recognition during latency I, when only EBNA-1 is expressed (40). EBNA-1 is expressed in all EBV-positive malignancies but its role in oncogenesis has not been well characterized. Expression of EBNA-1 in the B cells of

transgenic mice results in the formation of B cell lymphomas, suggesting EBNA-1 may play a direct role in tumourigenesis, however, EBNA-1 is not required for LCL formation (20, 41).

EBNA-2 is unable to bind DNA directly but interacts with EBNA-2 response elements through its association with the cellular DNA binding protein, recombination signal-binding protein Jκ (RBP-Jκ), which binds to a specific DNA sequence found within all EBNA-2 responsive promoters (42). EBNA-2 upregulates the expression of *CD21*, *CD23* and *c-myc*, as well as, *LMP1* and *LMP2* (33) and is essential for the immortalization of B cells *in vitro* (43). The P3HR-1 strain of EBV, in which the EBNA-2 gene is deleted, is unable to transform B cells *in vitro* (43-45). However, repairing the deletion by homologous recombination restores the ability of the virus to immortalize B cells in culture, thereby confirming the importance of EBNA-2 in transformation (43-45).

EBNA-3A (EBNA3), -3B (EBNA4) and -3C (EBNA6) consist of 944, 937 and 992 amino acids respectively and are produced by the alternative splicing of transcripts initiated at the EBV C promoter (Cp) (33) (Figure 1.3). All EBNA-3 proteins are similar to EBNA-2 in that they associate with RBP-Jκ (46, 47). The EBNA-3s compete with EBNA-2 for binding to RBP-Jκ and, therefore, also control RBP-Jκ activity and regulate gene expression initiated at cellular and viral promoters containing Jκ sequences. Through its interaction with RBP-Jκ, EBNA-3A functions mainly as a repressor, repressing EBNA-2 induced activity of the Cp promoter in EBV-infected B and epithelial cells (33). EBNA-3B, on the other hand, induces the expression of several cellular genes including *vimentin*, *Bcl-2* and *CD40* (48). EBNA-3C induces *CD21* and *LMP1* gene transcription and may also interact with the retinoblastoma protein (pRb) to promote

transformation (49-52). In addition to interacting with RBP-Jκ, EBNA-3C also interacts with histone deacetylases (HDACs) to repress gene transcription (53). Studies using EBV recombinants have determined that EBNA-3A and -3C are required for *in vitro* B cell transformation, whereas EBNA-3B is not (54).

EBNA-LP is encoded by the leader of each EBNA mRNA, hence the name EBNA-leader protein (LP) (33). EBNA-LP cooperates with EBNA-2 in transcriptional regulation by enhancing its transactivation potential (55). It is believed that this effect on EBNA-2 activity is due to the ability of EBNA-LP to bind HDACs and prevent them from entering the nucleus, where they might otherwise inhibit EBNA-2-induced transcription (56). A role for EBNA-LP in cell-cycle regulation has been suggested by the finding that EBNA-LP, together with EBNA-2, can induce cyclin D2 expression and the G_0 to G_1 transition in primary B cells (57). EBNA-LP is also known to colocalize with pRb and p53 but there is no indication that this association has any effect on pRb or p53 function (58). EBNA-LP is not required for B cell transformation *in vitro* but is important for the efficient outgrowth of LCLs (59). The ability of EBNA-LP to enhance EBNA-2 transactivation, however, alludes to an important role for EBNA-LP in EBV-induced transformation.

1.2.3.2 LMP1

LMP1 is an integral membrane protein consisting of 386 amino acids (33) that has six hydrophobic transmembrane domains and cytoplasmic N- and C- termini (24). The C-terminal domain of LMP1 has extensive functional homology to CD40, a co-stimulatory receptor expressed on the surface of B cells (24). LMP1 functions as a

constitutively active CD40 by interacting with a set of molecules called tumour necrosis factor receptor associated factors (TRAFs) by way of its TRAF-binding domain (60, 61) (Figure 1.4). TRAFs act as intermediaries in signalling by members of the tumour necrosis factor receptor (TNFR) family of cell surface receptors (24). CD40 is a member of this receptor family and is expressed on GC B cells where its interaction with CD154, on the surface of T helper cells, initiates signals required for B cell survival and differentiation (24). Both LMP1 and CD40 interact with Janus kinase 3 (JAK3) to promote the activation of NF-κB and signal transducers and activators of transcription (STATs) (62, 63) (Figure 1.4). They also activate the JNK signalling pathway which, in turn, activates the activator protein-1 (AP-1) family of transcription factors (64, 65) (Figure 1.4). Signalling from LMP1 and CD40 rescues B cells from apoptosis and drives proliferation (24). By mimicking CD40 signals, LMP1 provides the surrogate T cell help required for the development and differentiation of EBV-infected B cells.

LMP1 is expressed in many EBV-positive malignancies and there is abundant evidence to support a link between LMP1 expression and EBV-associated transformation. When transfected into EBV-negative BL cells, LMP1 upregulates the expression of a number of adhesion molecules, activation markers, haematopoietic growth factor receptors and anti-apoptotic proteins (52, 66-69). LMP1 expression has similar effects in primary B lymphocytes, and also alters the growth of immortalized human epithelial cells in culture by inhibiting their differentiation (70, 71). Transgenic mice expressing LMP1 in either their B cell compartment or keratinocytes form B cell lymphomas and experience epithelial hyperproliferation, respectively (41, 72). Lastly,

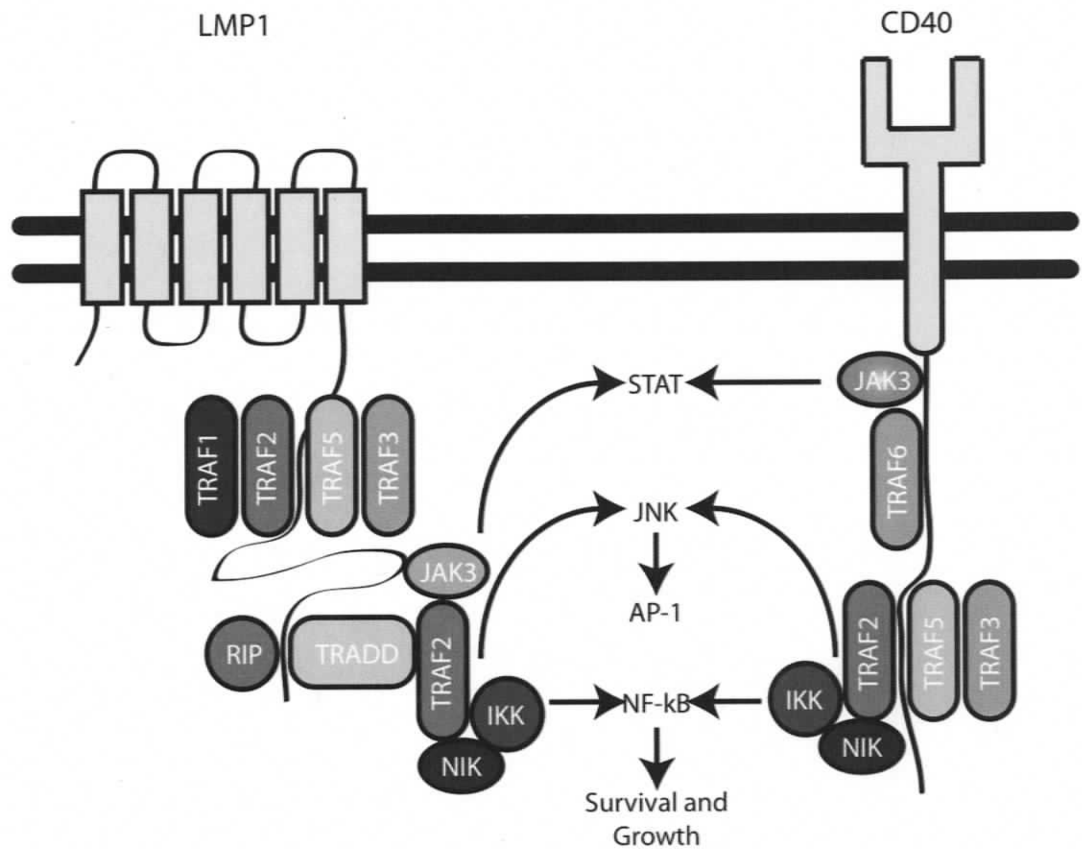

Figure 1.4 – The signalling relationship between LMP1 and CD40. A schematic illustrating the parallels between LMP1 and CD40 signalling in B lymphocytes. Both LMP1 and CD40 contain TRAF-binding domains and interact with multiple members of the TRAF family. Downstream signalling from LMP1 and CD40 via TRAFs results in the activation of NF-κB. LMP1 and CD40 also interact with JAK3 and JNK resulting in the activation of STAT proteins and AP-1 transcription factors, respectively. Downstream signalling from LMP1 and CD40 promotes survival of B cells and drives their proliferation. Adapted from (24).

LMP1 is required for the establishment and continued proliferation of EBV-infected LCLs (73).

1.2.3.3 LMP2A and -2B

LMP2A and -2B are transcribed from two different promoters and their transcripts differ only in the first exon (33). The first exon of LMP2A encodes a 119 amino acid, N-terminal tail, which is absent in LMP2B. LMP2A is a 12-transmembrane domain-containing protein with cytosolic N- and C-termini (24). The N-terminal tail contains several short protein-protein interaction motifs, including a YEEA site and an immunoreceptor tyrosine-based activation motif (ITAM), involved in the recruitment of cellular signalling molecules (Figure 1.5). These molecules include the Syk tyrosine kinase and members of the Src family of tyrosine kinases (SFK), particularly Lyn. The ITAM of LMP2A is similar to those found in the α- and β- chains of the BCR and each recruits similar signalling molecules (24) (Figure 1.6). It is through the recruitment of these signalling molecules that LMP2A is able to mimic the tonic, non-proliferative signals provided by the BCR in the absence of antigen, signals required for the positive selection of B cells in the bone marrow and for survival of mature B cells in the periphery (24) (Figure 1.6). Expression of LMP2A as a transgene in the B cell lineage, promotes B cell proliferation and survival in mice lacking a functional BCR, demonstrating that LMP2A can replace the survival signals usually provided by the BCR (27, 74, 75). LMP2A also inhibits BCR signalling by sequestering BCR-associated tyrosine kinases away from the BCR, a process that is important for the maintenance of EBV latency and discussed further in Section 1.3.2.2 (76-79). LMP2A is expressed in many EBV-

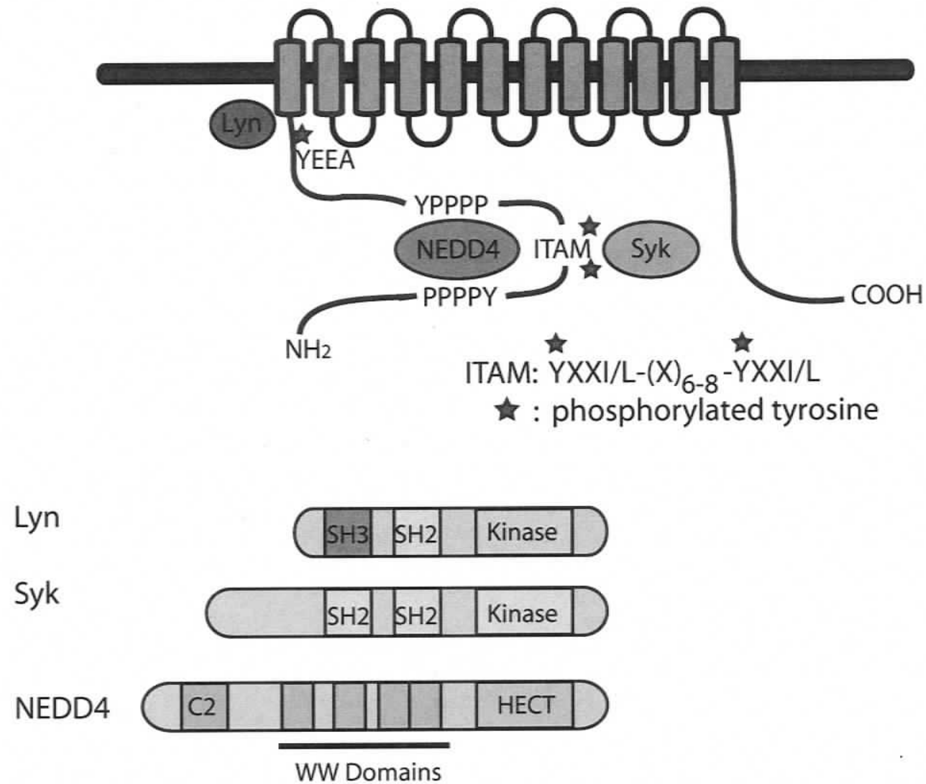

Figure 1.5 - Schematic of LMP2A and associated signalling proteins. LMP2A is a 12-transmembrane domain-containing protein with cytosolic N- and C- termini. The N-terminal tail consists of 119 amino acids and contains several short protein-protein interaction motifs involved in the recruitment of cellular signalling molecules. These motifs include a YEEA motif, that when phosphorylated on tyrosine residue Y112, recruits SFKs such as Lyn via their SH2 domain. LMP2A also possesses an ITAM, which when phosphorylated on its two tyrosine residues, Y74 and 85, recruits Syk tyrosine kinase via its tandem SH2 domains. LMP2A also has two PPPY motifs that bind the WW domains of NEDD4 family E3 ubiquitin ligases.

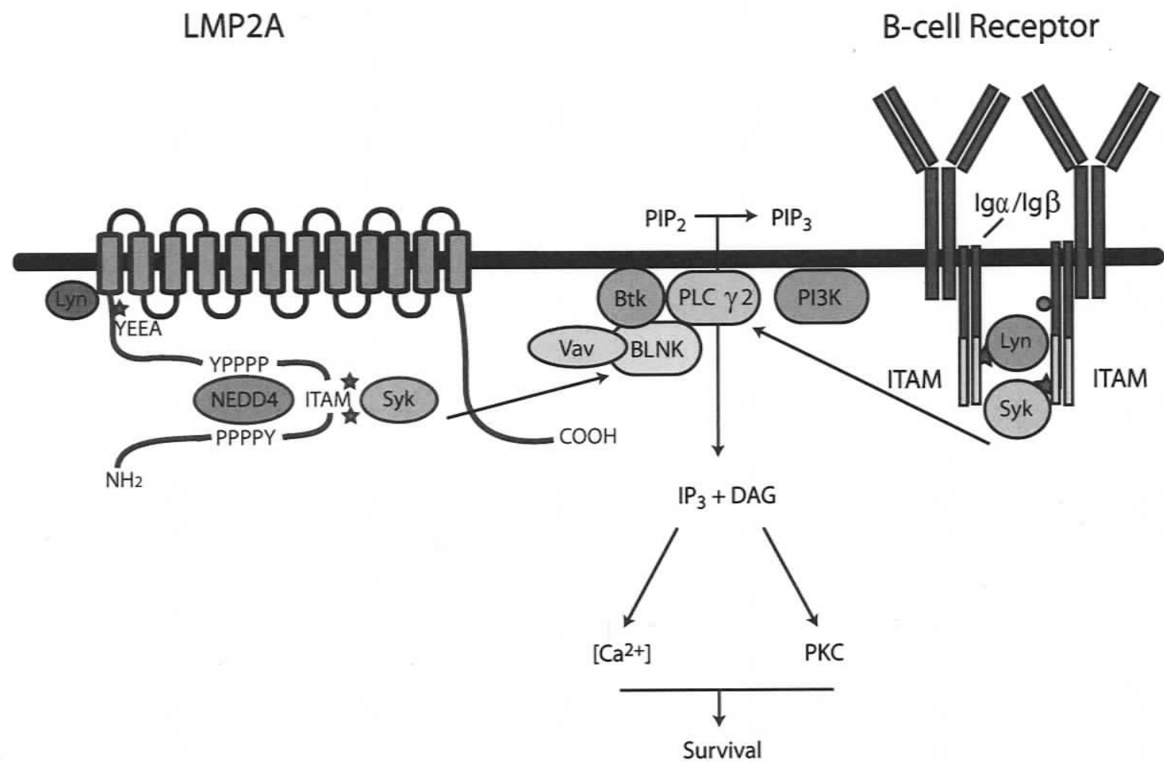

Figure 1.6 – The signalling relationship between LMP2A and the BCR. A schematic illustrating the parallels between LMP2A and BCR initiated signals. The N-terminus of LMP2A contains a similar ITAM to those found within the Igα/Igβ chains of the BCR. The signalling proteins recruited to LMP2A are the same as those recruited to the BCR and include members of the Src family of tyrosine kinases, primarily Lyn, and Syk tyrosine kinase. LMP2A signalling mimics the non-proliferative, tonic signal provided by the BCR in the absence of antigen which is required for B cell survival. To maintain viral latency, LMP2A also inhibits BCR signalling by sequestering tyrosine kinases used by the BCR. ★ = phosphorylated tyrosine residues. Adapted from (24).

positive malignancies of lymphoid and epithelial origin. Although LMP2A is thought to be dispensable for EBV-induced B cell transformation, its ability to activate pro-survival and pro-migratory pathways implies an important role for this viral protein in the pathogenesis of EBV-positive tumours (25, 33). Thus, understanding how LMP2A uses host cell signalling molecules to alter BCR signalling and to initiate its own signals, and determining how these signals affect B cell biology, may prove invaluable to understanding the link between EBV infection and cancer. LMP2A structure, function and contribution to EBV biology and pathogenesis will be covered further in Section 1.3.

LMP2B differs from LMP2A in that it lacks the 119-amino acid N-terminal tail encoded by the first exon of LMP2A (33). LMP2B, like LMP2A, is not required for B cell transformation *in vitro* and the exact function of LMP2B is unknown. LMP2B forms aggregates with LMP2A and it has recently been suggested that, through the formation of these aggregates, LMP2B is able to modulate LMP2A function (80, 81). In support of this, overexpression of LMP2B in EBV-positive BL cells increases the switching of EBV infection from latent to lytic upon BCR cross-linking, while silencing LMP2B in these same cells decreases lytic activation (82, 83). This data suggests that LMP2B may negatively regulate the ability of LMP2A to maintain EBV latency.

1.2.3.4 Other latency gene products

Other EBV-latent gene products include the BamA rightward transcripts (BARTs) and the small non-polyadenylated EBV-encoded RNAs (EBERs), EBER-1 and EBER-2 (33). The exact function of these EBV gene products and their contribution to EBV-associated diseases are not well understood. The EBERs are expressed in all three forms

of latency and associate with several cellular proteins including the interferon-inducible double-stranded RNA-activated protein kinase (PKR), a key mediator of the anti-viral effect of interferon (IFN) (33, 84-86). EBERs directly bind PKR and inhibit its phosphorylation (87), which confers resistance to IFN-α-induced apoptosis in BL cells and is likely important for viral persistence (87, 88). When expressed in EBV-negative BL cells, EBERs promote growth in soft agar, tumourgenicity in SCID mice, resistance to apoptotic inducers and upregulation of Bcl-2 expression (89). EBERs also induce transcription of IL-10 in B cells, IL-9 in T cells and insulin-like growth factor-1 (IGF-1) in epithelial cells, all of which act as autocrine growth factors (90-92). This data suggests a role for these virus-encoded RNA molecules in promoting and maintaining EBV-associated oncogenesis.

BARTs are differentially spliced mRNAs with a common 3' open reading frame and polyadenylation signal (33). BARTs are expressed during all types of EBV infection and all EBV-associated malignancies (93-98). The BARTs contain several open reading frames, BARF0, RK-BARF0, A73, and RPMS1, but whether these encode for protein remains controversial as detection of the protein products of these open reading frames in EBV-infected cells is difficult to demonstrate (99, 100). Deletion of the BamA region of the EBV genome does not affect the immortalization of B cells *in vitro*, which implies that BARTs are dispensable for transformation (101).

1.2.4 EBV-associated diseases

EBV infection is associated with several human diseases of B cell origin and, despite its preference for the B cell compartment, EBV is also able to infect epithelial and

T cells where its presence is associated with several malignancies. In B cells, EBV is associated with some non-malignant diseases such as IM and the genetic disorder, X-linked lymphoproliferative syndrome (XLP) (20). EBV infection is also associated with several B cell malignancies including Burkitt's lymphoma, Hodgkin's disease and immunoblastic lymphomas. Immunoblastic lymphomas are a heterogeneous group of EBV-positive B cell tumours that arise in individuals who are immunocompromised (25). These lymphomas are commonly seen in post-transplant patients taking immunosuppressive drugs, or in patients suffering from acquired immunodeficiency syndrome (AIDS) or genetic disorders of the immune system (102, 103). Infection of epithelial cells with EBV is also associated with several human cancers including NPC and gastric carcinoma (20). In addition to infecting B cells and epithelial cells, EBV is also able to infect T cells and has been linked to a number of lymphomas arising in this cell type. Most EBV-associated T-cell lymphomas are extranodal and have a cytotoxic phenotype (104). One such T cell lymphoma, almost always associated with EBV, presents in the nasal cavity and is thus referred to as Nasal NK/T-cell lymphoma (105). Similar EBV-positive T cell lymphomas also occur at other sites including the skin, gastrointestinal tract or gonads, but at a much lower frequency (105). EBV-associated diseases display different patterns of viral gene expression (Table 1.2) and the more well characterized of these diseases are discussed below.

1.2.4.1 Nasopharyngeal carcinoma

NPC shows the most consistent association with EBV worldwide (105). There are two forms of NPC, the keratinizing form and the non-keratinizing, undifferentiated

form. Tumours of the latter form are classified as lymphoepitheliomas due to their high levels of lymphocytic infiltration. In most of the world, NPC is a rare disease and both forms are observed, however, in Chinese, Southeast Asian and Inuit populations NPC is much more common (105). In these high-risk groups most NPC tumours are of the non-keratinizing form and association with EBV is 100% (106). Incidence rates are high in individuals of Chinese descent, particularly Cantonese males, irrespective of where they live suggesting a genetic link for the development of EBV-associated NPC (107). In addition to a genetic predisposition, environmental factors such as diet (i.e. salted fish) are believed to be important in the development of this disease (108). These tumours arise from the clonal proliferation of EBV-infected cells expressing a latency II pattern of EBV gene expression (109) (Table 1.2). The expression of LMP1 and LMP2A in EBV-infected epithelial cells of the nasopharynx may initiate inappropriate signals and alter the biological properties of these cells. In fact, LMP2A expression increases the invasive and migratory capabilities of primary tonsillar epithelial cells in culture suggesting that signals initiated by LMP2A may be responsible for the high metastatic rate of EBV-positive NPC (110).

1.2.4.2 Infectious Mononucleosis

Individuals infected with EBV during adolescence or early adulthood often develop a self-limiting, lymphoproliferative disease known as infectious mononucleosis (IM) (24). The symptoms associated with IM generally last two weeks to one month and most commonly include fever, sore throat and lymphadenopathy but may be accompanied by headache, fatigue, splenomegaly and hepatomegaly (111). Several

serious complications may arise during IM. Airway obstruction, due to massive enlargement of the tonsils and adenoids, and splenic rupture, due to enlargement of the spleen, are major concerns (111). During IM, EBV-infected lymphoblasts, which resemble LCLs and express all of the latency genes (Latency III), are present in large numbers in the tonsils and lymph nodes (112) (Table 1.2). Also, EBV replication is detected at high levels in the oropharynx of IM patients (105). The symptoms of IM, however, are not due to viral replication but are instead caused by the high levels of pro-inflammatory cytokines, such as IL-1, IFN-γ and TNF-α, produced by EBV-reactive CTLs cells as the number of activated T cells along with the ratio of CD8+ CTLs to CD4+ T cells is increased during IM (105).

In normal healthy individuals, primary EBV infection is controlled by EBV-reactive CTLs but in patients who suffer from a rare familial condition, known as XLP, primary EBV infection is often fatal (105). XLP presents in young boys as an extreme sensitivity to EBV with IM-like symptoms. Some XLP patients die of uncontrolled B cell proliferation during acute EBV infection, suggesting their immune system response is ineffective. Other XLP patients develop symptoms suggestive of an excessive, overactive immune response (20). XLP is caused by mutations in the SLAM-associated protein (SAP) gene (113). SAP is a SH2 domain containing protein involved in the regulation of lymphocyte signalling and is preferentially expressed in T and natural killer (NK) cells (113). The role of SAP in lymphocyte signalling, immune system responses and EBV infection remains unclear but its mutation in XLP patients highlights the importance of the immune system in controlling acute EBV infection (20).

1.2.4.3 Burkitt's Lymphoma

As mentioned previously, endemic BL was the tumour in which EBV was first discovered (7). Endemic BL occurs at an annual incidence of approximately 5-10 cases per 100,000 children in equatorial Africa and parts of Papua New Guinea (105). Sporadic cases also occur worldwide but at a much lower frequency. Both forms of BL differ in their association with EBV, where almost every endemic BL tumour is EBV-positive, only about 15% of sporadic BL tumours carry the virus (105). The areas associated with endemic BL overlap with areas of endemic *Plasmodium falciparum* malaria infection (105). Malaria is an immunosuppressive factor and infection with malaria presents an increased risk for development of endemic EBV-positive BL. A feature of all BL tumours is that they have undergone a chromosomal translocation that positions the *c-myc* locus in the vicinity of one of the three constitutively expressed immunoglobulin loci (114). This translocation deregulates *c-myc* expression, thus driving cells through the cell cycle and activating anti-apoptotic pathways to promote cell survival (20). This translocation event is rare and resulting BL tumours are clonal in nature, arising from a single cell expressing a single type of immunoglobulin (115). Constitutive *c-myc* expression drives the proliferation of EBV-infected B cells exhibiting a Latency I pattern of viral gene expression, meaning they express only EBNA-1 and not the latency genes associated with promoting B cell growth (116) (Table 1.2). Since EBNA-1 is less immunogenic than the other latency proteins, its sole expression may benefit these tumours by allowing EBV-positive BL cells to escape immunosurveillance by CTLs (117).

1.2.4.4 Hodgkin's Disease

IM is a predisposing factor for the development of Hodgkin's disease (HD) (105). In developed countries, approximately 40% of HD tumours are EBV positive, whereas this percentage increases to around 80% in developing countries. Classic HD is characterized by the presence of multinucleated Reed-Sternberg cells (HRS) in a background of reactive inflammatory cells including granulocytes, lymphocytes, plasma cells and histocytes (20, 105). HRS tumours are monoclonal and arise from a post-GC B cell with crippling mutations in its Ig genes (118). HRS tumour cells are therefore unique in that they lack surface immunoglobulin expression, a state inconsistent with B cell survival (119). All malignant cells of EBV-positive HRS tumours carry the EBV genome and express a latency II program of viral gene transcription (105) (Table 1.2). How EBV-positive HRS cells escape the CTL response is not well understood, but disruption of the immune system following IM and/or the release of CTL-suppressive cytokines by HRS cells may contribute to CTL evasion (24).

The exact contribution of EBV to HRS tumours is unknown. It has been speculated that the key role of EBV in HD is to induce survival and proliferation of BCR-deficient GC B cells through the expression of LMP1 and LMP2A (119, 120). LMP1 likely provides these cells with a proliferative signal while LMP2A contributes to their survival by mimicking BCR signals (74, 75, 121). The rescue of these pre-apoptotic, BCR-negative GC B cells by LMP2A may be a crucial step in the evolution of B cells to HRS cells (120). Thus, understanding how EBV latency proteins, such as LMP2A, are able to substitute for specific molecular changes critical to tumourigenesis may prove invaluable to characterizing the role of EBV in human malignancy.

1.3 LMP2A

Latent Membrane Protein 2A (LMP2A) is an integral membrane protein crucial to the establishment and maintenance of EBV latency. LMP2A serves to both mimic and inhibit BCR signalling. By mimicking BCR signals LMP2A is able to provide cells with pro-survival and pro-migratory signals that may contribute to the pathogenesis of EBV-positive cancers. The structure, function and role of LMP2A in EBV biology are reviewed in detail below.

1.3.1 Structure

LMP2A is a 54 kilodalton protein comprised of a 119 amino acid amino-terminal tail, 12 hydrophobic transmembrane domains and a 27 amino acid carboxy-terminal tail (122) (Figure 1.5). Both the N- and C- termini of LMP2A are located within the cytosol. The N-terminal tail contains eight tyrosine residues, some of which are constitutively phosphorylated. These tyrosine residues are located at amino acid positions 23, 31, 60, 64, 74, 85, 101 and 112 (123). Tyrosine residues, 74, 85 and 112, are essential for LMP2A to initiate signalling, inhibit BCR signalling and maintain viral latency, while tyrosines 60, 64 and 101 are not required for these LMP2A-mediated effects (123). The N-terminus of LMP2A contains several protein-protein interaction motifs involved in the recruitment of cellular signalling proteins and the three essential tyrosine residues, 74, 85 and 112, are found within these motifs. Y112 is located within the YEEA motif and, when phosphorylated, recruits Src family tyrosine kinases (SFK) to LMP2A via their Src homology 2 (SH2) domain (124-127) (Figure 1.5). In B cells, Lyn is the primary SFK recruited to this site but Src, Fyn and Lck are also recruited to a lesser extent (124, 127).

Y74 and Y85 are located within the immunoreceptor tyrosine-based activation motif (ITAM) and, when phosphorylated in B cells, provide a binding site for the tandem SH2 domains of Syk tyrosine kinase (76, 77, 128) (Figure 1.5). The LMP2A ITAM has the consensus sequence YXX(L/I)X_{6-9}-YXX(L/I) (where X is any amino acid) and this ITAM is similar to those found within the cytoplasmic domains of the Igα/Igβ chains of the BCR (129, 130). The N-terminus of LMP2A also possesses two PPPPY (PY) motifs that interact with the WW domains of NEDD4 family E3 ubiquitin ligases (126, 131-133) (Figure 1.5). The recruitment of NEDD4 family proteins to LMP2A leads to the ubiquitination of LMP2A and its associated tyrosine kinases, thus targeting these proteins for proteasomal degradation (126, 131-134). SH3 domains recognize and bind to proline-rich protein sequences, making the PY motifs potential SH3-domain binding sites (135). However, no SH3-domain containing proteins have been reported to interact with LMP2A at the PY motifs (136). A clustering signal located within the C-terminus of LMP2A is believed to mediate its aggregation in lipid rafts of the plasma membrane (137-140). The exact cellular location of LMP2A, however, remains an issue of debate. Some research groups have observed abundant LMP2A expression in intracellular organelles, including perinuclear regions, the trans-Golgi network, early endosomes and lysosomes, and very little in the plasma membrane (80, 141, 142). As mentioned previously, LMP2B is believed to associate with LMP2A and regulate its function and the association of these two proteins is thought to be achieved through their shared C-terminal domains (81). LMP2A contains several sites of palmitoylation, most of which are within the C-terminus of the protein (138-140). However, palmitoylation of these sites is not required for LMP2A raft association (139, 140) and it is believed that LMP2A

is localized to lipid rafts through its interaction with a constitutively raft-associated protein such as Lyn.

1.3.2 Signalling

LMP2A was initially proposed to initiate signalling in B cells via a two-step process (125). First, Lyn phosphorylates Y112 of the YEEA site either through an undefined weak interaction with another site in LMP2A or by colocalizing with LMP2A in lipid rafts (125, 137). Phosphorylation of the YEEA site allows the SH2 domain of Lyn to bind the YEEA motif, and this stable association with LMP2A permits Lyn to phosphorylate Y74 and Y85 of the LMP2A ITAM. Syk is then recruited via its two SH2 domains to the phosphorylated ITAM and activated to initiate downstream signalling. This model was proposed because mutation of Y112 to phenylalanine (Y112F) abolished Lyn binding and drastically attenuated LMP2A tyrosine phosphorylation in LCLs (125). This mutation also diminished the small level of constitutive Syk tyrosine phosphorylation seen in LMP2A-expressing LCLs (125). Recent findings by Lu et al. suggest that this model may need revision. When expressed in 293 and HaCaT epithelial cell lines, Y112F LMP2A promoted a level of Syk tyrosine phosphorylation equivalent to that seen in LMP2A-expressing cells (128). Y112F LMP2A was also still capable of recruiting Syk in these cells, albeit slightly less efficiently (128). Since LMP2A generates signals that may contribute to the pathogenesis of EBV-positive malignancies, it is important to understand how these signals are initiated.

1.3.2.1 Mimicking the B cell receptor

The N-terminus of LMP2A contains an ITAM similar to those found within the intracellular regions of the BCR Igα/Igβ chains. Also, the kinases recruited to LMP2A when it is tyrosine phosphorylated are the same as those recruited to the BCR (24). It is through the recruitment of these kinases that LMP2A is believed to mimic BCR signalling (Figure 1.6). Lyn is recruited to the BCR and tyrosine phosphorylates the ITAMs of the BCR Igα/Igβ chains resulting in subsequent Syk recruitment and activation and the initiation of downstream signalling (143). The BCR can transduce different levels of signalling and the biological outcome of BCR signalling depends on the strength of the signal transduced. Engagement of the BCR by specific antigen, along with the appropriate T cell help, results in the activation of potent growth-promoting signalling cascades. In the absence of antigen, the BCR delivers a non-proliferative, tonic signal required for the survival of all peripheral B cells and it is this signal that is mimicked by LMP2A (24, 143) (Figure 1.6). A complete lack of BCR signal, on the other hand, is not conducive to B cell survival and results in apoptosis. The signals provided by LMP2A can substitute for the tonic signal normally provided by the BCR and promote the survival of B cells lacking a functional BCR. When expressed as a transgene in the B cells of RAG-deficient mice, LMP2A is able to serve as a surrogate BCR activating ERK/MAPK and PI3K pathways to rescue these B cells from apoptosis and promote their development (74, 75, 144, 145). The transgenic expression of LMP2A in the B cells of RAG-competent mice inhibits the Ig heavy chain rearrangements necessary for the expression of a functional BCR, providing further evidence that LMP2A can function as a surrogate BCR (74, 75, 145). Syk binding to the LMP2A ITAM is required for

propagation of LMP2A-induced survival signals, as the ability of LMP2A to promote B cell survival in these transgenic mice was abolished when Y74 and Y85 of the LMP2A ITAM were mutated to phenylalanine (146). The ability of LMP2A to promote B cell development and survival is also dependent on the BCR signalling molecules B cell linker protein (BLNK or SLP-65) and Bruton's tyrosine kinase (BTK) as LMP2A is unable to promote B cell survival in mice deficient in these two proteins (147, 148) (Figure 1.6).

1.3.2.2 Maintaining viral latency

LMP2A can block BCR signalling in LCLs and requires an intact YEEA site and ITAM to do so (76, 149). Following BCR cross-linking, BCR-induced signal transduction is measured by calcium mobilization, cellular kinase activation, induction of tyrosine phosphorylation and activation of transcription, each of which is blocked by expression of LMP2A (77, 150, 151). The two PY motifs located within the N-terminus of LMP2A recruit NEDD4 family E3 ubiquitin ligases by way of their WW domains (126, 131-133) (Figure 1.5). Recruitment of NEDD4 proteins to LMP2A results in the ubiquitination and subsequent proteasomal degradation of LMP2A and its associated tyrosine kinases (126, 131-134). It has been suggested that LMP2A exerts its negative effect on BCR signalling by sequestering SFKs and Syk away from the BCR and, through its interaction with NEDD4 family proteins, promoting their ubiquitination and degradation. The PY motifs of LMP2A, however, do not appear to be essential for blocking BCR signalling in LCLs (132). The negative effect on BCR signalling could also be mediated by the localization of LMP2A to lipid rafts as LMP2A has been shown

to exclude the BCR from lipid rafts where it would otherwise initiate normal B cell signalling (137). The ability of LMP2A to inhibit BCR signalling is likely important for the maintenance of viral latency as the stimulation of B cells via the BCR results in reactivation of EBV and induction of the lytic cycle (152, 153). Studies using EBV recombinants have shown that LMP2A expression blocks activation of the lytic cycle in LCLs, as measured by induction of the EBV immediate early gene *BZLF1*, and that an intact YEEA site and ITAM are required for this block (76-79).

1.3.2.3 Oncogenic signals

LMP2A is not considered a true viral oncogene, as its expression alone is unable to transform B cells *in vitro* (33). However, evidence that LMP2A activates cellular signalling pathways that promote cell survival and migration suggest it may be involved in the maintenance and progression of EBV-positive malignancies.

1.3.2.3.1 Pro-survival signals

Genetic analyses of EBV indicate that LMP2A is not essential for B cell transformation (33) despite its ability to promote the survival of BCR-negative B cells (74, 75). The ability of LMP2A to promote B cell survival under normally apoptotic conditions is attributed to its effect on Akt activation, as LMP2A expression induces PI3K-dependent Akt activation in both B and epithelial cells (154-163). In B cells, the serine-threonine kinase Akt promotes survival by preventing apoptosis through inhibition of the pro-apoptotic factor Bad, prevention of procaspase cleavage, and inhibition of Forkhead transcription factor family members (164-166) (Figure 1.7). LMP2A-mediated

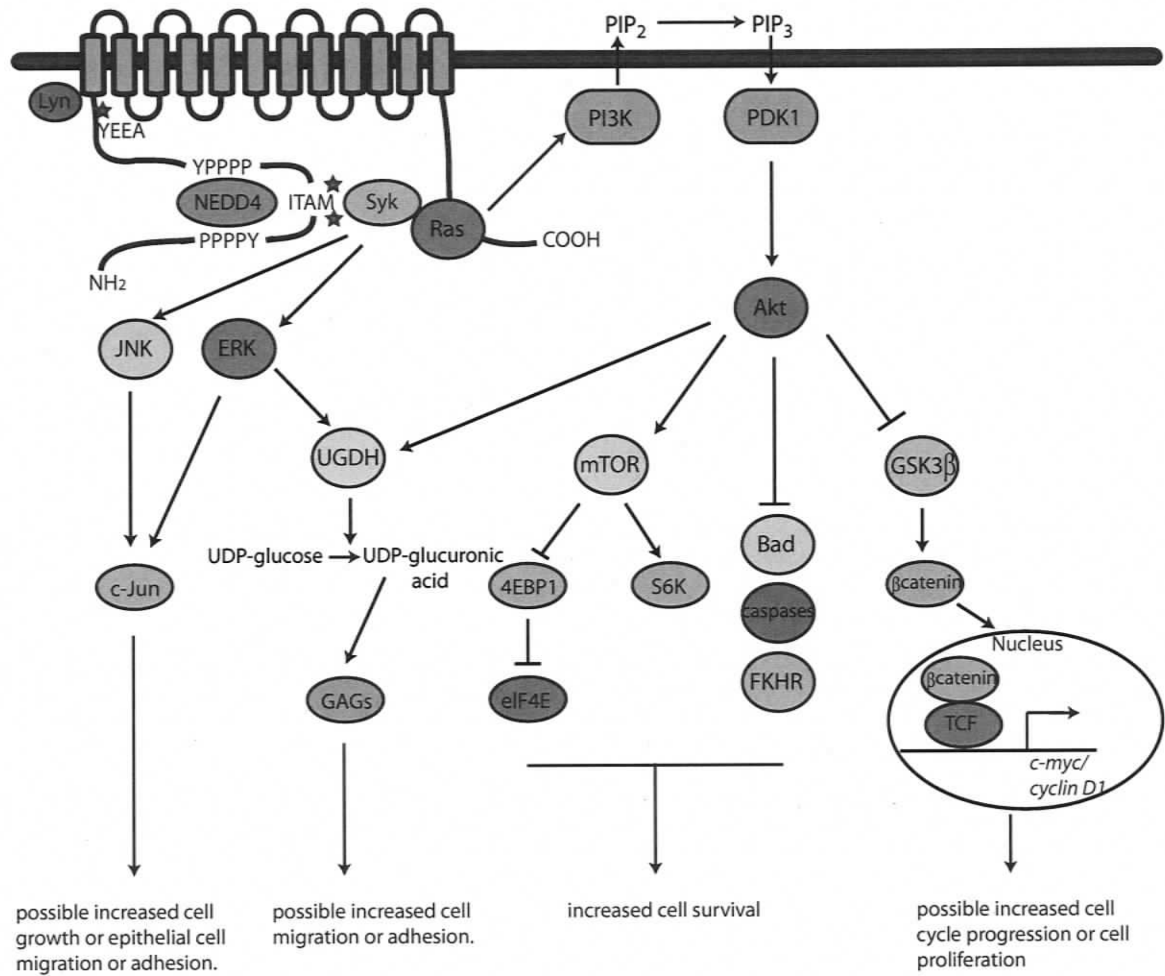

Figure 1.7 – Signalling pathways activated by LMP2A that may contribute to the pathogenesis of EBV-associated malignancies. LMP2A is involved in the activation of several signalling pathways that promote the survival of both lymphoid and epithelial cells and enhance epithelial cell adhesion and migration. ★ = phosphorylated tyrosine.

Akt activation has a preventative role in apoptosis, as demonstrated by the PI3K/Akt-dependent resistance of LMP2A-expressing cells to TGFβ1-induced apoptosis (154). The multifunctional cytokine, TGFβ1, functions as an inducer of apoptosis in some BL and gastric carcinoma cell lines and TGFβ1 levels are elevated in patients with EBV-associated malignancies (167-169). LMP2A inhibits TGFβ1-induced apoptosis in EBV-negative BL cells by preventing TGFβ1-induced DNA fragmentation and caspase 3 activation (154). The recruitment of Lyn and Syk to LMP2A is thought to be essential for LMP2A-mediated Akt activation, as mutation of the tyrosine residues within the YEEA site and ITAM abolish this activation (163). Ras may also play a role in LMP2A-mediated Akt activation in B cells. In BCR-negative B cells from LMP2A transgenic mice, Ras is constitutively activated and these cells undergo apoptosis in the presence of a Ras, PI3K or Akt inhibitor, suggesting that activation of the Ras/PI3K/Akt pathway by LMP2A provides the anti-apoptotic signal these cells require to survive without a functional BCR (161). Increased Ras activation in these cells also correlates with elevated levels of the anti-apoptotic protein, Bcl-xL, alluding to a possible role for Bcl-2 family members in mediating the anti-apoptotic effects of LMP2A (161).

In epithelial cells, LMP2A expression also promotes the PI3K-dependent activation of Akt (155, 162). Again, the recruitment of Syk appears to be necessary for this activation as mutating the tyrosine residues of the ITAM, or reducing Syk levels by shRNA, abolishes LMP2A-mediated Akt activation (156). Downstream of PI3K/Akt, LMP2A expression in epithelial cells promotes activation of the cellular kinase mammalian target of rapamycin (mTOR) (157). Through its role in the regulation of

protein synthesis, mTOR has emerged as a principal mediator of cell growth and proliferation. mTOR phosphorylates and activates the translational activator, S6K, and phosphorylates and inactivates the translational repressor, 4E-BP1, to promote translation and accelerate cell cycle progression (170) (Figure 1.7). In epithelial cells, LMP2A expression promotes the phosphorylation of both these downstream mTOR effectors (157) suggesting that LMP2A-mediated Akt activation may not only promote cell survival by inhibiting apoptosis but may also contribute to epithelial cell proliferation.

Surprisingly, expression of LMP2A in EBV-negative BL cells does not increase their survival or tumourigenic capacity in SCID mice (163). However, nude mice injected with LMP2A-expressing keratinocytes form highly vascularized, highly metastatic tumours (162). Similarly, in cultured keratinocyte and human gastric carcinoma cell lines, LMP2A expression promotes proliferation, inhibits differentiation and confers anchorage-independent growth (155, 162). The increased level of Akt activation in these LMP2A-expressing cells is believed to contribute to their transformed phenotype, as the chemical inhibition of proteins within the Ras/PI3K/Akt pathway abolishes LMP2A-induced anchorage-independent growth (155). The ability of LMP2A to promote constitutive activation of the Ras/PI3K/Akt pathway, a common event during cell survival and tumourigenesis, suggests that this viral protein may play a key role not only in the maintenance of EBV latency but also in the development and maintenance of EBV-associated malignancies.

1.3.2.3.2 Pro-migratory signals

Several EBV-positive cancers, particularly NPC, are associated with a high rate of metastasis, a property often associated with a poor prognosis. *In vitro*, LMP2A has been demonstrated to increase the adhesive, invasive and migratory capabilities of epithelial cells (110, 128, 171, 172). The first indication of LMP2A's involvement in cell migration and adhesion came from a study analyzing the expression of cell adhesion molecules in response to LMP2A expression in keratinocytes (173). LMP2A expression resulted in the dramatic upregulation of suprabasal laminin$_5$, integrin $\alpha_6\beta_4$ and integrin β_1 expression. ICAM-1, α_3 and α_5 integrins were also upregulated in LMP2A-expressing cells, whereas E-cadherin and desmoplakin expression patterns were unchanged. Subsequently, LMP2A was shown to induce the transcription of integrin α_6 mRNA in primary tonsillar epithelial cells as well (110). LMP2A promotes the spreading of epithelial cells on extracellular matrix (ECM) proteins and the ability of LMP2A to promote this spreading is integrin dependent and PI3K, ERK and PKC independent (171). Similarly, LMP2A expression increases the invasiveness of epithelial cell lines and primary tonsillar epithelial cells (110, 172). The invasive property of LMP2A-expressing cells is also likely dependent on integrin expression as treatment of LMP2A-expressing primary tonsillar epithelial cells with integrin α_6 blocking antibodies impairs their movement into Matrigel (110). ERK signalling may also play a role in promoting this invasive phenotype (Figure 1.7). The ERK pathway is important for migration of breast cancer, fibrosarcoma and endothelial cells and LMP2A expression is known to enhance ERK activation (172, 174-176). The treatment of LMP2A-expressing epithelial

cells with the ERK inhibitor PD98059 results in the loss of the invasive phenotype of these cells (172).

LMP2A expression also increases the migratory capabilities of epithelial cell lines and primary tonsillar epithelial cells as has been demonstrated using transwell and scratch wound assays (110, 128, 171). LMP2A's association with Syk is important for mediating its effect on cell migration as mutating the tyrosine residues of the LMP2A ITAM, and reducing Syk levels by siRNA, impairs the migration of LMP2A-expressing cells (128). Migrating LMP2A-expressing primary tonsillar epithelial cells, but not stationary cells, upregulate expression of integrin α_6, indicating that changes in integrin expression also likely have a role in LMP2A-induced migration (110). LMP2A expression in EBV-negative LCLs enhances integrin α_5 promoter activity and upregulates α_5 mRNA expression suggesting that LMP2A may have a role in B cell migration as well, however, this has yet to be investigated (177).

This data supports a role for LMP2A in the metastasis of EBV-positive epithelial cancers as integrin expression generally correlates with metastasis. NPC is highly metastatic and LMP2A and integrin α_6 mRNA expression levels correlate strongly in EBV-positive NPC (110). Thus, it is likely that LMP2A is able to increase the adhesive, invasive and migratory properties of epithelial cells through the upregulation of integrin expression.

1.3.2.3.3 Other signalling pathways

Mitogen-activated protein kinase (MAPK) members, ERK, JNK and p38, have a great influence on the control of metabolic processes such as proliferation, migration,

differentiation and apoptosis. ERK and JNK are activated by LMP2A expression in epithelial cells and activation of these MAPKs results in the subsequent phosphorylation and activation of the downstream effector c-Jun (172) (Figure 1.7). The phosphorylation of c-Jun inhibits its ubiquitination (178, 179) and c-Jun stability is increased in LMP2A-expressing cells (172). The increased c-Jun phosphorylation and stability observed in LMP2A-expressing epithelial cells indicate that c-Jun-mediated signalling occurs within these cells, but the consequence of this signalling has yet to be elucidated. c-Jun is a widely used cellular transcription factor that controls the regulation of many genes involved in cell growth and proliferation (180). In B cells, c-Jun is activated in response to BCR cross-linking and is important for B cell proliferation and differentiation (180). In other cell types, c-Jun inhibits differentiation, as does LMP2A, suggesting c-Jun may play a role in this LMP2A-induced phenotype (162, 181). Furthermore, c-Jun harbours oncogenic potential. It is capable of partially transforming chicken embryo fibroblasts (182) and can cooperate with other factors, such as activated Ras, in mammalian cells to carry out effective transformation (183). As mentioned previously, ERK activation plays a role in promoting LMP2A-induced epithelial cell migration but whether downstream activation of c-Jun plays a role in this process has yet to be determined (172). In BJAB cells, an EBV-negative BL cell line, LMP2A associates with and serves as a substrate for ERK (184). In these cells ERK phosphorylates LMP2A on two serine residues within its N-terminus, S15 and S102, suggesting that ERK and c-Jun may play a role in LMP2A signalling in B cells as well, however, this has yet to be investigated (184).

Another protein affected by LMP2A expression is UDP-glucose dehydrogenase (UGDH), a protein that catalyzes the oxidation of UDP-glucose to UDP-glucuronic acid an essential precursor for the synthesis of glycosaminoglycans (GAGs) (Figure1.7). The expression of LMP2A in epithelial cells results in UGDH promoter activation and increased UGDH protein expression (110, 160). The ability of LMP2A to promote UGDH expression is dependent on activation of the PI3K/Akt and ERK pathways and requires an intact LMP2A ITAM and YEEA site (160). Elevated UGDH levels in LMP2A-expressing epithelial cells results in increased GAG production (160). GAGs are important structural and recognition elements and are believed to participate in numerous cellular phenomena such as development, adhesion, motility, transformation and angiogenesis (185). It is hypothesized that LMP2A expression increases GAG production, thereby enhancing the motility, proliferation and transformed phenotype of EBV-infected epithelial cells, however, this has yet to be examined (160).

As mentioned previously, LMP2A expression in both B cells and epithelial cells also results in increased Akt activation (154-156, 161-163). We have discussed the role of Akt in cell survival, but haven't addressed its role in other signalling pathways. Akt phosphorylates and inactivates glycogen synthase kinase 3β (GSK3β), a serine/threonine kinase that contributes to the regulation of glycogen metabolism (186) and functions in the Wnt signalling pathway (187). The Wnt signalling pathway is deregulated in a high percentage of colon cancers and GSK3β functions in this pathway as a regulator β-catenin (187). In the absence of a Wnt signal GSK3β phosphorylates β-catenin resulting in its ubiquitination and proteasomal degradation, while in the presence of a Wnt signal the

ability of GSK3β to phosphorylate β-catenin is blocked. The inability of GSK3β to phosphorylate β-catenin results in its stabilization, cytoplasmic accumulation and nuclear translocation. Once in the nucleus, β-catenin interacts with members of the T-cell factor (TCF) family of transcription factors to promote the expression of target genes (188) (Figure 1.7). In epithelial cells, LMP2A expression promotes PI3K dependent activation of Akt which subsequently phosphorylates and inactivates GSK3β (158). In these LMP2A-expressing cells cytoplasmic and nuclear levels of β-catenin are increased along with the activity of TCF-sensitive luciferase reporters (158). Both intact LMP2A ITAM and PY motifs are required for nuclear LMP2A-mediated β-catenin accumulation (159). β-catenin/TCF target genes include *cyclin D1* and *c-myc*, whose increased expression may enhance cell cycle progression, increase the proliferation of LMP2A-expressing cells and contribute to the pathogenicity of EBV-positive epithelial cell malignancies (189, 190) (Figure 1.7). However, whether the enhanced TCF-promoter activity induced by LMP2A has such an effect has yet to be examined.

1.4 Hypothesis and Objectives

LMP2A is able to insert itself into host cell signalling pathways to inhibit normal BCR signalling and initiate pro-survival and migratory signals. Thus, understanding how LMP2A uses cellular tyrosine kinases to initiate these signals may prove beneficial to understanding the role of EBV in cancer pathogenesis. It has been appreciated for some time that LMP2A recruits the tyrosine kinases Lyn and Syk in B cells, however, recent data published by Lu et al. (128) suggest that our current understanding of how LMP2A

uses these kinases to initiate signalling (125) needs to be revised. Therefore, we set out to further characterize the requirement of the LMP2A signalling motifs and the cellular tyrosine kinases, Lyn and Syk, for the initiation and propagation of LMP2A signals. We first used LMP2A point mutants to determine whether the SFK-binding YEEA site was required for LMP2A to recruit and activate Syk in B cells. We then assessed the relative contribution of Lyn to the initiation of LMP2A signalling by examining its requirement for LMP2A-mediated Syk activation by using siRNA to reduce Lyn levels in human B cells and a Lyn-deficient DT40 chicken B cell line. Finally, we reduced Lyn levels in B cells using siRNA and evaluated the role of Lyn in downstream LMP2A-mediated Akt activation. These experiments were carried out to test our hypothesis that LMP2A can initiate and propagate signals independently of Lyn in B cells. We found that the YEEA site was not absolutely required for tyrosine phosphorylation of the LMP2A ITAM, or for LMP2A to activate Syk. Using siRNA to silence Lyn expression in LCLs, we found Lyn was required for LMP2A to promote Syk tyrosine phosphorylation. In contrast, DG75 B cells or Lyn-deficient DT40 B cells transiently expressing higher levels of LMP2A did not require Lyn for LMP2A-mediated Syk phosphorylation. Lyn was also not required for LMP2A-mediated Akt activation in DG75 B cells, rather Akt activation was significantly enhanced in LMP2A-expressing cells where Lyn was reduced. We provide evidence that Lyn likely inhibits LMP2A-mediated Akt activation by phosphorylating Syk on Y323, which recruits the c-Cbl E3 ubiquitin ligase to Syk and targets Syk for degradation. This work demonstrates that the requirements for LMP2A to initiate signalling in B cells correlate with the amount of LMP2A expressed. It also reveals a novel role for Lyn in limiting the magnitude of LMP2A signalling.

Chapter 2: Materials and Methods

2.1 Antibodies, constructs, siRNA and other reagents

The anti-Flag M2 monoclonal antibody (mAb) was purchased from Sigma-Aldrich (Oakville, ON). The anti-phosphotyrosine mAbs, PY20 and PY99, and the anti-c-Cbl polyclonal antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The 4G10 anti-phosphotyrosine mAb was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The mouse mAb to Syk (4D10.1) was obtained from Abcam (Cambridge, MA). Polyclonal antibodies to Akt and pSyk (Y323), as well as the mAb against pAkt (S473), were purchased from Cell Signalling Technology (Danvers, MA). The anti-Lyn mAb antibody was obtained from BD Biosciences (Mississauga, ON) and the anti-LMP2A rat mAb, 14B7, was purchased from ITN GmbH (Neuherberg, Germany). Human Lyn, Syk and non-targeting (control) siGENOME SMARTpool siRNA oligonucleotides were obtained from Dharmacon RNA Technology (Lafayette, CO). The Src family kinase inhibitor, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1) was purchased from BioMol (Plymouth Meeting, PA) and its inactive analog 4-Amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3) was purchased from Calbiochem (San Diego, CA).

4xFlag-mutant LMP2A constructs have been previously described (126). The 4xFlag constructs were generated by cloning singly Flag-tagged LMP2A constructs into the pCMV 3xFlag vector (Sigma-Aldrich). The resulting proteins consist of three Flag epitopes, a spacer of 14 amino acids, an additional Flag epitope, and a spacer of 6 amino acids, followed by the LMP2A protein. Hemagglutinin (HA) tagged human Syk in

pBluescript II was a gift from Dr. Andre Veillette (IRCM, Montreal, QC). A eukaryotic expression vector was generated by removing HA-Syk from pBluescript II by digestion with *EcoRI/Sal I* (New England Biolabs, Ipswich, MA) and cloning it into the pcDNA3 mammalian expression vector (Invitrogen, Burlington, ON) which had been digested with *EcoRI/XhoI* (Invitrogen). The synthesis and sequence of the biotinylated pITAM and pYEEA peptides have been previously described (133). The GST-Syk tandem SH2, GST-c-Cbl N-terminus and GST-c-Cbl N-terminus G306E constructs were obtained from Dr. Tony Pawson (SLRI, Toronto, ON).

2.2 Cell lines and tissue culture

The DG75, BJAB, and Daudi human B cell lines as well as the LCLs (a gift from Dr. Giovanna Tosato, NIH, Bethesda, MD) were maintained in RPMI 1640 media (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 2mM L-glutamine (Invitrogen), 50μM 2-mercaptoethanol (Sigma-Aldrich), and 1mM sodium pyruvate (Sigma-Aldrich). 293T cells were grown in Dulbecco Modified Eagle's Medium (DMEM) (Invitrogen) supplemented as described above. DT40 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 1% chicken serum (Sigma-Aldrich), 4mM L-glutamine, and 50μM 2-mercaptoethanol. The BOSC 23 293T cells were transfected as previously described with an LMP2A cDNA cloned into the pMX-pie retroviral expression vector (191). A stable, bulk population of LMP2A-expressing cells was obtained by selecting the cells in media containing 0.5μg/ml puromycin (Sigma-Aldrich).

2.3 Purification of GST fusion proteins

GST and GST fusion proteins were purified from BL21 *E. coli* cell lysates by incubating 1ml of cleared bacterial lysate with 100μl of packed Glutathione-Agarose beads (Sigma-Aldrich) on a nutator at 4°C for 1hr. Beads were collected by centrifugation at ~950x*g* and the lysate was re-extracted as described above. Beads were then combined and washed three times with phosphate-buffered saline (PBS). Bound proteins were eluted from the beads by incubation in 1ml 50mM Tris Base (Sigma-Aldrich), 25mM reduced glutathione (Sigma-Aldrich) for 2 min at 37°C. Eluted proteins were then dialyzed in PBS at 4°C overnight. The integrity and quantity of purified fusion proteins were estimated by comparing the fusion proteins to BSA standards of known concentration on Coomassie Blue-stained SDS-PAGE gels. Glycerol was added to the fusion proteins to a final concentration of 50% before they were stored at -20°C.

2.4 Transfections

DG75, BJAB, and Daudi B cells were transfected by electroporation using a BTX ECM 830 square wave electroporator (BTX, San Diego, CA) with three 8ms pulses of 225V spaced at 1s intervals. For DG75 and BJAB cells, $1x10^7$ cells were transfected with 10μg or 15μg of DNA, respectively, and for Daudi cells, $1.5x10^7$ cells were transfected with 20μg of DNA. After electroporation, cells sat for 5min at room temperature before being resuspended in 10ml fresh RPMI 1640 media. Cells were then incubated in a 37°C, 5% CO_2 incubator for 24hrs. $2x10^7$ or $4x10^7$ DT40 chicken B cells were co-transfected as described above, with the indicated 4xFlag-LMP2A plasmid DNA (30μg or 50μg, respectively) and HA-Syk plasmid DNA (20μg or 30μg, respectively).

An equivalent amount of 3xFlag-pCMV and pcDNA3 were used in the controls lacking 4xFlag-LMP2A or HA-Syk plasmid, respectively. After electroporation, cells sat for 5min at room temperature before being resuspended in 30ml fresh RPMI 1640 media. Cells were then incubated in a 37°C, 5% CO_2 incubator for 24hrs.

A calcium phosphate transfection protocol was employed for transfection of 293T cells. The night before transfection, cells were split 1:5 in $10cm^2$ plates. For each plate of 293T cells to be transfected, 5μg of plasmid DNA was pipetted into a 17x100mm polystyrene tube to which 800μl of sterile 250mM $CaCl_2$ was added. To this mixture 800μl of sterile HEPES-buffered saline (HBS) (50mM HEPES, pH 7.05, 10mM KCl, 12mM dextrose, 280mM NaCl, 1.5mM Na_2HPO_4-$7H_2O$) was added dropwise over 10 seconds while vortexing at medium speed. The mixture was then vortexed for an additional 10 seconds. The media was then aspirated from the cells and replaced with 4ml fresh DMEM to which the DNA/$CaCl_2$/HBS mixture was added dropwise. Plates were gently rocked to disperse the DNA-$CaPO_4$ precipitate evenly over the cells before being incubated at 37°C, 5% CO_2 for ~8hrs. The media containing the DNA-$CaPO_4$ precipitate was then aspirated from the cells and replaced with 10ml fresh DMEM and cells were incubated at 37°C, 5% CO_2 for 24hrs.

2.5 siRNA transfection experiments

DG75 cells and LCLs were transfected using the electroporation conditions described above. $4x10^6$ DG75 cells were co-transfected with 4xFlag-PY^--LMP2A plasmid DNA (4μg) and either Lyn (1.4μM final concentration) or Syk (600nM final concentration) siRNA oligonucleotides (Dharmacon), whereas LCLs were transfected

with siRNA alone. An equivalent amount of 3xFlag-pCMV and non-silencing siRNA (Dharmacon) were used in the controls. In experiments where the effects of Lyn and Syk siRNA were being compared to one another, the total amount of siRNA in each sample was made equivalent by the addition of control siRNA. After electroporation, cells sat for 5min at room temperature before being resuspended in 10ml fresh RPMI 1640 media. Cells were then incubated in a 37°C, 5% CO_2 incubator for 48hrs.

2.6 Cell lysis, immunoprecipitations and GST pulldowns

Transfected cells were collected by centrifugation at ~1500x*g* for 5 min at 4°C. Cells were then lysed in 500μl 1% Nonidet P-40 (NP-40) lysis buffer (1% Nonidet P-40, 50mM Tris pH 7.4, 150mM NaCl, 2mM EDTA, 10% glycerol, protease inhibitor cocktail (Sigma-Aldrich), 1mM sodium orthovanadate, and 1mM phenylmethylsulfonyl fluoride) on a nutator for 10min at 4°C. Lysates were cleared of detergent-insoluble material by centrifugation at ~21,000x*g* for 10min at 4°C. For experiments involving SFK inhibition with PP1, DG75 cells transfected with 3xFlag-pCMV or 4xFlag-LMP2A were treated with 1μM, 10μM or 50μM PP1, or with 10μM PP3 as a negative control, at 37°C for 30min prior to lysis. Cleared lysates were incubated with 1-2 μg of antibody, as indicated in the figure legends, and 10μl packed Protein G-Sepharose beads (Sigma-Aldrich) on a rocker for 1hr at 4°C. Immunoprecipitates were then washed three times with 500μl 1% NP-40 lysis buffer and bound proteins were eluted by boiling in SDS-PAGE sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 125mM Tris pH 6.8).

For precipitation of GST fusion proteins with biotinylated phosphopeptides, 10μl of Streptavidin-Agarose beads (Sigma-Aldrich) were incubated with ~3nmol biotinylated phosphopeptide in 500μl 1% NP-40 lysis buffer supplemented with 2mM dithiothreitol (DTT) on a nutator for 1hr at 4°C. After incubation, peptide-coated beads were washed three times with 500μl 1x NEB Buffer 3 (New England Biolabs, Ipswich, MA). Dephosphorylation of the phosphorylated ITAM peptide was achieved by incubating phosphopeptide-coated beads with 2 units of calf intestinal phosphatase (CIP) (New England Biolabs) for 30min at 37°C, after which, an additional 2 units of CIP was added, and the reaction was allowed to proceed at 37°C for an additional 30min. Beads were then washed three times with 500μl 1% NP-40 lysis buffer, before being incubated with 2 μg GST fusion protein in 200μl of 1% NP-40 lysis buffer on a nutator for 1hr at 4°C. Beads were again washed three times with 500μl 1% NP-40 lysis buffer and precipitated proteins were eluted by boiling in SDS-PAGE sample buffer.

For pulldowns using GST fusion proteins, cells were lysed as described above with the exception that the lysis buffer was supplemented with 2mM DTT. Cleared lysates were then incubated with 10μg of the indicated GST fusion protein and 10μl of Glutathione-Agarose beads (Sigma-Aldrich) on a nutator for 1hr at 4°C. Beads were then washed three times with 500μl of 1% NP-40 lysis buffer and bound proteins were eluted by boiling in SDS-PAGE sample buffer.

2.7 Western blotting

After boiling at 95°C for 5 min to elute proteins, samples were run on SDS-PAGE gels at 30mA constant current before being transferred to BioTrace nitrocellulose

membranes (Pall Corporation, Ann Arbor, MI) at 15V constant voltage for 30min using a semi-dry transfer apparatus (Bio-Rad, Mississauga, ON). Membranes were blocked in 5% skim milk powder, in Tris-buffered saline (TBS) containing 0.02% sodium azide. Blots were then incubated in primary antibody, at 1μg/ml in TBS containing 0.02% sodium azide, at 4°C overnight on a shaker. Blots were rinsed with shaking three times for 10min with TBS containing 0.05% Tween-20 (TBST) before incubation with either goat anti-mouse or goat anti-rabbit IgG horse radish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) (1:10,000 in TBS) at room temperature for 30 min on a shaker. Blots were again rinsed with shaking three times for 10min in TBST before being exposed to the chemiluminescent substrate, Supersignal (Pierce, Rockford, IL). Bands were visualized by exposing the membranes to autoradiography film (Interscience, Markham ON). Blots were stripped with TBS pH 2.0 for 30 min at room temperature before being reprobed as described above. For quantification of Akt activation, western blots were preformed as described above, with the exception that blots were incubated with shaking with a IR Dye 700DX-conjugated goat anti-rabbit secondary antibody (Rockland Immunochemicals, Gilbertsville, PA) (1:20,000 in TBS) at room temperature for 1hr. Blots were then rinsed with shaking three times for 10min in TBST, and fluorescence intensities were determined using a LI-COR Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE). Data was analyzed with Microsoft Excel Software.

Chapter 3: Results

3.1 LMP2A-mediated tyrosine phosphorylation of Syk is independent of the LMP2A YEEA motif when LMP2A is transiently expressed in epithelial and B cells

Since LMP2A initiates signals that promote the migration and survival of EBV-positive malignancies, it is important to understand how LMP2A co-opts cellular tyrosine kinases to initiate these signals. LMP2A's ability to initiate signalling was originally thought to proceed via a two step mechanism whereby recruitment of Lyn to the YEEA site was required for tyrosine phosphorylation of the LMP2A ITAM, which allowed the subsequent recruitment and activation of Syk and the initiation of downstream signalling (125). The YEEA motif and Lyn were argued to be required for LMP2A to recruit and tyrosine phosphorylate Syk in LCLs (125), whereas in 293 epithelial cells this was found to be YEEA-motif independent (128). Therefore, we wanted to clarify the role of the YEEA motif and Lyn in initiating LMP2A signalling in B lymphocytes. To this end, the DG75 human B cell line was transiently transfected with cDNAs encoding for a quadruply Flag-tagged LMP2A (4x Flag-LMP2A) or several mutant LMP2A proteins. These mutants included one in which tyrosine 112 within the YEEA site had been mutated to phenylalanine (4x Flag-LMP2A Y112F) to disrupt Lyn binding (126), and one in which the two tyrosine residues within the ITAM were mutated to phenylalanine (4x Flag-LMP2A Y74/85F) to abolish Syk binding (126). Immunoprecipitation of Syk followed by immunoblotting with an anti-phosphotyrosine antibody showed that Syk was inducibly tyrosine phosphorylated in LMP2A-expressing DG75 cells (Figure 3.1A). It

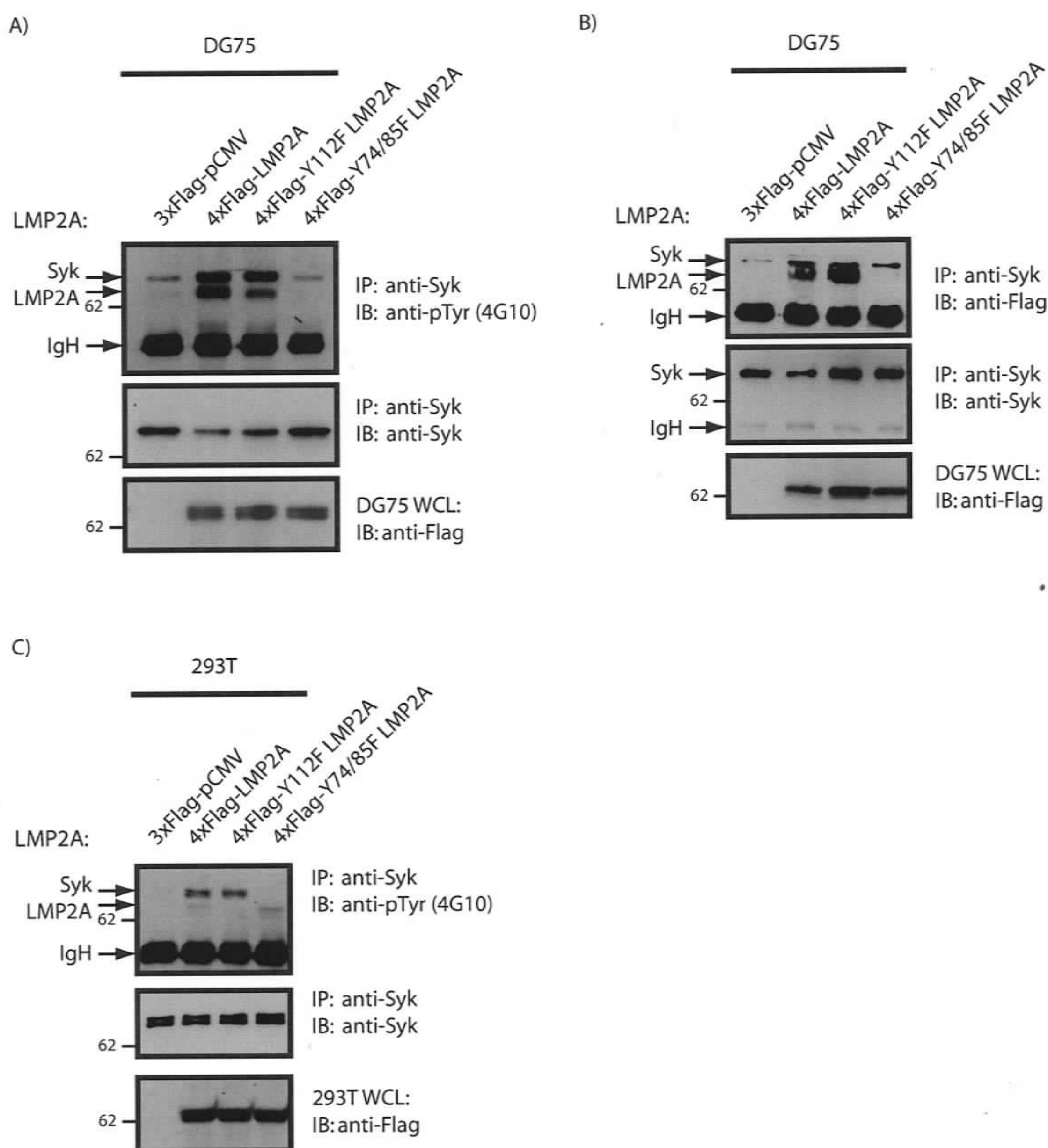

Figure 3.1 - LMP2A-mediated tyrosine phosphorylation of Syk is independent of the YEEA site in transiently transfected epithelial and lymphoid cells. ***A&B***, DG75 cells were transfected with plasmids encoding for 4xFlag-LMP2A or the indicated point mutants. Cell lysates were immunoprecipitated (IP) with the anti-Syk mAb and immunoprecipitated proteins were analyzed by immunoblotting (IB) with the 4G10 anti-pTyr antibody ***(A)*** or the anti-Flag M2 mAb ***(B)***. Blots were then stripped and reprobed with the anti-Syk mAb. ***C***, 293T cells were transfected, immunoprecipitated and immunoblotted as described in ***A***. Whole cell lysate (WCL) was included to show the level of Flag-tagged LMP2A in the respective lysates. Molecular mass markers (in kDa) are indicated to the left of the blots.

should be noted here that overall tyrosine phosphorylation of Syk correlates with its activation, allowing us to use Syk tyrosine phosphorylation as a surrogate measure for activation (128). Mutation of the ITAM, but not the YEEA site, abolished the ability of LMP2A to induce Syk tyrosine phosphorylation in these cells (Figure 3.1A). Moreover, we found that the Y112F mutant co-precipitated with Syk equivalently to the wild type LMP2A protein in DG75 B cells, whereas the ITAM mutant did not co-precipitate with Syk (Figure 3.1B). Similarly as reported by Lu et al. in 293 cells (128), we also found that in the 293T epithelial cell line Syk tyrosine phosphorylation was induced in a YEEA-independent manner (Figure 3.1C).

To show that the Y112F mutant's ability to utilize Syk in B cells is not restricted to DG75 cells, LMP2A-mediated Syk tyrosine phosphorylation was examined in the Daudi and BJAB human B cell lines. Daudi cells were transfected with 4xFlag PY^- LMP2A constructs, which have mutations in the two PY motifs (4xFlag-PY- LMP2A) and the YEEA motif and ITAM (4xFlag-PY-/Y112F LMP2A and 4xFlag-PY-/Y74/85F LMP2A, respectively) rendering them unable to associate with NEDD4 E3 ubiquitin ligases (126). These mutants were used because they are expressed at a higher level and are hyperphosphorylated allowing for increased expression in this difficult to transfect cell line. In these two cell lines we also found that LMP2A-mediated Syk tyrosine phosphorylation was dependent on the LMP2A ITAM, but independent of the YEEA site (Figures 3.2A and B respectively). Thus, our results demonstrate that when transiently expressed in B and epithelial cells, LMP2A-mediated Syk tyrosine phosphorylation occurs via an ITAM-dependent mechanism that does not require the YEEA motif.

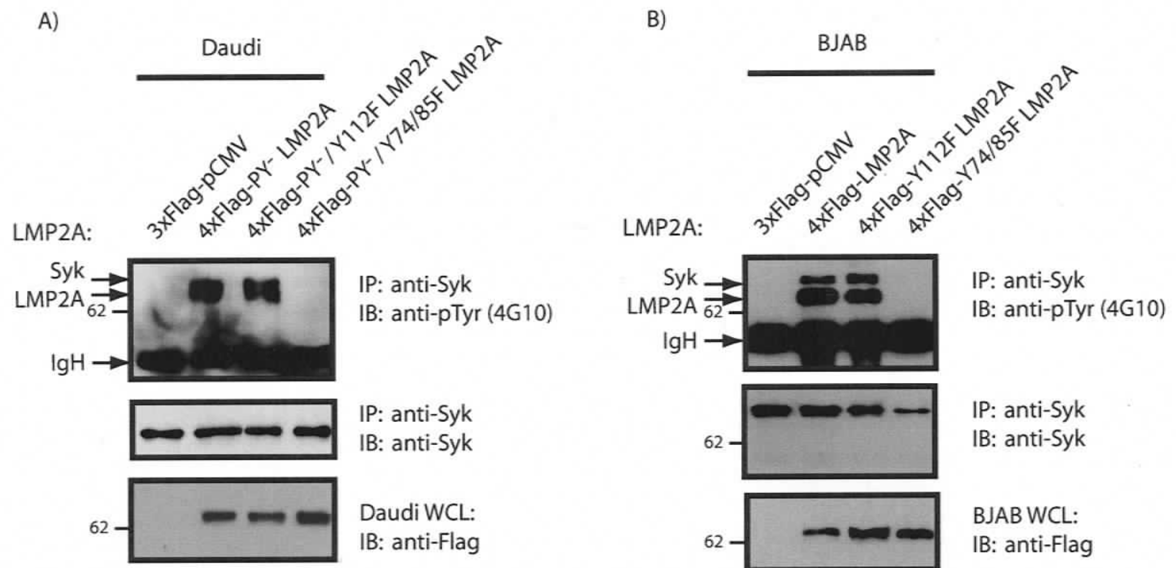

Figure 3.2 - LMP2A-mediated tyrosine phosphorylation of Syk is independent of the YEEA site in multiple transiently transfected B cell lines. ***A&B***, Daudi and BJAB cells were transfected with plasmids encoding for the indicated 4xFlag-LMP2A proteins. Cell lysates were immunoprecipitated (IP) with the anti-Syk mAb and immunoprecipitated proteins were analyzed by immunoblotting (IB) with the 4G10 anti-pTyr antibody. Blots were stripped and reprobed with the anti-Syk mAb. Whole cell lysate (WCL) was included to show the level of Flag-tagged LMP2A in the respective lysates. Molecular mass markers (in kDa) are indicated to the left of the blots.

3.2 Phosphorylation of the LMP2A ITAM does not require the YEEA site in DG75 B cells

Despite not being required for recruitment of Syk to LMP2A or LMP2A-mediated Syk tyrosine phosphorylation, we found that the YEEA site was important for overall LMP2A tyrosine phosphorylation in DG75 B cells and 293T epithelial cells (Figures 3.3A and B respectively). However, the ability of the LMP2A Y112F mutant to recruit and tyrosine phosphorylate Syk suggested that the ITAM was tyrosine phosphorylated in this mutant despite the drastic reduction observed in overall tyrosine phosphorylation. To determine if the ITAM was tyrosine phosphorylated in the Y112F mutant, but simply not recognized by the 4G10 anti-phosphotyrosine antibody, we performed similar western blotting experiments using the PY99 and PY20 anti-phosphotyrosine antibodies and obtained similar results (Figure 3.3C and D respectively). These results suggested that either the ITAM was tyrosine phosphorylated at very low stoichiometry and/or that three anti-phosphotyrosine antibodies do not efficiently recognize the LMP2A ITAM when tyrosine phosphorylated.

We wanted to conclusively show that the ITAM of the Y112F mutant was phosphorylated in DG75 cells and thus developed a functional assay to assess ITAM phosphorylation. The tandem SH2 domains of Syk specifically bind the LMP2A ITAM when doubly phosphorylated on tyrosines 74 and 85 (76, 126, 128, 147). In support of this, we found that a GST fusion protein containing the tandem SH2 domains of Syk (GST-Syk SH2s) was precipitated by a biotinylated, doubly phosphorylated ITAM peptide (pITAM), but not by a biotinylated, phosphorylated YEEA peptide (pYEEA) (Figure 3.4A). This association was dependent on phosphorylation of the ITAM as pre-treating the peptide with CIP prevented the pITAM peptide from precipitating the GST-

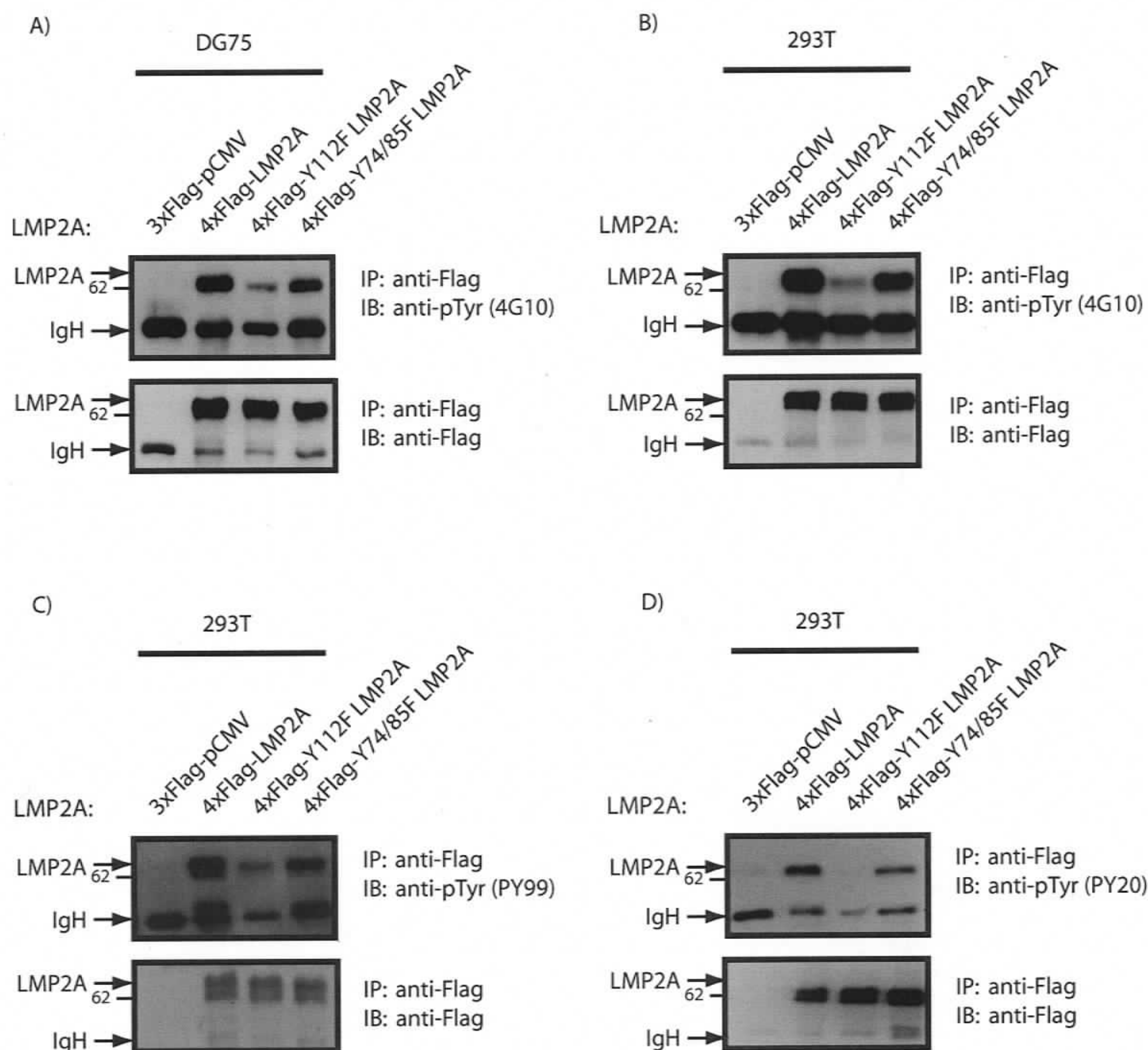

Figure 3.3 – Mutation of the YEEA motif decreases overall LMP2A tyrosine phosphorylation in transiently transfected epithelial and lymphoid cells. ***A&B***, DG75 and 293T cells were transfected with plasmids encoding for 4xFlag-LMP2A or the indicated point mutants. Flag-tagged LMP2A proteins were immunoprecipitated (IP) from cell lysates using the anti-Flag mAb and analyzed by immunoblotting (IB) with the 4G10 mAb or with the anti-Flag M2 mAb. ***C&D***, 293T cells were transfected and cell lysates immunoprecipitated as in ***A***. Immunoprecipitated proteins were analyzed by immunoblotting with the anti-phosphotyrosine antibodies PY99 (***C***) and PY20 (***D***) or with the anti-Flag M2 mAb. Molecular mass markers (in kDa) are indicated to the left of the blots.

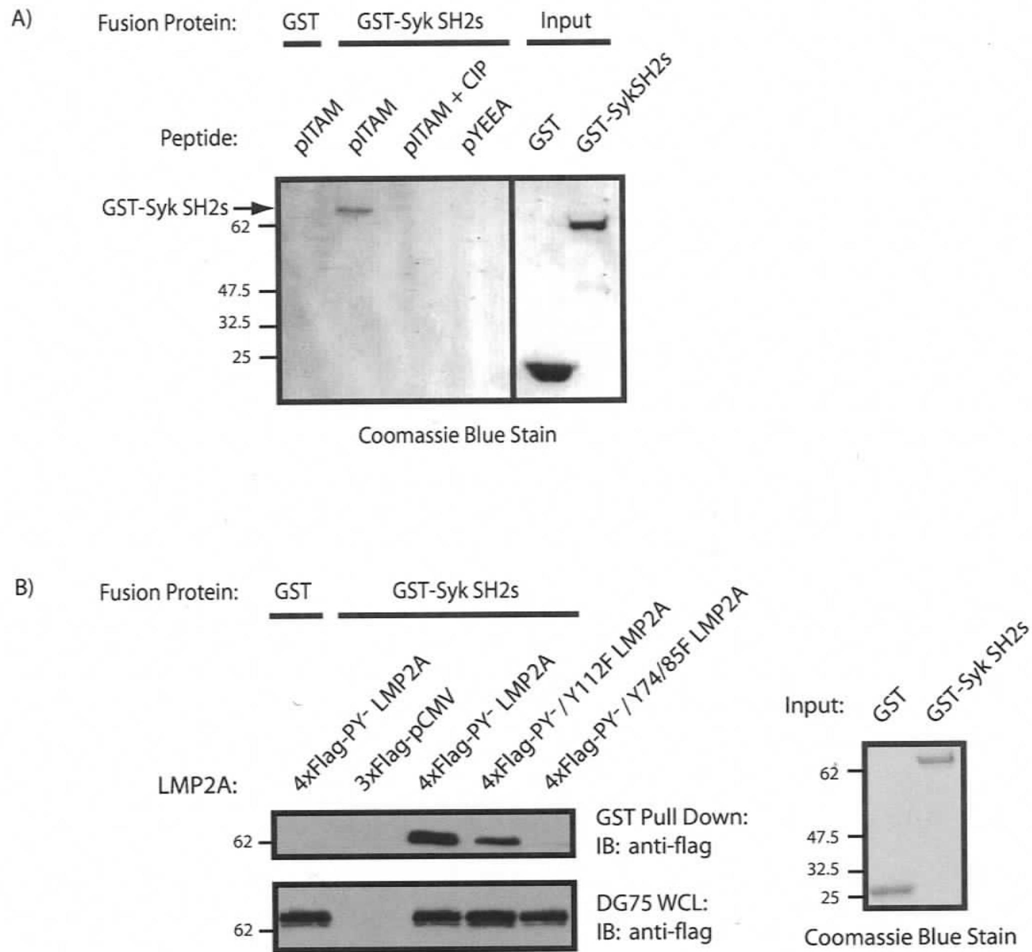

Figure 3.4 – Tyrosine phosphorylation of the LMP2A ITAM does not require the YEEA site in DG75 B cells. *A*, GST and GST-Syk SH2s fusion proteins were incubated with the indicated biotinylated peptides. In the pITAM+CIP lane, the pITAM peptide was pre-treated with calf intestinal phosphatase (CIP) to dephosphorylate the peptide prior to incubation with fusion protein. Precipitated proteins were run on SDS-PAGE gels and visualized by Coomassie Blue staining. Purified GST and GST-Syk SH2s fusion proteins show the amount of fusion protein added (Input). ***B***, DG75 cells were transfected with plasmids encoding for the indicated 4xFlag-LMP2A point mutants. The indicated GST fusion proteins were used to precipitate proteins from cell lysates and precipitated proteins were analyzed by immunoblotting (IB) with the anti-Flag mAb. Whole cell lysate (WCL) was included to show the level of Flag-tagged LMP2A in the respective lysates. Purified GST and GST-Syk SH2s fusion proteins were run on a SDS-PAGE gel and Coomassie Blue stained to show the amount of fusion protein added to the reactions (right panel). Molecular mass markers (in kDa) are indicated to the left of the blots.

Syk SH2 domains (Figure 3.4A). These results show that the GST-Syk SH2 domains can be used as a tool to assess the tyrosine phosphorylation status of the LMP2A ITAM.

The GST-Syk SH2 domains were then used to assess ITAM phosphorylation of LMP2A and LMP2A point mutants expressed in DG75 B cells. Figure 3.4B shows that GST-Syk SH2 domains can precipitate both LMP2A and Y112F mutant LMP2A from DG75 cell lysates, albeit the YEEA mutant was precipitated to a lesser extent than the wild type protein. Thus, our results demonstrate that the YEEA site is not required for tyrosine phosphorylation of the LMP2A ITAM in LMP2A-expressing DG75 cells, but it does enhance it. Our results also suggest the reduction in overall tyrosine phosphorylation observed for the LMP2A Y112F mutant is likely not due to a lack of ITAM phosphorylation, but rather the inability of the anti-phosphotyrosine antibodies we used to recognize the phosphorylated LMP2A ITAM.

3.3 Phosphorylation of the LMP2A ITAM and LMP2A-mediated Syk tyrosine phosphorylation are not dependent on Lyn in B cells transiently expressing LMP2A

The fact that the SFK binding-YEEA site did not appear to be required for LMP2A-mediated ITAM and Syk tyrosine phosphorylation suggested these events may be occurring independent of SFKs themselves. To address this, we next investigated whether SFKs were required for tyrosine phosphorylation of the LMP2A ITAM and LMP2A-mediated Syk activation.

As Lyn is the primary SFK recruited to LMP2A in B lymphocytes (124, 127), we wanted to begin by investigating whether Lyn was important for overall LMP2A tyrosine phosphorylation or for Syk recruitment and activation. We used siRNA to reduce Lyn

expression in DG75 B cells and examined LMP2A and Syk tyrosine phosphorylation. We found that significantly reducing both the p56 and p53 isoforms of Lyn, did not significantly alter overall tyrosine phosphorylation of the PY^- LMP2A mutant (Figure 3.5A). We also found that reducing Lyn levels did not affect overall LMP2A-dependent Syk tyrosine phosphorylation (Figure 3.5B) nor the ability of the GST-Syk SH2 domains to precipitate LMP2A from DG75 cell lysates (Figure 3.6A). Similarly, the ability of Syk and LMP2A to co-immunoprecipitate was unaffected by reducing Lyn expression with siRNA (Figure 3.6B). Taken together, these data are consistent with LMP2A recruiting and tyrosine phosphorylating Syk independently of Lyn.

Our siRNA experiments lead to a reduction in Lyn expression but did not completely eliminate it. This was especially true of the smaller p53 Lyn isoform (see Figure 3.5 and 3.6). Human B cells express multiple SFKs in addition to Lyn (192-194), and several including Lck, Fyn, and Src, are known to associate with LMP2A (124). Therefore, to rule out that our findings in Figure 3.5 and 3.6 were not due to incomplete silencing of Lyn, or the presence of additional SFKs, overall LMP2A and LMP2A ITAM tyrosine phosphorylation was assessed in LMP2A-expressing DG75 cells treated with the tyrosine kinase inhibitor PP1. PP1 is known to specifically inhibit SFKs at a concentration of 10μM (195). We first examined the effect of SFK inhibition on overall LMP2A tyrosine phosphorylation and found that PP1 treatment reduced overall LMP2A tyrosine phosphorylation in a dose dependent manner (Fig 3.7A). We then used our GST-Syk SH2 domains to precipitate LMP2A from DG75 cell lysates to examine the effect of SFK inhibition specifically on LMP2A ITAM phosphorylation. Treatment of LMP2A expressing cells with PP1 reduced the amount of LMP2A precipitated by the

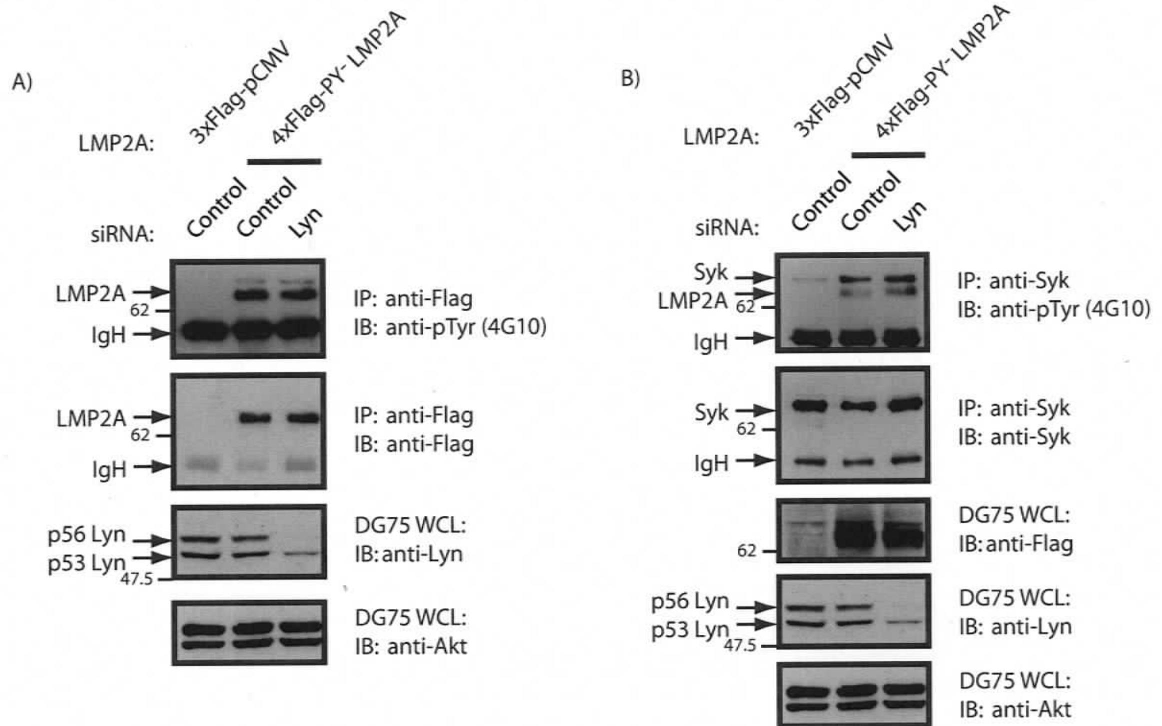

Figure 3.5 – Reducing Lyn levels does not impair LMP2A-mediated Syk tyrosine phosphorylation in DG75 B cells. ***A,*** DG75 cells were co-transfected with a plasmid encoding for the 4xFlag-PY$^-$ LMP2A point mutant or vector alone (3xFlag-pCMV) and the indicated siRNA oligonucleotides. Cell lysates were immunoprecipitated (IP) with the anti-Flag M2 mAb and precipitated proteins were analyzed by immunoblotting (IB) with the 4G10 anti-pTyr mAb or the anti-Flag M2 mAb. ***B,*** DG75 cells were transfected with the plasmids described in ***A***, and Syk was immunoprecipitated (IP) from cell lysates with the anti-Syk mAb. IPs were then immunoblotted (IB) with the 4G10 anti-pTyr mAb. The blot was then stripped and reprobed with the anti-Syk mAb. Whole cell lysates (WCL) were probed with the anti-Lyn mAb to demonstrate the efficacy of siRNA silencing, the anti-Flag mAb to show the expression of 4xFlag-LMP2A proteins and the anti-Akt antibody to demonstrate that overall protein levels in the lysates were equivalent. Molecular mass markers (in kDa) are indicated to the left of the blots.

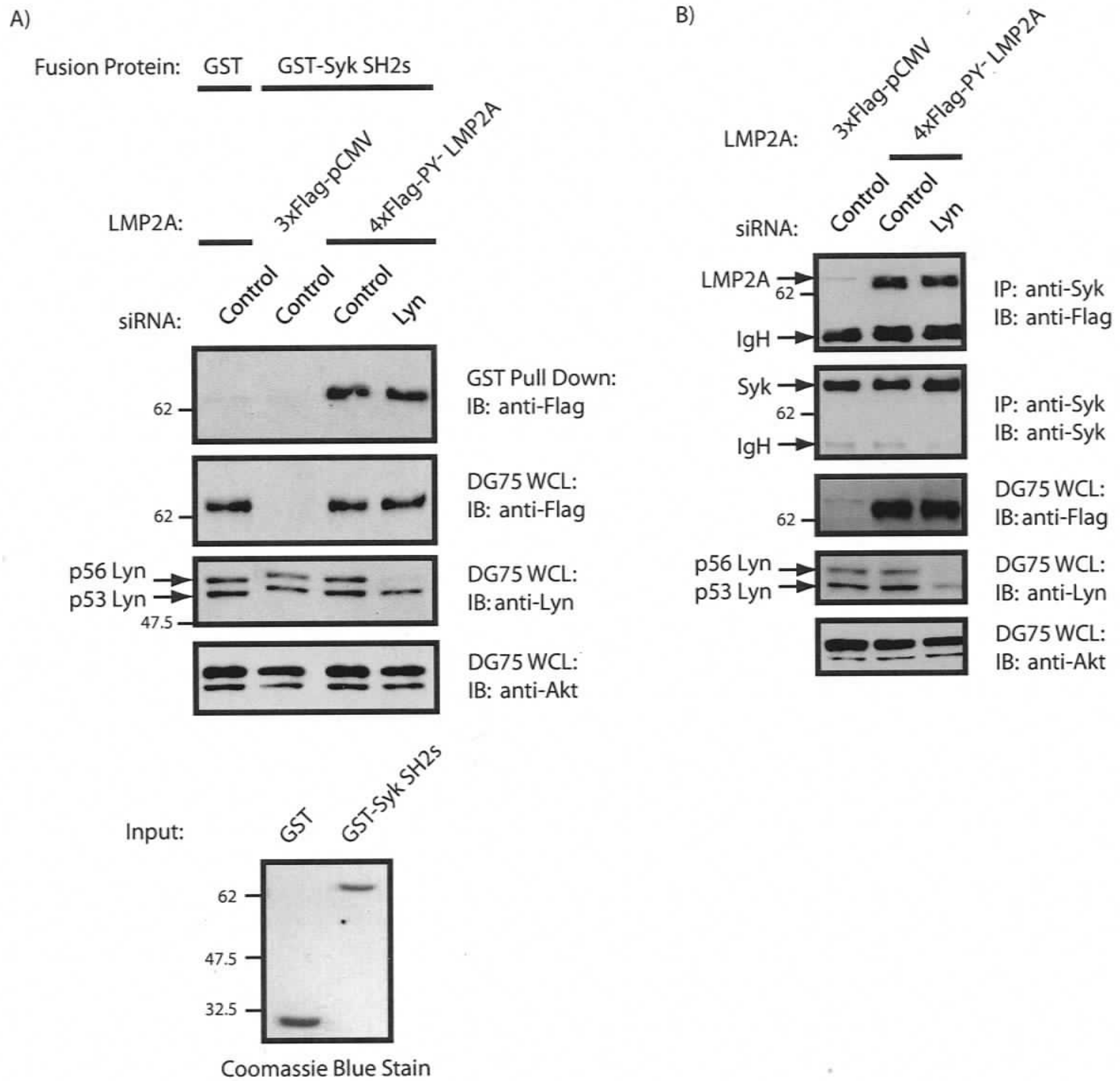

Figure 3.6 – Reducing Lyn levels does not impair tyrosine phosphorylation of the LMP2A ITAM in DG75 B cells. ***A,*** DG75 cells were co-transfected with a plasmid encoding for the 4xFlag-PY$^-$ LMP2A point mutant or vector alone (3xFlag-pCMV) and the indicated siRNA oligonucleotides. The indicated fusion proteins were used to precipitate 4xFlag-PY$^-$ LMP2A from cell lysates. Precipitated proteins were analyzed by immunoblotting with the anti-Flag mAb. Purified GST and GST-Syk SH2s fusion proteins were run on a SDS-PAGE gel and Coomassie Blue stained to indicate the amount of fusion protein added to the reactions (lower panel). ***B,*** DG75 cells were transfected with the plasmids described in ***A***, and Syk was immunoprecipitated (IP) from cell lysates with the anti-Syk mAb. IPs were then immunoblotted (IB) with the anti-Flag mAb before the blot was stripped and reprobed with the anti-Syk mAb. Whole cell lysates (WCL) were probed with the anti-Lyn mAb to demonstrate the efficacy of siRNA silencing, the anti-Flag mAb to show the expression of 4xFlag-LMP2A proteins and the anti-Akt antibody to demonstrate that overall protein levels in the lysates were equivalent. Molecular mass markers (in kDa) are indicated to the left of the blots.

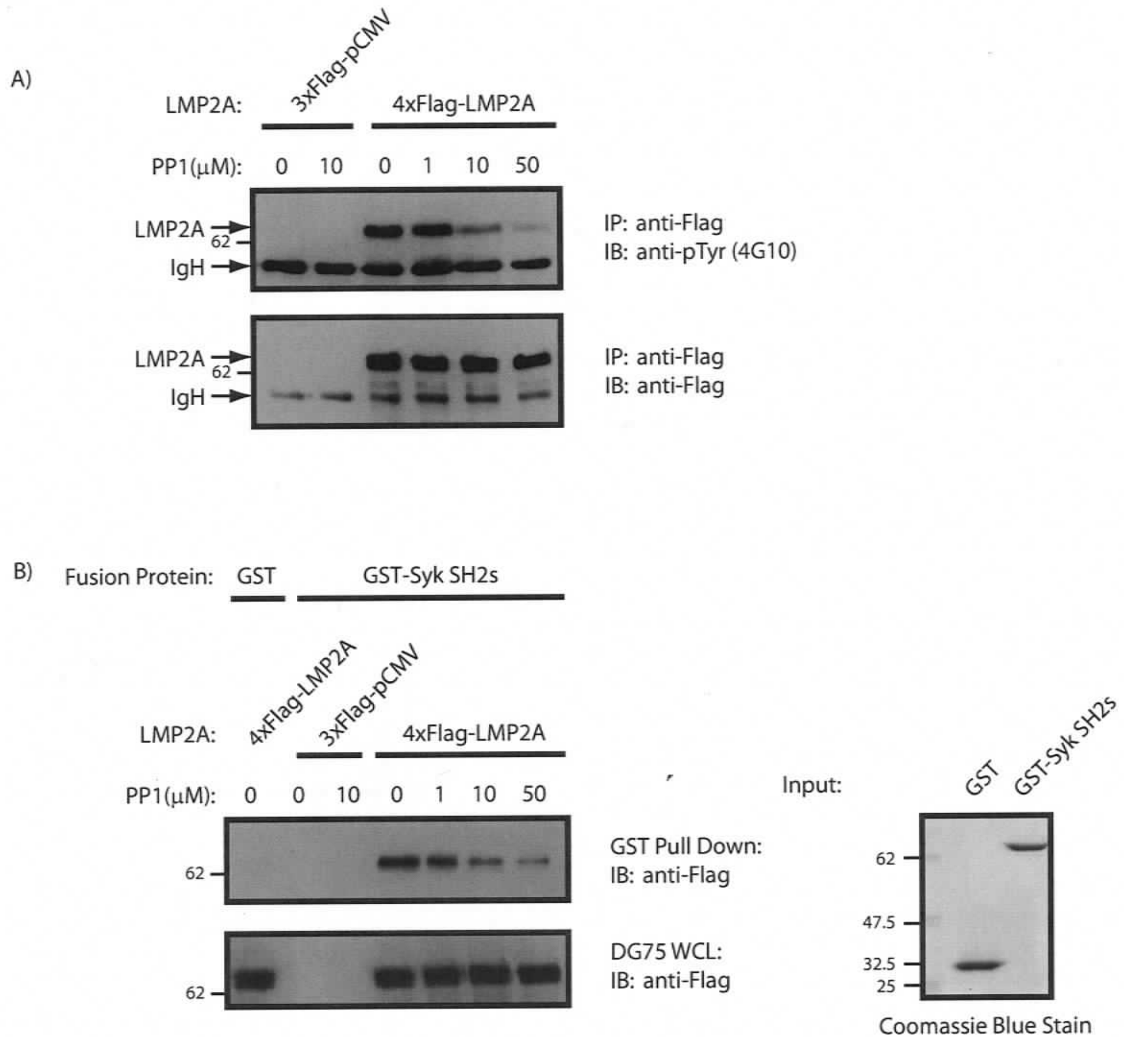

Figure 3.7 – The SFK inhibitor PP1 reduces overall LMP2A and LMP2A ITAM tyrosine phosphorylation in DG75 B cells. ***A,*** DG75 cells were transfected with plasmids encoding for 4xFlag-LMP2A and then treated with the indicated concentrations of PP1. Flag-tagged LMP2A proteins were immunoprecipitated (IP) from cell lysates using the anti-Flag mAb and analyzed by immunoblotting (IB) with the 4G10 mAb or with the anti-Flag M2 mAb. ***B,*** DG75 cells were transfected and PP1 treated and cell lysates immunoprecipitated as in ***A***. The indicated GST fusion proteins were used to precipitate proteins from cell lysates and precipitated proteins were analyzed by immunoblotting (IB) with the anti-Flag mAb. Whole cell lysate (WCL) was included to show the level of Flag-tagged LMP2A in the respective lysates. Purified GST and GST-Syk SH2s fusion proteins were run on a SDS-PAGE gel and Coomassie Blue stained to show the amount of fusion protein added to the reactions (right panel). Molecular mass markers (in kDa) are indicated to the left of the blots.

GST-Syk SH2 domains in a dose dependent manner (Fig 3.7B). These results suggest that other SFKs may be compensating for Lyn in our siRNA experiments, however, it should be noted that while overall LMP2A and LMP2A ITAM tyrosine phosphorylation were slightly reduced by PP1 treatment they were not completely abolished. Also, we could not rule out the possibility that PP1 may non-specifically inhibit other tyrosine kinases which may have affected the results of the above experiments.

To rule out that our findings in Figure 3.7 were not due to non-specific inhibition of other tyrosine kinases, we analyzed LMP2A-mediated Syk tyrosine phosphorylation in DT40 chicken B cells and DT40 cells rendered deficient in Lyn, the only SFK present in these cells (196). LMP2A was equivalently tyrosine phosphorylated in parental and Lyn-deficient DT40 cells (Figure 3.8A) so we examined how loss of Lyn affected the ability of Syk to be recruited to LMP2A and become tyrosine phosphorylated. To this end, we expressed 4xFlag-LMP2A and human HA-tagged Syk in DT40 and Lyn-deficient DT40 cells. Human Syk was used in these experiments as we lacked an antibody that recognized chicken Syk. We found that human Syk was tyrosine phosphorylated equivalently in both DT40 cells and Lyn-deficient DT40 cells expressing LMP2A (Figure 3.8B). Furthermore, the ability of LMP2A to recruit Syk was not compromised in the Lyn-deficient DT40 cells (Figure 3.8C) and, consistent with what we observed in human B cells, the LMP2A YEEA motif was not required for LMP2A to promote Syk tyrosine phosphorylation in DT40 cells (Figure 3.8D). Not unexpectedly, however, we found that the LMP2A ITAM was required to promote Syk tyrosine phosphorylation in these cells (Figure 3.8D). These data corroborate the results from our siRNA experiments and suggest the results of our PP1 experiments may be due to non-specific inhibition of other

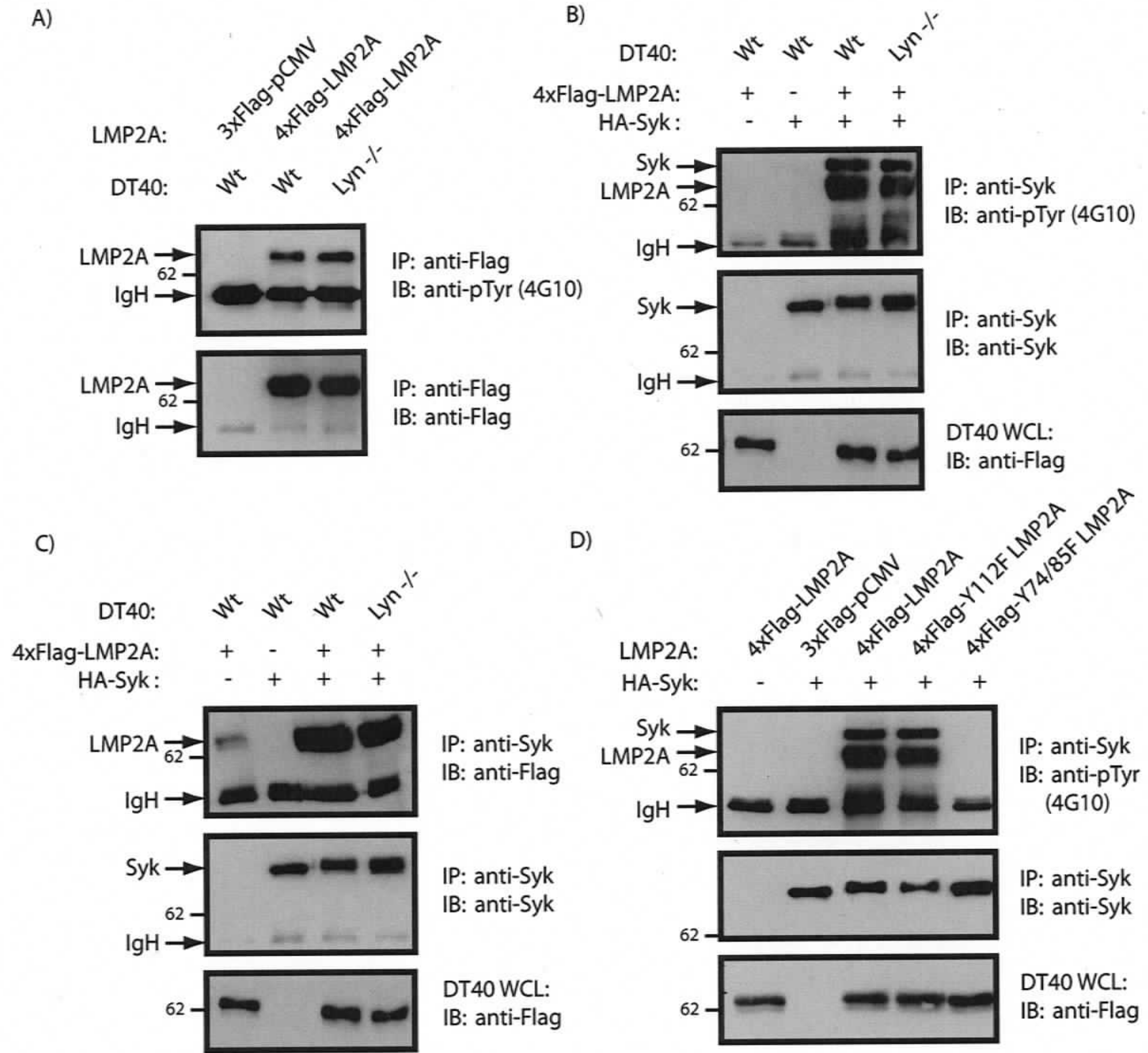

Figure 3.8 - LMP2A-mediated Syk recruitment and activation are independent of Lyn in DT40 chicken B cells. ***A,*** Wt or Lyn-/- DT40 cells were transfected with plasmid encoding 4xFlag-LMP2A. LMP2A was immunoprecipitated (IP) from cell lysates with the anti-Flag M2 mAb and precipitated proteins were analyzed by immunoblotting (IB) with the 4G10 anti-pTyr mAb or the anti-Flag M2 mAb. ***B,*** Cells described in ***A***, were co-transfected with (+) or without (-) plasmids encoding for 4xFlag-LMP2A or human HA-Syk. Syk was immunoprecipitated from cell lysates (IP) with the anti-Syk mAb and analyzed by immunoblotting (IB) the 4G10 mAb. The blot was stripped and reprobed with the anti-Syk mAb. ***C,*** Cells were transfected and Syk immunoprecipitated as described in ***B.*** An immunoblot (IB) of the immunoprecipitates was performed using the anti-Flag mAb. The blot was stripped and reprobed with the anti-Syk mAb. ***D,*** DT40 cells were co-transfected with a plasmid encoding for the indicated 4xFlag-LMP2A proteins or vector alone (3xFlag-pCMV) with (+) or without (-) human HA-Syk as indicated. Immunoprecipitations (IP) and immunoblots (IB) were preformed as in ***B.*** Lysates were probed with the anti-Flag mAb to indicate the level of 4xFlag-LMP2A protein expression in the each transfection. Molecular mass markers (in kDa) are indicated to the left of the blots.

tyrosine kinases in addition to SFKs. These results further demonstrate that Lyn is not required for LMP2A to recruit and activate Syk.

3.4 Lyn is required for LMP2A-mediated Syk tyrosine phosphorylation in LCLs

Our experiments examining the ability of LMP2A to promote Syk tyrosine phosphorylation in DG75 and DT40 cells relied on the transient expression of LMP2A using a CMV-based eukaryotic expression vector. We wanted to determine if Lyn was also required for LMP2A to promote Syk tyrosine phosphorylation when LMP2A expression was driven by its own viral promoter. To that end, we examined if Lyn was required for Syk tyrosine phosphorylation in LCLs, where Syk is highly tyrosine phosphorylated in a LMP2A-dependent manner (76, 77, 125). We found that reducing Lyn expression in two LCL lines resulted in a reduction in Syk tyrosine phosphorylation (Figure 3.9A). As well, LMP2A tyrosine phosphorylation was also reduced when Lyn was silenced in these cell lines (Figure 3.9B and data not shown). To determine if the observed differences in Syk tyrosine phosphorylation between DG75 cells and LCLs might be due to differences in LMP2A expression levels, we compared LMP2A expression in the two cell lines. Our results show that considerably more LMP2A was expressed in the transfected DG75 cells compared to the LCLs (Figure 3.9C). Thus, Lyn-dependent signalling is a characteristic of cells expressing lower levels of LMP2A, whereas cells expressing higher levels of LMP2A do not require Lyn to initiate signalling.

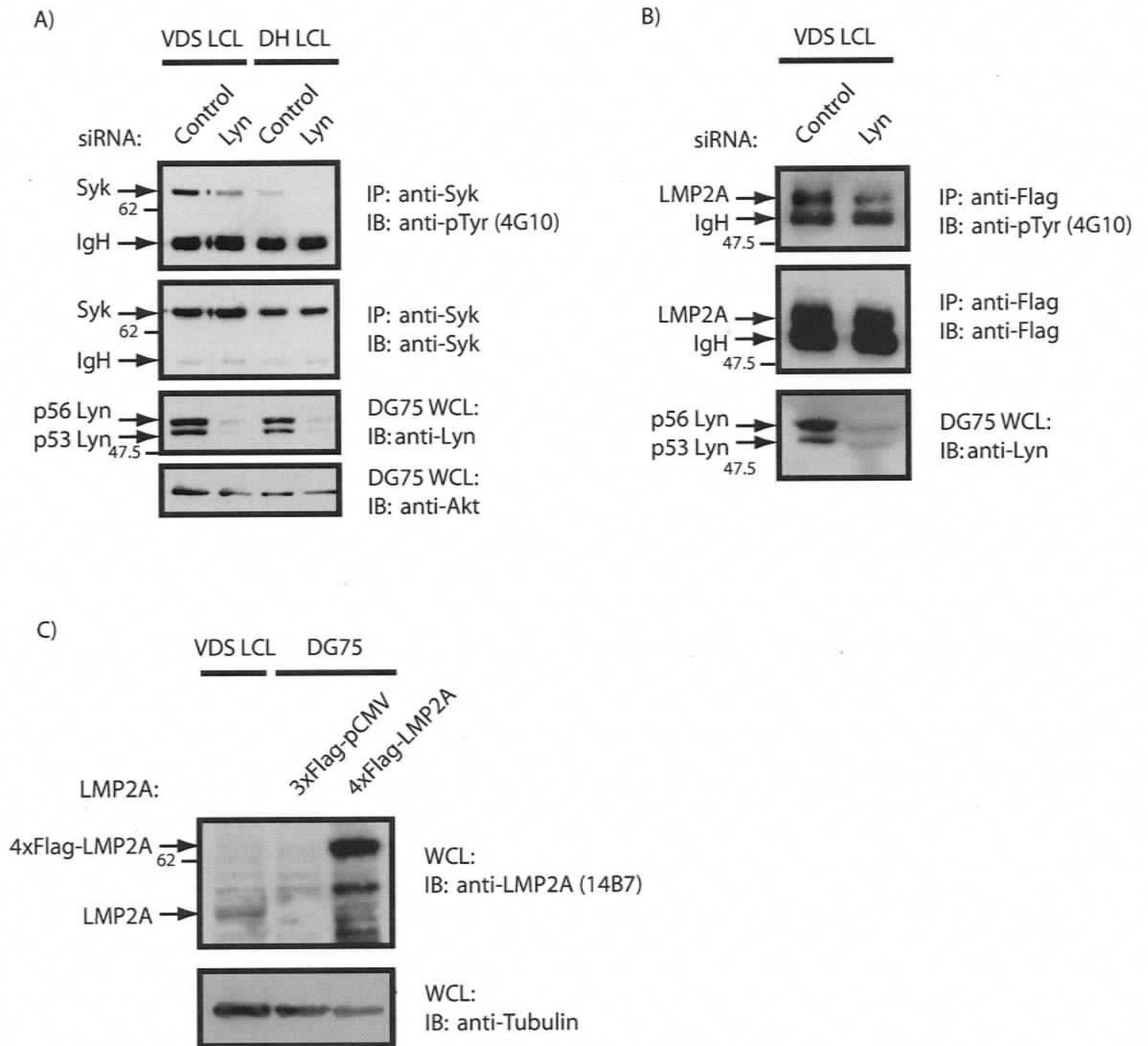

Figure 3.9 – Reducing Lyn levels inhibits Syk tyrosine phosphorylation in LCLs. ***A***, LCL lines, VDS and DH, were transfected with the indicated siRNA oligonucleotides. Cell lysates were immunoprecipitated (IP) with the anti-Syk mAb and precipitated proteins were analyzed by immunoblotting (IB) with the 4G10 anti-pTyr mAb. The blot was then stripped and reprobed with the anti-Syk mAb. Whole cell lysates (WCL) were probed with the anti-Lyn mAb to demonstrate the efficacy of siRNA silencing. ***B***, LCLs were transfected as described in ***A***, and LMP2A was immunoprecipitated (IP) from cell lysates with the anti-LMP2A mAb. IPs were then immunoblotted (IB) with the 4G10 anti-pTyr mAb or the anti-LMP2A mAb. Whole cell lysates (WCL) were probed with the anti-Lyn mAb to demonstrate the efficacy of siRNA silencing. ***C***, DG75 cells transfected with 4xFlag-LMP2A or vector or untransfected LCLs were lysed and equivalent amounts of whole cell lysate (WCL) were immunoblotted with the anti-LMP2A mAb. The blot was then reprobed with an anti-tubulin mAb to demonstrate that overall protein levels in the lysates were equivalent. Molecular mass markers (in kDa) are indicated to the left of the blots.

3.5 Lyn is not required for LMP2A-mediated Akt activation in DG75 B cells

Our data show that LMP2A can recruit Syk independently of the YEEA motif and Lyn in B cells transiently expressing LMP2A. The recruitment of Syk to LMP2A and its activation initiates downstream signalling pathways including those regulated by Akt (151, 154-159, 162, 163), Jnk (172), Erk (145, 172) and β-catenin (158, 159). In particular, the activation of Akt provides pro-survival signals to LMP2A-expressing transgenic B cells (161). Therefore, we examined whether LMP2A-mediated Akt activation was independent of Lyn using a phospho-specific antibody that recognizes active Akt (197). Akt was activated in DG75 B cells expressing the 4xFlag-PY$^-$ mutant LMP2A, and reducing Syk expression with siRNA significantly reduced LMP2A-mediated Akt activation in these cells (Figure 3.10A). In contrast, reducing Lyn levels significantly enhanced Akt activation in LMP2A-expressing cells (Figure 3.10A). The quantification of Akt activation in Figure 3.10A showed that expressing the PY$^-$ mutant LMP2A or reducing Lyn expression with siRNA on their own in DG75 cells, resulted in a 1.66-fold increase in Akt activation compared to control DG75 cells co-transfected with 3xFlag-pCMV and control siRNA (Figure 3.10B). However, co-transfection of cells with the PY$^-$ mutant LMP2A and Lyn siRNA resulted in Akt activation over four-fold higher than observed in control cells. Akt activation was also examined in DG75 B cells expressing 4xFlag-PY$^-$ LMP2A and 4xFlag-PY$^-$ LMP2A with additional mutations in the ITAM or YEEA motif (4xFlag-PY$^-$/Y112F LMP2A and 4xFlag-PY$^-$/Y74/85F LMP2A, respectively). We found that LMP2A-mediated Akt activation was ITAM and YEEA motif-dependent (Figures 3.11A and B) as was previously observed in LCLs (163). Taken together, these data show that Syk, the LMP2A ITAM and the LMP2A YEEA

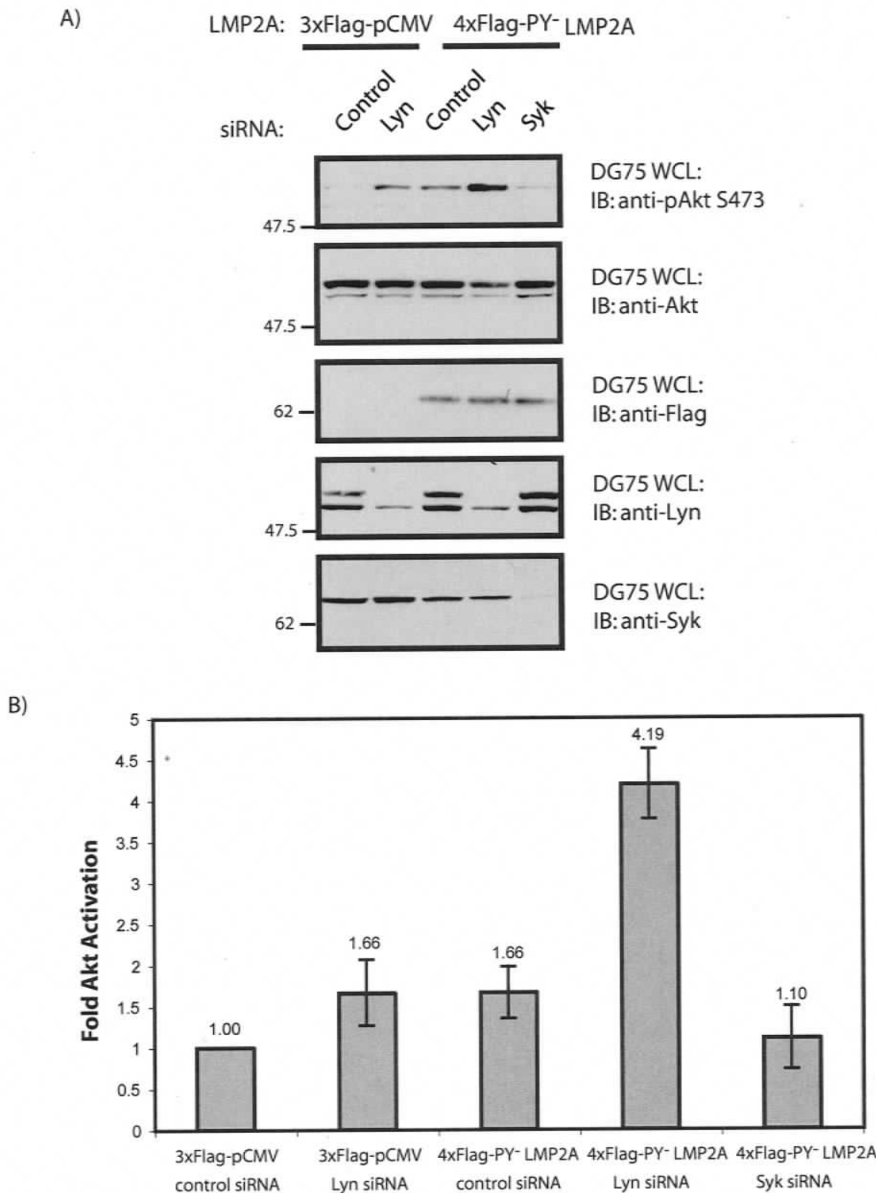

Figure 3.10 - Lyn negatively regulates LMP2A-mediated Akt activation in LMP2A-expressing B cells. ***A,*** DG75 cells were co-transfected with a plasmid encoding for the 4xFlag-PY⁻ LMP2A point mutant or vector alone (3xFlag-pCMV) and the indicated siRNA sequences. Activation of Akt was analyzed by immunoblotting (IB) whole cell lysates (WCL) with the anti-pAkt S473 mAb. The blot was stripped and reprobed to show Akt and 4xFlag-PY⁻LMP2A levels in the respective lysates. Lysates were also probed with the anti-Lyn or anti-Syk mAbs to demonstrate the efficacy of siRNA silencing. ***B,*** Lysates shown in ***A***, were rerun and Akt activation was quantified using a LI-COR Odyssey infrared imager. Akt activation was measured as the ratio of anti-pAkt S473/total Akt levels and expressed relative to the cells transfected with vector and control siRNA (3xFlag-pCMV; control siRNA). Error bars indicate the standard deviation of three independent experiments. Molecular mass markers (in kDa) are indicated to the left of the blots.

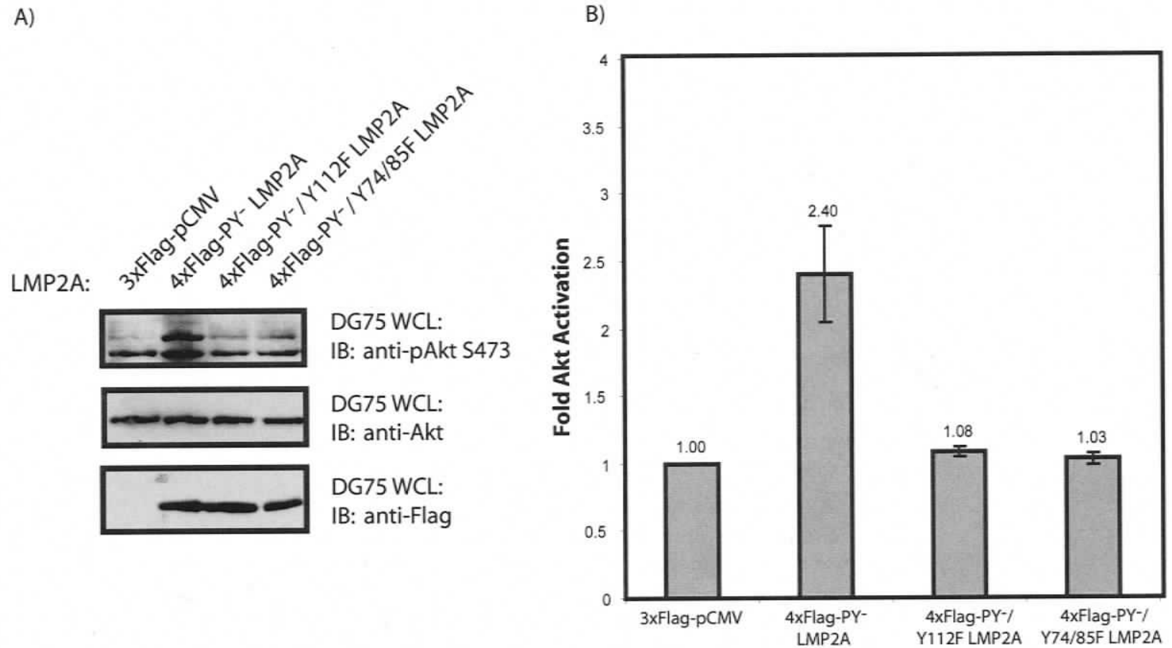

Figure 3.11 – Mutation of the YEEA motif abolishes LMP2A-mediated Akt activation. ***A,*** DG75 cells were transfected with constructs encoding for 4xFlag-PY$^-$ LMP2A or the indicated mutants. Akt activation was analyzed by immunoblotting (IB) whole cell lysates (WCL) with the anti-pAkt S473 mAb. The blot was stripped and reprobed with the anti-Akt polyclonal antibody and the anti-Flag M2 mAb to show the levels of Akt and 4xFlag PY$^-$ LMP2A in the respective lysates. Molecular mass markers (in kDa) are indicated to the left of the blots. ***B,*** The lysates shown in ***A***, were rerun and Akt activation of was quantified using a LI-COR Odyssey infrared imager. Akt activation was measured as a ratio of anti-pAkt S473/total Akt levels and expressed relative to the cells transfected with vector alone (3xFlag-pCMV). The error bars indicate the standard deviation of three independent experiments and the numbers indicate the mean fold Akt activation.

motif are required for LMP2A-mediated Akt activation. Moreover, our data show that Lyn is not required for, and likely inhibits, LMP2A-mediated Akt activation in DG75 B cells. We next sought to investigate the mechanism by which Lyn could limit Akt activation by LMP2A.

3.6 Lyn promotes phosphorylation of Syk on Y323 in LMP2A-expressing cells, which serves to recruit the c-Cbl E3 ubiquitin ligase to Syk

Lyn functions as both a positive and negative regulator of BCR signalling (reviewed in (198) and (199)). Lyn contributes to the initiation of BCR signalling by phosphorylating the ITAMs of the Igα and β chains of the BCR, thereby facilitating the recruitment of Syk. Lyn can also phosphorylate negative signalling receptors in B cells, including FcγRIIB and CD22. These receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that recruit signalling proteins, such as the SHIP inositol phosphatase and SHP-1 tyrosine phosphatase, that antagonize BCR signalling (reviewed in (200)). Lyn can also phosphorylate Syk within its linker region, between the kinase domain and the two SH2 domains, on Y323 (Y317 in mouse) (201). Phosphorylation of this tyrosine residue facilitates the recruitment of the E3 ubiquitin ligases c-Cbl and Cbl-b to Syk via their SH2-like domains. E3 ubiquitin ligases mark proteins for degradation with the polypeptide ubiquitin, and the recruitment of c-Cbl and Cbl-b to Syk facilitates its ubiquitination and targets it for degradation (202-204).

To investigate whether Lyn negatively regulated Syk in LMP2A-expressing cells, phosphorylation of Syk on Y323 was examined using a phospho-specific antibody. Syk was found to be phosphorylated on Y323 in DG75 B cells transiently expressing LMP2A and in 293T cells stably expressing LMP2A (Figures 3.12 A and B). Y323

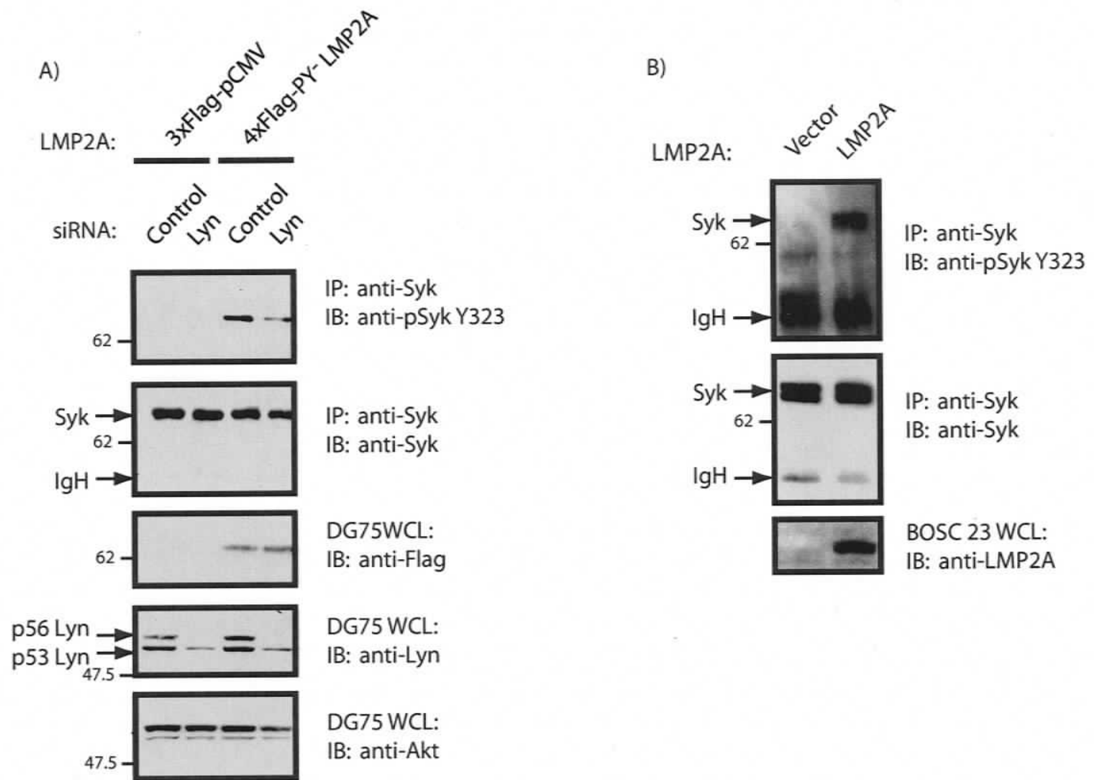

Figure 3.12 - Lyn phosphorylates Syk on Y323 in LMP2A-expressing cells. ***A,*** DG75 cells were co-transfected with a plasmid encoding for 4xFlag-PY⁻ LMP2A point mutant or vector alone (3xFlag-pCMV) and the indicated siRNA sequences. Phosphorylation of Syk on Y323 was analyzed by immunoblotting (IB) anti-Syk immunoprecipitates (IP) with the anti-pSyk Y323 mAb. The blot was stripped and reprobed with the anti-Syk mAb. Whole cell lysate (WCL) was probed with the anti-Flag M2 mAb to show the level of 4xFlag-PY⁻ LMP2A in the respective lysates and with the anti-Akt antibody to show that protein levels were equivalent. Whole cell lysate (WCL) was also probed and with the anti-Lyn mAb to demonstrate the efficacy of siRNA silencing. ***B,*** BOSC23 293T cells stably-expressing LMP2A were lysed and Syk Y323 phosphorylation was examined as described in ***A***. Whole cell lysate (WCL) was probed with the anti-LMP2A mAb to show expression of LMP2A in the respective lysates. Molecular mass markers (in kDa) are indicated to the left of the blots.

phosphorylation was reduced in LMP2A-expressing DG75 B cells in which Lyn levels were reduced by siRNA-treatment (Figure 3.12A). These results demonstrate that Lyn promotes the phosphorylation of Syk on a known negative regulatory site in LMP2A-expressing cells. This finding prompted us to examine whether c-Cbl was recruited to Syk in LMP2A-expressing cells.

To determine if c-Cbl could be recruited to Syk in LMP2A-expressing cells, we asked whether a GST fusion protein of the N-terminus of c-Cbl, that contains the SH2-like domain, was able to precipitate Syk from these cells. The GST-c-Cbl N-terminus could precipitate Syk and LMP2A from stable LMP2A-expressing, but not vector alone-transfected, 293T cells (Figures 3.13A) and from DG75 cells transiently transfected with 4xFlag-PY$^-$ LMP2A (Figure 3.13C). The ability of the GST-c-Cbl N-terminus to precipitate Syk and LMP2A was dependent on the SH2-like domain of c-Cbl, as a fusion protein with a mutation in the SH2-like domain (GST-c-Cbl G306E) (205) was unable to precipitate Syk or LMP2A (Figure 3.13A and C). In support of c-Cbl being recruited to Syk/LMP2A complexes, we found that c-Cbl was tyrosine phosphorylated in cells stably expressing LMP2A (Figure 3.13B) and DG75 cells transiently expressing LMP2A (Figure 3.13D). Furthermore, in DG75 cells this phosphorylation was ITAM-dependent (Figure 3.13D) which fits with our idea that c-Cbl is recruited to LMP2A via its interaction with Syk Y323. Taken together, these data are consistent with Lyn functioning as negative regulator of Syk in LMP2A-expressing cells by phosphorylating Y323 in the Syk linker region and facilitating the recruitment of the c-Cbl E3 ubiquitin ligase to Syk.

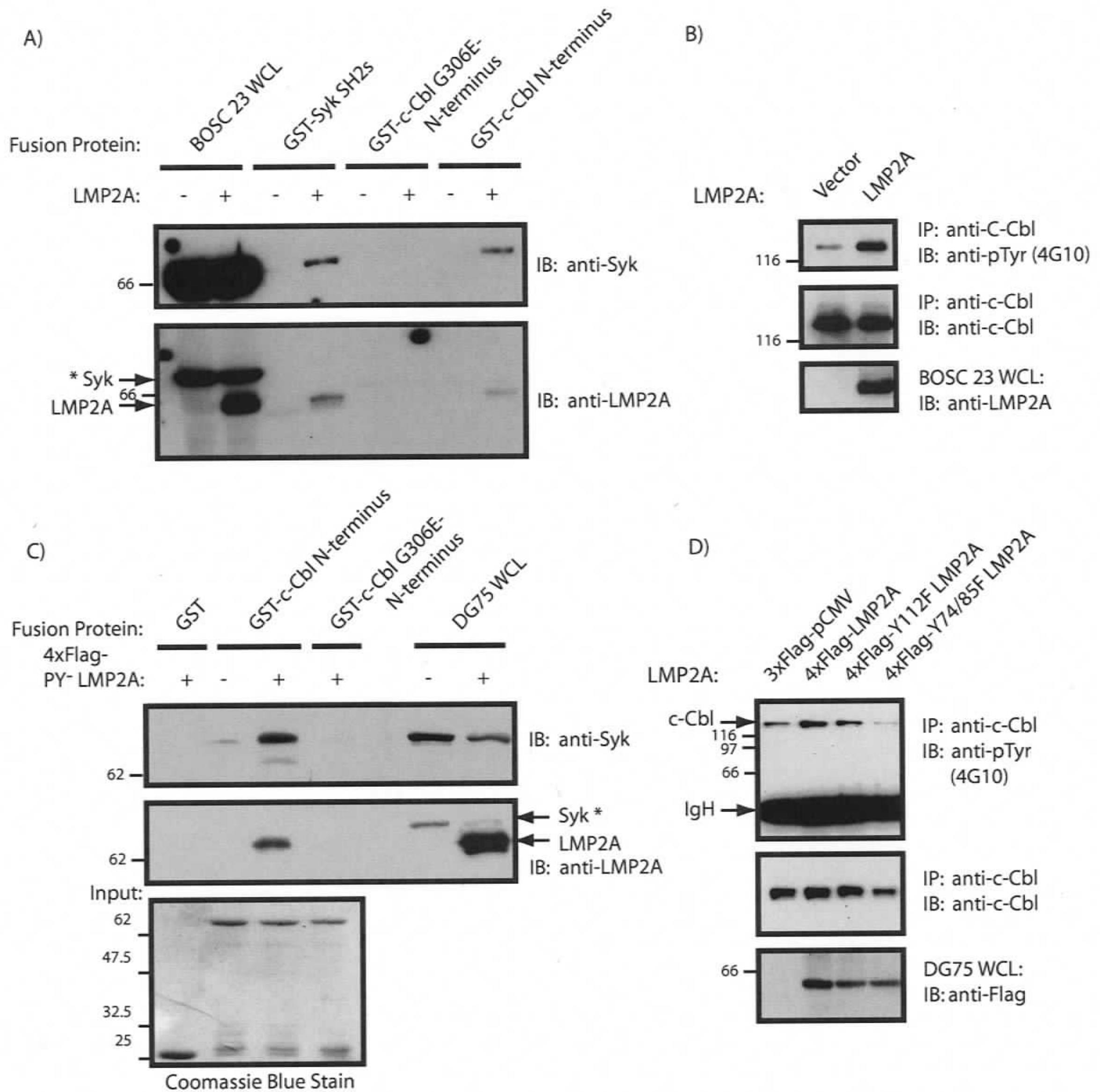

Figure 3.13 – The c-Cbl E3 ubiquitin ligase is recruited to Syk by way of its SH2-like domain. ***A&C,*** Lysates derived from BOSC23 293T cells stably-expressing LMP2A (***A***) or DG75 cells transiently transfected with 4xFlag-PY⁻ LMP2A (***C***) were precipitated with the indicated GST fusion proteins and immunoblotted (IB) with the anti-Syk or LMP2A mAbs. Whole cell lysate (WCL) was probed with the anti-Syk mAb and then reprobed with anti-LMP2A mAb (***A***) or anti-Flag M2 mAb (***C***) to show expression of these proteins in the respective lysates. Syk bands in the anti-LMP2A reprobes are due to residual signal from the inefficient removal of the anti-Syk mAb during stripping (*). ***B&D,*** Lysates derived from BOSC23 293T cells stably-expressing LMP2A (***B***) or DG75 cells transiently transfected with 4xFlag-LMP2A or the indicated point mutants (***D***) were immunoprecipitated (IP) with the anti-c-Cbl pAb and precipitated proteins were analyzed by immunoblotting (IB) with the 4G10 mAb. Blots were then stripped and reprobed with the anti-c-Cbl pAb. Whole cell lysates (WCL) were probed with the anti-LMP2A mAb (***B***) or the anti-Flag M2 mAb (***D***) to show expression of LMP2A in the respective lysates. Molecular mass markers (in kDa) are indicated to the left of the blots.

Chapter 4: Discussion & Future Research

4.1 Discussion

Epstein-Barr virus infection is associated with several human malignancies of B, T and epithelial cell origin. Understanding how proteins expressed from the EBV genome interfere with or co-opt host cell signalling molecules to initiate their own signals is the key to understanding the role of this virus in cancer pathogenesis. LMP2A is one viral protein able to insert itself into host cell signalling pathways to inhibit normal BCR signalling and initiate pro-survival and pro-migratory signals. Thus, understanding how LMP2A uses cellular tyrosine kinases to initiate these signals may prove beneficial to understanding the role of EBV in tumour formation.

While it has been appreciated for some time that LMP2A recruits the Lyn and Syk tyrosine kinases in B cells, the relative contribution of these enzymes to the initiation of LMP2A signalling has not yet been fully elucidated. In this thesis, I have made several important observations regarding the role of Lyn and the Lyn-binding YEEA motif in the initiation of signalling by LMP2A in B lymphocytes. Here, we have shown that the LMP2A YEEA site is not absolutely required for tyrosine phosphorylation of the LMP2A ITAM, or for LMP2A to recruit and tyrosine phosphorylate Syk in B cells transiently expressing LMP2A. In contrast, reducing Lyn levels in LCLs, which express lower levels of LMP2A, attenuated Syk tyrosine phosphorylation. In addition, we have shown that Lyn was not required for LMP2A-mediated Akt activation in B cells, but rather, reducing Lyn levels in LMP2A-expressing cells augmented Akt activation. Finally, our data suggests that Lyn negatively regulates LMP2A signalling in part through

phosphorylation of Syk on Y323. Phosphorylation of Syk on this site likely recruits the c-Cbl E3 ubiquitin ligase to Syk, which we believe targets Syk for ubiquitin-mediated degradation.

Through the transient expression of 4xFlag-LMP2A and LMP2A point mutants in the human DG75 B cell line, we were able to show that the LMP2A YEEA motif was required for overall LMP2A tyrosine phosphorylation (Figure 3.3) but not for phosphorylation of the LMP2A ITAM (Figure 3.4). Furthermore, we found that the YEEA motif and Lyn were not required for LMP2A-mediated Syk recruitment and activation in DG75 cells (Figures 3.1, 3.5, 3.6 and 3.8). These results do not fit with the two-step model for initiating LMP2A signalling originally proposed by Fruehling et al. (76). This disparity may be due to intrinsic differences in the requirement for SFKs between the LCL cells used by Fruehling et al. and the human B lymphoma cell lines used in our study. Alternatively, the difference in YEEA motif requirement may be due to differences in how LMP2A was expressed in the two cell types. In the study by Fruehling et al., recombinant virus containing the Y112F mutation was used to infect primary B cells and resulted in stable expression of the Y112F LMP2A protein, whereas in our study Y112F LMP2A was transiently expressed in B lymphoma cell lines from a vector under the control of a CMV promoter. For this reason we chose to examine the requirement of Lyn for LMP2A-mediated Syk activation in LCLs. Reducing Lyn levels with siRNA in two LCL cell lines decreased overall Syk and LMP2A tyrosine phosphorylation (Figure 3.9A and B). Our data demonstrating that more LMP2A was expressed in the DG75 cells compared to the LCLs, suggests that the requirement for Lyn may be related to the amount of LMP2A expressed in the cell. In LCLs where Lyn is

expressed at lower levels, there is likely a greater requirement for Lyn to "prime" the ITAM in order for it to recruit and activate Syk. In cells expressing higher levels of LMP2A there is likely less of a requirement for this. Thus, while Lyn is not absolutely required to initiate LMP2A signalling, its recruitment to Y112 improves the efficiency of LMP2A to recruit and activate Syk. Our results showing that an intact YEEA motif was required for optimal ITAM phosphorylation (Figure 3.4B), and the data of Lu et al. showing that Y112F LMP2A was slightly less efficient than wild type LMP2A at recruiting Syk (128), support this assertion. Thus, these findings argue that the discrepancy between the findings of Fruehling et al. (125) and Lu et al. (128), regarding the requirements for LMP2A to initiate signalling, is due to differences in the amount of LMP2A expressed. Our findings also suggest that the lack of, or reduction in, overall LMP2A tyrosine phosphorylation observed for the Y112F mutant in previous studies (76) is likely due to the inability of several commercially available antibodies to recognize the LMP2A ITAM when it is phosphorylated as opposed to there being a lack of tyrosine phosphorylation at this site (Figure 3.3).

Our results argue that not only is the SFK-binding YEEA site not required, but the SFKs themselves are not required for LMP2A-mediated Syk tyrosine phosphorylation in B cells transiently expressing LMP2A. Through the use of siRNA to reduce Lyn levels in DG75 cells, along with the Lyn-/- DT40 cell line, we were able to show that overall LMP2A tyrosine phosphorylation, LMP2A ITAM phosphorylation and Syk recruitment and activation are not dependent on Lyn (Figures 3.5, 3.6 and 3.8). The results of these experiments contradict the results of our PP1 experiments, however, which demonstrated that inhibition of SFKs with PP1 decreased overall LMP2A and ITAM tyrosine

phosphorylation (Figure 3.7). The discrepancy between these results may be due to the non-specific action of PP1 on other tyrosine kinases. Our finding that the activation of Syk by LMP2A was Lyn independent, is consistent with other studies reporting that ITAM-containing receptors can activate Syk independently of SFKs. In the Jurkat T cell line JCaM1.6, which is deficient in the SFK Lck and has reduced expression of Fyn, T cell receptor (TCR) signalling is impaired (206). However, overexpression of Syk in these cells can restore TCR-mediated NF-AT luciferase activity (207). Likewise, in COS-7 cells expressing a chimera consisting of the extracellular and transmembrane domains of CD8 and the intracellular, ITAM-containing γ subunit of the FcεRI receptor, Syk was able to promote tyrosine phosphorylation of this chimera independently of Lyn (208). Thus, these studies and our own data demonstrate that ITAM-containing receptors such as LMP2A can utilize Syk independently of SFKs.

Our data also show that Syk is required for LMP2A-mediated Akt activation in DG75 B cells, but Lyn is not (Figure 3.10). In fact, Akt activation was significantly enhanced in LMP2A-expressing DG75 cells where Lyn was reduced by siRNA. While reducing Lyn levels or expressing LMP2A on their own were sufficient to activate Akt in DG75 cells, the combined treatment resulted in a slightly greater than additive effect. Lyn-deficient B cells also exhibit enhanced BCR-mediated Akt activation (209, 210) and one target of Lyn in BCR-stimulated cells is Y323 of Syk (201). Phosphorylation of this site in BCR-stimulated cells, serves to recruit the SH2-like domain of c-Cbl/Cbl-b E3 ubiquitin ligases, which target Syk for ubiquitination-mediated degradation (202-204). We have determined that Lyn is similarly required for phosphorylation of Syk on Y323 in LMP2A-expressing cells B cells (Figure 3.12A). Since Lyn is required for Syk Y323

phosphorylation, we propose that reducing Lyn levels in LMP2A-expressing B cells by siRNA enhances Akt activation by failing to target active Syk for ubiquitin-mediated degradation by c-Cbl/Cbl-b. This model is supported by the fact that the c-Cbl N-terminus precipitates LMP2A and Syk from LMP2A-expressing epithelial and DG75 B cells in a SH2-like domain-dependent manner (Figure 3.13 A and C respectively).

One surprising finding was that, although Lyn was not required for LMP2A-mediated Akt activation, the YEEA motif was (Figure 3.11). Swart et al. also reported that the YEEA motif was required for LMP2A-mediated Akt activation in LCL B cells (163). It is not clear how LMP2A activates Akt in a YEEA-dependent, Lyn-independent fashion, but it is possible that the YEEA motif may recruit some other protein that is required for LMP2A-mediated Akt activation.

The data presented in this thesis provides new insight into how LMP2A utilizes tyrosine kinases to initiate and control the magnitude of LMP2A signalling in B lymphocytes. Since the YEEA site is not required for Syk recruitment and activation in B cells transiently expressing LMP2A, we speculate that Syk itself is able to tyrosine phosphorylate the ITAM in cells expressing higher levels of LMP2A. This theory is supported by data from other research groups demonstrating that ITAM-containing receptors can utilize Syk independently of SFKs (207, 208). While the YEEA site does not appear to be absolutely required, our data and that of others, argue that the YEEA site improves the efficiency of LMP2A ITAM phosphorylation and Syk recruitment and activation (Figure 3.4 and (128)). It is possible that the binding of SFKs to the YEEA site serves to "prime" the LMP2A ITAM by phosphorylating it on tyrosine residues 74 and 85 allowing Syk to associate and become activated. This, however, would first

require tyrosine phosphorylation of the YEEA site. Others have speculated that SFKs promote this phosphorylation by associating with LMP2A via a YEEA-independent mechanism, either through direct binding to another site within LMP2A or through co-localization of the two proteins in lipid rafts (125). Once Syk is recruited and activated, we hypothesize that Syk itself tyrosine phosphorylates the ITAMs of adjacent LMP2A proteins and activates other Syk molecules. The LMP2A C-terminal clustering signal and LMP2A lipid raft aggregation likely bring LMP2A molecules in close enough proximity to one another to support such a scenario (138-140). Our hypothesis is supported by studies in SFK-deficient mice, which showed that SFKs are not required for, but rather enhance, BCR signalling ((211) and reviewed in (212)). In B cells, Lyn associates with the BCR either through co-localization in lipid rafts or by binding to the ITAMs of the Igα/β chains of the BCR via a weak interaction. Upon BCR cross-linking the associated Lyn molecules phosphorylate the ITAMs of adjacent receptor molecules resulting in Syk recruitment and activation. However, since B cells purified from Lyn deficient mice exhibit only a delay in the tyrosine phosphorylation of Igα and Syk upon BCR cross-linking (213), it has been speculated that Syk itself might initiate the signalling response in the B cells of SFK-deficient mice (212). This data suggests that the primary function of SFKs in BCR signalling is to speed up the response to immunoreceptor engagement and supports our hypothesis that association of SFKs with LMP2A might simply serve to "prime" the LMP2A ITAM and enhance the ability of Syk to promote LMP2A signalling.

While it appears as though the YEEA site is not required for the initiation of LMP2A signalling in cells expressing higher levels of LMP2A, it does appear to be

important for downstream LMP2A signalling. The LMP2A YEEA site is required for LMP2A-mediated Akt activation, inhibition of BCR signalling and maintenance of viral latency (Figure 3.11, (125, 163)). We speculate that the association of some other protein with the YEEA site may inhibit or override the negative regulatory signal of Lyn to promote LMP2A-mediated Akt activation. It is also possible that the LMP2A YEEA motif might simply serve to bind Lyn and promote its degradation, thereby alleviating the negative regulatory effect of Lyn on LMP2A-mediated Akt activation. As mentioned previously, the two PY motifs located within the N-terminus of LMP2A recruit NEDD4 family E3 ubiquitin ligases by way of their WW domains (126, 131-133). In support of this latter hypothesis, LMP2A expression in B cells has been shown to reduce steady-state levels of Lyn (77, 125, 131, 133, 214) and promote Lyn ubiquitination (133) and degradation (131, 133), effects dependent on the LMP2A PY and YEEA motifs (125, 132). However, mutation of the PY motifs in epithelial cells does not appear to have an effect on LMP2A-mediated Akt activation (159). It is clear from our findings that the exact manner in which the LMP2A YEEA site is used to promote downstream LMP2A signalling requires further investigation (see Section 4.2.1).

The data presented here also highlight a novel role for Lyn as a negative regulator of LMP2A-mediated Akt activation. We have shown that Lyn promotes phosphorylation of Syk on Y323, which we argue serves as the mechanism by which Lyn regulates LMP2A signalling. Syk Y323 phosphorylation likely recruits c-Cbl to Syk, which we believe targets Syk for ubiquitin-mediated degradation. In addition to promoting Syk Y323 phosphorylation, Lyn may also promote the phosphorylation of negative signalling receptors in LMP2A-expressing cells to inhibit downstream LMP2A

signalling. As mentioned previously, Lyn also has a negative regulatory role in BCR signalling (reviewed in (198, 199)). In B cells, Lyn phosphorylates the ITIMs of the negative signalling receptors, FCγRIIB and CD22, resulting in the recruitment of cellular phosphatases, such as SHIP and SHP-1, that antagonize BCR signalling (reviewed in (200)). CD22 is a member of the sialoadhesion family of molecules that bind sialic acid (215). It is expressed on the surface of B cells and has an important role in mediating B cell activation and adhesion (215). After BCR ligation, CD22, along with Lyn and SHP-1, form a negative regulatory loop that inhibits calcium flux and MAPK activation in BCR-stimulated B cells (213, 216). Similarly, co-ligation of FcγRIIB, the B cell Fc receptor for IgG, with the BCR also inhibits calcium flux and MAPK activation by inducing Lyn-mediated phosphorylation of the FcγRIIB ITIM and subsequent SHIP recruitment (213, 217). A negative regulatory role for Lyn in BCR-mediated Akt activation that is independent of CD22 or FcγRIIB phosphorylation has been demonstrated using Lyn-deficient DT40 cells (209). Whether the phosphorylation of these negative signalling receptors by Lyn might play a role in its ability to inhibit LMP2A-induced Akt activation in B cells, however, has yet to be addressed.

LMP2A provides tonic, pro-survival signals to EBV-positive malignancies, and has evolved mechanisms for regulating the strength of these signals. The recruitment of NEDD4 E3 ubiquitin ligases to LMP2A regulates the strength of LMP2A signalling by ubiquitinating LMP2A and its associated kinases and targeting them for degradation (126, 131-133, 214). The NEDD4 family proteins Itch (AIP4), NEDD4-2 and WWP2 are known to associate with the PY motifs of LMP2A (131-133). In LMP2A-expressing Jurkat T cells, Itch promotes LMP2A ubiquitination and degradation in a PY motif-

dependent manner (126). In these cells, mutation of the LMP2A PY motifs stabilizes LMP2A levels and the formation of LMP2A-kinase complexes suggesting that the PY motifs might regulate LMP2A-induced signals (126). Similarly, In B cells, the binding of Itch to the PY motifs of LMP2A enhances LMP2A, Lyn and Syk ubiquitination and decreases the stability of LMP2A and Lyn (132, 133). Furthermore, crossing mice homozygous for a deficiency in Itch into an LMP2A transgenic background results in the hyperphosphorylation of LMP2A, Lyn and Syk suggesting LMP2A signalling is enhanced in the B cells of these mice (214). The novel negative regulatory role of Lyn in LMP2A signalling presented in this thesis is important as it likely presents another strategy by which LMP2A-induced signals are regulated. The phosphorylation of Syk by Lyn, and its subsequent c-Cbl-mediated degradation may serve as another example by which the magnitude of LMP2A signalling is controlled. The regulation of LMP2A signalling is vital to the persistence of EBV, as maintaining these signals at a tonic, low level is necessary for promoting B cell survival while avoiding viral reactivation.

This thesis provides new insight into how LMP2A utilizes cellular tyrosine kinases to initiate signalling in B lymphocytes and highlights a novel role for Lyn as a negative regulator of this signalling. Since these signals likely contribute to the pathogenesis of EBV-positive malignancies, it is important to determine the precise mechanisms by which they are initiated in order to understand how best LMP2A signalling could be interfered with to treat these disorders. Importantly, our findings argue that Lyn may not be a good therapeutic target, as inhibiting Lyn could enhance pro-survival signalling in EBV-positive tumours expressing high levels of LMP2A. Our data

also suggest it may be worthwhile to examine the status of Lyn in these tumours as mutations inhibiting Lyn expression or activation may promote tumour growth.

4.2 Future Research

While the findings presented in this thesis shed light on how LMP2A uses cellular tyrosine kinases to initiate and propagate signals in B lymphocytes, we still do not have a complete picture as to exactly how these processes are being carried out. The data presented here have left us with some unanswered questions, which we are interested in addressing in the future.

4.2.1 Further characterization of the LMP2A YEEA motif in LMP2A-mediated Akt activation

The ability of LMP2A to constitutively activate Akt, a protein commonly deregulated in human cancer, suggests this viral protein may be involved in the development or maintenance of EBV-associated malignancies. Thus, understanding how LMP2A uses cellular proteins to promote the activation of Akt may prove invaluable to understanding the link between EBV-infection and cancer. We have determined that the LMP2A YEEA site is required for LMP2A-mediated Akt activation (Figure 3.11) and are interested in further characterizing its involvement in this process. The two PY motifs located within the N-terminus of LMP2A recruit NEDD4 family E3 ubiquitin ligases by way of their WW domains (126, 131-133). In B cells, LMP2A expression reduces steady-state levels of Lyn (77, 125, 131, 133, 214) and promotes Lyn ubiquitination (133) and degradation (131, 133) in a PY motif-dependent manner. Therefore, it is possible that the LMP2A YEEA motif might simply serve to recruit Lyn and bring it in close enough proximity to NEDD4 family E3 ubiquitin ligases to promote its ubiquitination and degradation, thereby alleviating its negative regulatory function. However, mutation of the PY motifs in epithelial cells does not appear to have an effect on LMP2A-mediated

Akt activation (159). Therefore, we speculate that the LMP2A YEEA motif contributes to LMP2A-mediated Akt activation through the recruitment of an as-yet-undetermined protein that either inhibits or overrides the negative regulatory signal of Lyn. We could explore this idea using affinity chromatography and mass spectrometry to identify proteins that bind to the LMP2A YEEA motif. We could use an immobilized pYEEA peptide to precipitate proteins from DG75 cell lysates and then determine their identities by mass spectrometry, as was previously described for the LMP2A PY motifs (133). Once other YEEA site-binding proteins have been identified, we could systematically examine their requirement for LMP2A-mediated Akt activation by inhibiting their activity chemically or reducing their levels with siRNA. We could then assess the effects of these treatments on Akt activation in LMP2A-expressing cells by Western blotting. Alternatively, we could examine the requirements of identified YEEA-site binding proteins on LMP2A-mediated Akt activation by knocking out their expression in the DT40 chicken B cell line. DT40 cells would be practical to use in this experiment as they undergo homologous recombination at a relatively high frequency. We could then examine the ability of LMP2A to promote Akt activation in these cell lines by Western blotting. If we identify a protein that is functioning in this pathway, we expect that inhibiting its function or expression will impair the ability of LMP2A to activate Akt in B cells. These experiments will not only further elucidate the requirements of host cell proteins for LMP2A-mediated Akt activation but may identify potential therapeutic targets for the treatment of EBV-associated disorders.

4.2.2 Further investigation into the role of Lyn as a negative regulator of LMP2A-mediated Akt activation

Understanding how LMP2A-induced signals are regulated is important to our understanding of EBV biology, as LMP2A signalling must be maintained at a tonic, low level to promote B cell survival while avoiding viral reactivation (24, 143). As mentioned previously, the role of the LMP2A PY motifs and NEDD4 family E3 ubiquitin ligases in the regulation of LMP2A signalling has been appreciated for sometime (Section 4.1). However, the negative regulatory role of Lyn in LMP2A signalling presented in this thesis is a novel finding (Figure 3.10). Further characterization of the negative regulatory role of Lyn in LMP2A signalling is important as it presents another strategy by which LMP2A-induced signals are regulated. We have demonstrated that Lyn expression is required for the phosphorylation of Syk on Y323, a c-Cbl E3 ubiquitin ligase binding site, in LMP2A-expressing B cells (Figure 3.12A). We have also shown that c-Cbl is able to interact with Syk via its SH2-like domain in LMP2A-expressing epithelial and B cells (Figure 3.13A and C). We speculate that Lyn negatively regulates LMP2A-mediated Akt activation by phosphorylating Syk on Y323, promoting c-Cbl recruitment and subsequent ubiquitination and degradation of Syk. To determine if this is the case, we would first like to show that Y323 is required for the interaction of c-Cbl with Syk. To address this, we will express HA-Syk and HA-Syk Y323F in DG75 cells and then use our GST-c-Cbl N-terminus to precipitate proteins from cell lysates. We expect that the N-terminus of c-Cbl will be able to precipitate HA-Syk but not HA-Syk Y323F, indicating that phosphorylation of Syk on Y323 is required for its interaction with c-Cbl. Secondly, we would like to assess the requirement of Lyn for Syk and c-Cbl to interact with one another and will examine this using siRNA to reduce Lyn levels in

DG75 cells. This experiment may be complicated by the fact that our siRNA treatments are not 100% effective at eliminating Lyn expression and by the expression of other SFKs in DG75 cells. Therefore, we will also perform this experiment in Lyn-/- DT40 cells to get around these issues, as Lyn is the only SFK expressed in this cell type. Since Lyn promotes phosphorylation of Syk on Y323 in LMP2A-expressing cells (Figure 3.12A), we expect that reducing or eliminating Lyn expression will inhibit the ability of our GST-c-Cbl N-terminus to precipitate Syk from cell lysates. Finally, we would like to determine if the ubiquitination of Syk is decreased in DG75 cells co-transfected with 4xFlag-LMP2A and Lyn siRNA compared to cells co-transfected with 4xFlag-LMP2A and control siRNA. If our hypothesis is correct, we expect that ubiquitination of Syk will be decreased in DG75 cells with reduced Lyn expression.

Lyn also functions as a negative regulator of BCR signalling by phosphorylating the ITIMs of negative signalling receptors including FcγRIIB and CD22 (reviewed in (198) and (199)). In B cells, phosphorylation of the ITIMs of these negative signalling receptors results in the recruitment of cellular phosphatases, such as SHIP inositol phosphatase and SHP-1 tyrosine phosphatase, which antagonize BCR signalling (reviewed in (200)). A negative regulatory role for Lyn in BCR-mediated Akt activation has been previously described in Lyn-deficient DT40 cells (209). This negative effect of Lyn on Akt activation is likely not dependent on CD22 or FcγRIIB phosphorylation, as BCR-mediated Akt activation was inhibited in the absence of SHIP and SHP-1 (209). To date, no work has been published that examines the role or tyrosine phosphorylation status of CD22 or FcγRIIB in LMP2A-expressing cells. We would like to determine whether, in addition to Syk Y323 phosphorylation, the phosphorylation of these negative

signalling receptors by Lyn also plays a role in its ability to modulate LMP2A-mediated Akt activation in B cells. We could address this by comparing the tyrosine phosphorylation status of CD22 and FcγRIIB in LMP2A-expressing DG75 cells transfected with either control or Lyn siRNA by Western blotting. Furthermore, in these cells we could examine the recruitment of the phosphatases SHP-1 and SHIP to CD22 and FcγRIIB respectively (213, 216, 217). If negative signalling receptors are involved in mediating the negative regulatory effect of Lyn on LMP2A signalling, we expect that reducing Lyn levels by siRNA will decrease their tyrosine phosphorylation and subsequent phosphatase recruitment in LMP2A-expressing cells. We could further investigate the role of the phosphatases SHIP and SHP-1 in LMP2A-mediated Akt activation by transiently expressing 4xFlag-LMP2A in Lyn, SHIP and SHP-1 deficient DT40 cells and performing Western blots on cell lysates (209). If the inhibition of Akt activation by Lyn in LMP2A-expressing cells is dependent on these phosphatases, we expect Akt activation to be increased equivalently in these three cell types compared to wild-type LMP2A-expressing DT40 cells. These experiments will help us better understand how LMP2A signalling is regulated in B lymphocytes to allow propagation of pro-survival signals while maintaining viral latency.

4.2.3 Examining LMP2A-mediated Akt activation in the context of EBV-positive tumours

Here, we have demonstrated that transient expression of LMP2A in DG75 cells results in an increase in Akt activation (Figure 3.10). Akt is a cellular serine/threonine kinase that promotes cell survival by inhibiting apoptosis and whose activation is deregulated in many human cancers. Since LMP2A expression promotes the constitutive

activation of Akt we speculate that LMP2A functions through Akt to contribute to the pathogenesis of EBV-associated malignancies. Thus, we are interested in examining the role of LMP2A and Lyn in Akt signalling in the context of EBV-positive tumours. Up until this point we have primarily been expressing LMP2A and our LMP2A point mutants transiently in EBV-negative B lymphoma cell lines from a vector under the control of a CMV promoter. The level of LMP2A expression achieved in our experiments, therefore, may not be indicative of the level of LMP2A expression that occurs within EBV-positive tumours. Also, the possibility that other viral proteins expressed during EBV latency may affect LMP2A's signalling capabilities was not taken into account in most of our experiments. Therefore, we would like to examine the role of LMP2A in Akt activation under more physiologically relevant conditions and will do so using EBV-positive B cell lymphoma lines. EBV-positive Hodgkin's lymphomas are B cell lymphomas that exhibit a latency II pattern of viral gene expression (Table 1.1 and 1.2), meaning they express EBNA1, LMP1, LMP2A/2B, and EBERs (105). EBV-positive Burkitt's lymphomas are also B cell lymphomas but express a latency I pattern of viral gene expression, meaning EBNA1 is the only protein expressed from the EBV-genome, making these tumour cells impractical to use for the study of LMP2A (105). We will, therefore, focus our efforts in EBV-positive Hodgkin's lymphoma cell lines. We will transfect these cells with control or LMP2A RNAi and examine the effect of these transfections on Akt activation by Western blotting. B cells infected with EBV *in vitro* exhibit a latency III pattern of viral gene expression (Table 1.1) and will proliferate continuously forming a lymphoblastoid cell line (LCL). Akt is constitutively activated in LCLs and this activation is dependent upon the continued expression of LMP2A (163).

Thus, we expect that reducing LMP2A levels by siRNA in EBV-positive Hodgkin's lymphoma cell lines will result in a decrease in Akt activation as well. We have determined that Syk is required for LMP2A-mediated Akt activation and that Lyn negatively regulates this activation (Figure 3.10) in DG75 cells and are interested in determining if the same is true in the context of EBV-positive tumours. To address this, we will transfect our EBV-positive HD cells with control, Lyn or Syk siRNA and examine the effects of these transfections on LMP2A-mediated Akt activation by Western blotting. To date, there have been no publications which take such an approach to examining the requirements of Lyn and Syk for LMP2A-induced signalling in EBV-positive B cell lymphomas or LCLs. We expect that reducing Syk levels in these cells by siRNA will significantly decrease Akt activation while reducing Lyn levels will result in an increase in Akt activation, as was observed in LMP2A-expressing DG75 cells (Figure 3.10).

Classic HD tumours are characterized by the presence of multinucleated Reed-Sternberg cells (HRS) (20, 105) and arise from post-GC B cells lacking surface immunoglobulin expression (118), a state inconsistent with B cell survival (119). It has been speculated that the key role of EBV in HD is to induce survival and proliferation of BCR-deficient GC B cells through the expression of LMP1 and LMP2A (119, 120). The ability of LMP2A to activate Akt likely rescues these GC B cells from apoptosis and contributes to the survival of EBV-positive HRS cells (74, 75, 121). Thus, we believe that this work will shed light on the contribution of EBV to HD tumours.

4.2.4 Assessing other downstream signalling pathways activated by LMP2A expression in B lymphocytes

In addition to cell survival, LMP2A signalling has been associated with increased epithelial cell adhesion and migration (Section 1.3.2.3.2). The role of LMP2A in cell migration is important to understand as several EBV-positive cancers, particularly NPC, are associated with a high rate of metastasis, a property often associated with poor prognosis. There are a number of published studies demonstrating the ability of LMP2A to enhance epithelial cell migration (110, 128, 171) but none addressing the effect of LMP2A expression on B cell migration. Changes in integrin expression likely have a role in LMP2A-induced epithelial cell migration, as migrating LMP2A-expressing primary tonsillar epithelial cells, but not stationary cells, upregulate integrin α_6 protein expression (110). Expression of LMP2A in the EBV-negative LCL, JR2B10, enhances integrin α_5 promoter activity and increases α_5 mRNA abundance suggesting that LMP2A may have a role in B cell migration as well (177).

We are interested in examining the effect of LMP2A signalling on B cell migration. For simplicity we will create a stable LMP2A-expressing B cell line and examine the effect of LMP2A expression on B cell migration using a transwell assay. The use of a stable LMP2A-expressing B cell line, as opposed to an LCL, allows us to examine the effect of LMP2A on B cell migration in isolation, without the expression of other EBV latency proteins. We have shown that DG75 cells are not a particularly migratory cell line, with only ~2.3% of cells migrating in response to the chemoattractant SDF-1α (data not shown), therefore, other B cell lines would need to be considered based on their migratory capabilities. We will examine the effect of LMP2A-expression on B cell migration both in the presence and absence of chemoattractant and migration will be

quantified using a flow cytometer. The B lymphocyte chemoattractant (BLC/CXCL13) and its receptor CXCR5 are required for B cell homing to follicles in the lymph nodes and spleen (218). BLC may be a good chemoattractant to use in these experiments as LMP2A has been speculated to promote the migration of EBV-infected B cells into mucosal lymphoid follicles during normal EBV infection (24, 25). As has been reported in epithelial cells (110, 128, 171), we expect that stable LMP2A expression will increase the migratory capability of B cells. If so, we will then address the requirements of the individual LMP2A signalling motifs in this process. We will create stable cell lines expressing LMP2A point mutants to determine if the ITAM or the YEEA motif are required for this process. We will then perform transwell migration assays to determine the requirements of the YEEA site and ITAM on LMP2A-induce B cell migration. The ITAM, but not the YEEA motif, is required for LMP2A-mediated migration of epithelial cells as mutation of the tyrosine residues of the LMP2A ITAM to phenylalanine decreased the migration of LMP2A-expressing 293 cells (128). We expect the same will be true in the case of B cell migration. If these experiments are successful we will then address the requirements of the individual kinases for LMP2A-induced B cell migration by reducing Lyn and Syk levels in our stable LMP2A-expressing cells by siRNA and performing transwell assays. Alternatively, we could express LMP2A transiently in wild-type, Lyn-/- and Syk-/- DT40 cells and quantify the migration of these cells using a transwell assay. We expect that Syk will be required for LMP2A-mediated B cell migration, since inhibiting Syk activity with the chemical inhibitor piceatannol or reducing Syk levels by siRNA impaired LMP2A-triggered epithelial cell motility (128).

We are interested, however, in determining if Lyn plays a positive or negative regulatory role in this process.

Finally, we would like to investigate the cellular signalling pathways involved in LMP2A-mediated B cell migration. Expression of LMP2A in the EBV-negative LCL, JR2B10, enhances integrin α_5 mRNA expression and promoter activity (177). In our LMP2A-expressing B cells we will examine the effect of LMP2A-expression on integrin α_5 levels. Similar to LCLs, we expect integrin α_5 levels to be increased in our LMP2A-expressing cells (177). We will then assess the role of this integrin in LMP2A-mediated B cell migration by treating cells with α_5 blocking antibodies (available from Chemicon International (219)) and performing a transwell assay. We expect that treatment with integrin α_5 blocking antibodies will inhibit LMP2A-induced B cell migration, as was seen for the treatment of primary tonsillar epithelial cells with integrin α_6 blocking antibodies (110). Understanding the role of integrins in LMP2A-induced migration is important as integrin expression correlates well with tumour metastasis (110). Thus, identifying and characterizing the function of LMP2A in B cell migration may highlight a role for EBV in the metastasis of EBV-positive B cell lymphomas.

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