AN INVESTIGATION OF THE REGULATION OF BLIMP1

BY THE EPSTEIN-BARR VIRUS IN B CELLS

By Kateřina Vrzalíková

A thesis submitted to the School of Cancer Sciences

of the University of Birmingham

for the degree of

DOCTOR OF PHILOSOPHY

School of Cancer Sciences

College of Medicine and Dental Sciences

The University of Birmingham

March 2011

ABSTRACT

BLIMP1 is a transcription factor that regulates plasma cell differentiation. In this thesis I explore the regulation of BLIMP1 by EBV and by the EBV oncogene, latent membrane protein-1 (LMP1).

In chapter 3, I show that BLIMP1α is down-regulated following the infection of germinal centre (GC) B cells with EBV. I also show that the ectopic expression of LMP1, was sufficient to decrease BLIMP1α expression in these cells and was accompanied by a partial disruption of the BLIMP1α transcriptional programme, including the aberrant induction of C-MYC. In chapter 4, I show that the ectopic expression of BLIMP1α in EBV-transformed cells and in EBV-positive Burkitt's lymphoma cells can induce the viral lytic cycle. Chapter 5 provides evidence that LMP1 drives a reciprocal regulatory loop in GC B cells involving BLIMP1α and C-MYC which ultimately leads to the activation of C-MYC and the repression of BLIMP1α. Finally, in chapter 6, I present preliminary evidence showing that the BLIMP1β isoform is up-regulated in EBV-transformed B cells and in Hodgkin's lymphoma cells; an effect which appeared to be mediated by hypomethylation of the BLIMP1β specific promoter.

In summary, my results suggest that EBV can subvert normal B cell differentiation by modulating expression of the different BLIMP1 isoforms. These effects appear to be important not only for the regulation of the viral lytic cycle in B cells, but also potentially for the block in differentiation characteristic of EBV-associated B cell lymphomas.

DEDICATION

This thesis is dedicated to the memory of my grandfather and my big supporter,

Jiří Valenta.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank everyone who has helped and supported me throughout my studies. In particular, I would like to thank Lymphoma and Leukemia Research Fund whose financial support has allowed me to accomplish all my work at the University of Birmingham.

Most importantly, special thanks must go to my supervisor, Prof Paul Murray, for his sustained support throughout my whole PhD. His guidance of my project, lab work and his help with the writing up of this thesis have been invaluable. Thank you for your faith in me, for giving me opportunity to realise my ideas and thoughts as well as your patience while putting up with my headstrongness. I would also like to say very special thanks to my supervisor Prof Ciaran Woodman who's 'dangerous influence' kept me motivated, enthusiastic and tirelessly working for years. I also wish to thank my supervisor Dr Martina Vockerodt, who's critical overlooking of my lab skills made me feel silly many times, but who was also there as a kind friend and even knew to pass me a glass of red wine after another long tonsil experiment in the lab at 3am. Although, Martina, you are never going to make me a fan of Blur! Many thanks also have to go to Prof Martin Rowe for his helpful advices throughout my whole PhD.

I also want to thank all the members of the 'PGM' and 'CBW' groups, past and present, for their generous help in the lab and for being great friends who made my time in Birmingham so enjoyable. I would like to say a huge thank you to Dr Sarah Leonard and Dr Jennifer Anderton, who have been helping me beyond the call of duty. Similar thanks go to Dr Stephanie Maloney and Dr Georgia Kapatai who were always there for me and especially at the beginning of my work in Birmingham. Thank you so much for your patience and kindness with what you repeated over and over every sentence when my English was so poor.

Personal thanks go to my family and friends, particularly my parents Jana and Jiří for their unconditional love and endless support throughout the years in all areas of my life, and to my little darling daughter Eileen Elizabeth who kept me smiling while writing up this thesis.

CONTENTS

Title of Section *Page Number*

LIST OF FIGURES

No. Title *Page Number*

Chapter 1.

No. Title *Page Number*

Chapter 4.

Chapter 5.

No. Title *Page Number*

Chapter 6.

LIST OF TABLES

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1. THE PHYSIOLOGY OF GERMINAL CENTRE

1.1.1. General introduction

Germinal centres (GCs) are histologically defined areas which form in secondary lymphoid organs in response to the entry of exogenous antigen. They represent a highly dynamic microenvironment where B cells undergo somatic hypermutation (SHM) and class switch recombination (CSR). The following sections summarize the formation and evolution of GCs, outline cellular phenotypes present within and generated by GCs, and provide a description of the processes of SHM and CSR.

1.1.2. Structure, development and function of germinal centres

The initial step in B cell ontogeny occurs in the bone marrow; where the recombination of immunoglobulin (*Ig*) heavy (*H*) and light (*L*) chain genes takes place in order to generate a functional B cell receptor (BCR). This is mediated by the recombination-activating genes, *RAG1* and *RAG2*, and is independent of antigen. Following antigen encounter, an immediate T cell-independent immune response results in the production of low affinity IgM antibodies. Subsequently, antigen-activated naïve B cells migrate into T cell rich areas of peripheral lymphoid tissues such as lymph nodes, tonsils, Peyer's patches and spleen (MacLennan *et al.*, 1990; Rajewsky, 1996; Hess *et al.*, 1998), where they become fully activated with the co-stimulatory help of T cells and antigen presenting cells (MacLennan, 1994). Some of the B cells develop into extrafollicular low affinity Ig-secreting cells,

whereas others undergo rapid clonal expansion and together with follicular dendritic cells (FDCs) form primary follicles. Following 3-5 days of vigorous proliferation, the characteristic structures of secondary follicles appear: naive IgM^+IgD^+ B cells gathered in follicular mantles surrounding polarized GCs comprised of dark zones with cycling centroblasts ($CD10^+CD77^+$) and light zones with smaller non-dividing centrocytes ($CD10^+CD77^-$) situated within a mesh of FDCs, T cells and macrophages (Figure 1.1.1). It is now apparent that GC B cells can migrate bidirectionally between dark and light zones as well as within individual dark and light compartments (Camacho *et al.,* 1998; Allen *et al.,* 2007; Schwickert *et al.,* 2007; Hauser *et al.,* 2007). The cycling patterns seem to depend upon gradients of chemokines, presumably established by stromal cells in the respective zones (Allen *et al.*, 2004).

1.1.3. Centroblasts and somatic hypermutation

Centroblasts are characterized by a high proliferation rate with an average cell cycle time between 6 to 12 hours (MacLennan, 1994; Allen *et al.*, 2007). GC B cells have high levels of telomerase activity, which prevents shortening of the telomeres in these highly proliferating cells (Hu *et al.*, 1997). Centroblasts express pro-apoptotic molecules such as Fas/CD95, but lack expression of anti-apoptotic genes, including BCL2 and its family members. Centroblasts do not receive CD40-mediated NF-κB-signals (MacLennan, 1994; Klein *et al.*, 2003; Liu *et al.*, 1991; Martinez-Valdez *et al.*, 1996; Basso *et al.*, 2004).

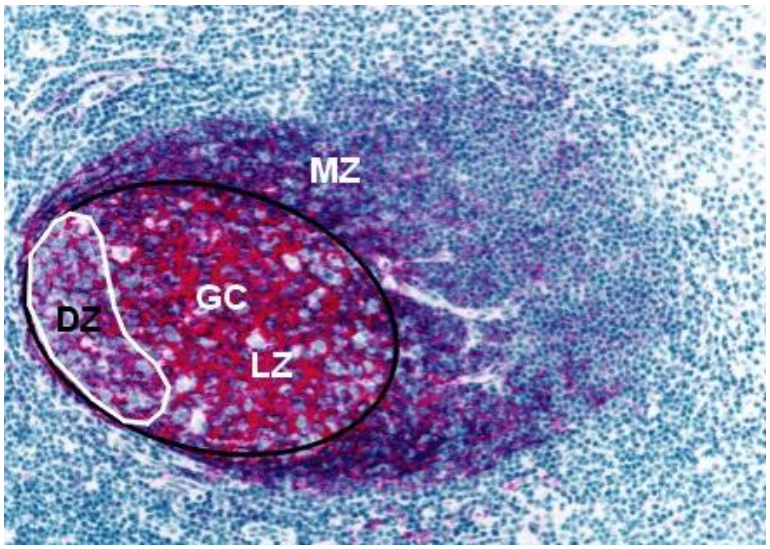


Figure 1.1.1. A fully developed GC in human tonsil. GC (black line) consists of dark zone (DZ) (white line) and light zone (LZ) comprises FDCs network (red). Cell nuclei are stained dark/blue. The GC is surrounded by follicular mantle (MZ) with high density of naïve B cells (figure provided by Kai-Michael Toellner).

Centroblasts undergo somatic hypermutation (SHM), a process in which mutations are introduced into variable regions of *Ig* genes (*IgV*) resulting in a change in the amino-acid sequence and diversification of *IgV* genes (Goossens *et al.*, 1998). These mutations are introduced into a region ~2 kb downstream of the transcriptional start site of the antibody genes, and include single nucleotide exchanges, deletions and duplications (Papavasiliou and Schatz, 2000; Bross *et al.*, 2000). SHM involves DNA strand breaks, though it is not clear exactly how they contribute to this process.

SHM is initiated by and requires the enzyme, activation-induced cytidine deaminase (AICDA also known as AID) (Revy *et al.,* 2000; Muramatsu *et al.,* 2000). AID deaminates cytosine (C) residues in DNA converting them to uracil (U); the resulting U:G (guanine) base pairs are subject to DNA mismatch repair (Chaudhuri *et al.*, 2003; Dickerson *et al.*, 2003; Ramiro *et al.*, 2003; Neuberger *et al*, 2005; Di Noia and Neuberger, 2007). Mutations can be generated either by C:G mutagenesis or by A:T mutagenesis (Figure 1.1.2) (Wilson *et al.*, 2005; Neuberger *et al.*, 2005). Several transcription factors regulate AID transcription in centroblasts including PAX5 (Gonda *et al.,* 2003), E proteins (Sayegh *et al.,* 2003) and IRF8 (Lee *et al.,* 2006).

Several transcription factors are involved in establishing the centroblast phenotype, these include BCL6, OBF1/POU2AF1, SPIB, BACH2 and IRF8 (Kosco-Vilbois *et al.*, 1997; Su *et al.*, 1997; Muto *et al.*, 2004; Lee *et al.*, 2006).

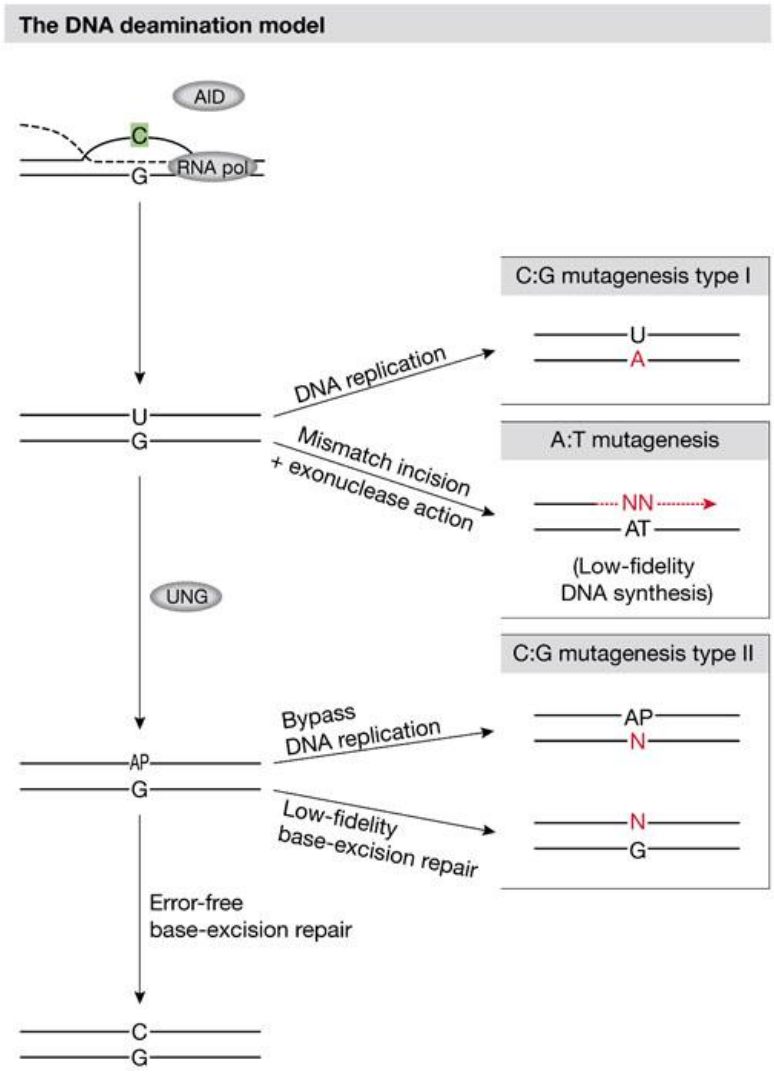


Figure 1.1.2. The DNA deamination model of SHM. AID deaminates C to U. As the U residue is treated as a T residue by DNA polymerases, copying of this base by a high-fidelity polymerase during chromosomal replication will cause a C:G (guanine) to T:A (adenine) transition (C:G mutagenesis type I). Alternatively, the U residues in DNA can be recognized by a uracil-DNA glycosylase (UNG) and excised (Larson and Maizels, 2004) to produce an apyrimidinic (AP) site. Such sites can be cleaved by several mechanisms involving an AP endonuclease in duplex DNA or an AP lyase activity in single-stranded DNA. During error-free base-excision repair, a gap of one or a few nucleotides is usually filled by DNA polymerase β. In SHM, error-prone base-excision repair can occur if other low-fidelity DNA polymerases are used in this step. Alternatively, mutations at C:G base pairs might arise (C:G mutagenesis type II) if a bypass DNA polymerase incorporates a base opposite an AP site before repair is initiated (taken from Seki *et al.,* 2005).

BCL6 is a transcriptional repressor which is required for the formation and maintenance of GCs (Ye *et al.*, 1997; Dent *et al.*, 1997). BCL6 recruits two co-repressor complexes; one composed of SMRT, NCoR and BCoR (Polo *et al.*, 2004), the other is referred to as the nucleosome remodelling and deacetylase (Mi-2-NuRD) complex (Fujita *et al.*, 2004, Parekh *et al.*, 2007). BCL6 extinguishes apoptotic and cell-cycle arrest responses by two distinct mechanisms: by inhibiting the transcription of *p53* (Phan and Dalla-Favera, 2004); and by binding to the transcriptional activator, MIZ-1 which suppresses MIZ-1 target genes including the cell cycle-arrest gene, *p21* (Phan *et al.*, 2005). BCL6 also represses DNA damage responses as well as the sensing of DNA damage by ATM and ATR proteins which allows centroblasts to tolerate the physiological DNA breaks required for SHM (Phan and Dalla-Favera, 2004; Ranuncolo *et al.*, 2007). In response to increasing levels of genomic stress, ATM targets BCL6 for phosphorylation and ubiquitin-mediated degradation (Phan *et al.*, 2007). BCL6 also prevents premature B cell activation induced in response to T cells, by down-regulating CD69, STAT1 and CD80 (Shaffer *et al.*, 2000; Niu *et al.*, 2003); the down-regulation of CD80 prevents interaction with the T cell ligand CD28, which should normally occur only in the light zone. BCL6 also blocks both plasma cell differentiation (by repressing the expression of BLIMP1) and memory cell differentiation (Parekh *et al.*, 2007; Shaffer *et al.*, 2000; Tunyaplin *et al.*, 2004; Vasanwala *et al.*, 2002; Kuo *et al.*, 2007).

1.1.4. Centrocytes and class switch recombination

Following SHM, centroblasts enter the light zones of GCs where they differentiate into a more heterogeneous population of centrocytes. Here, the centrocytes which have modified their antigen receptors during SHM are tested and selected for improved binding to the immunizing antigen. Only those B cells with favourable mutations will receive BCR stimulation coupled with co-stimulatory signals from antigen-enriched FDCs and T helper cells and will be rescued from apoptosis (Rothstein, 2000). These co-stimulatory signals include CD40 ligand, ICOS, TACI and BAFFR (Tafuri *et al.*, 2001; McAdam *et al.*, 2001; Castigli *et al.*, 2005; Hancz *et al.*, 2008).

Selected centrocytes undergo class switch recombination (CSR), a process of DNA recombination between two different switch regions located upstream of the constant (C) regions of *IgH* genes (Figure 1.1.3). Replacement of the initial Cμ (IgM) or Cδ (IgD) region by a downstream Cγ (IgG), Cα (IgA) or Cε (IgE) region results in the production of antibodies of different isotypes and effector functions (IgG, IgA, and IgE) with the same variable *IgV* region and thus the same antigen specificity and affinity (Toellner *et al.*, 1996). CSR also requires AID, which in centrocytes is regulated by IRF4 in response to the CD40/NF-κB signaling pathway (Muramatsu *et al.,* 2000; Klein *et al.,* 2006; Sciammas *et al.,* 2006).

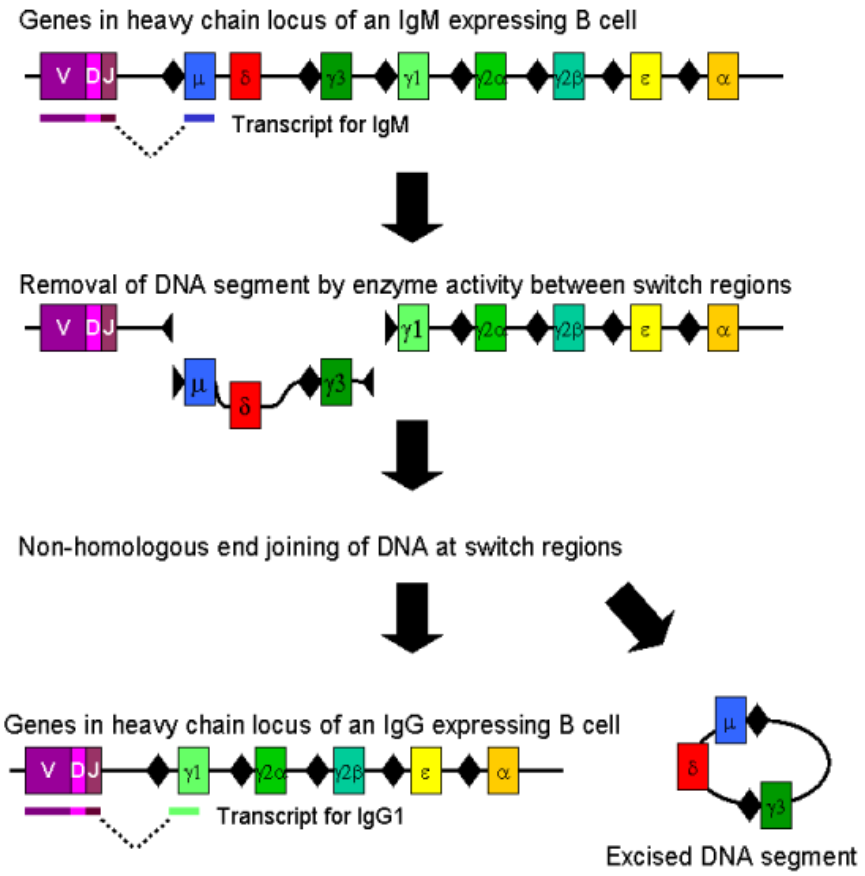


Figure 1.1.3. Mechanism of CSR. CSR involves DNA double-stranded breaks within conserved nucleotide motifs, called switch regions, which are upstream from gene segments that encode the C regions of IgH chains. This is catalysed by a series of enzymes including AID, UNG and AP-endonucleases, which introduce breaks into selected switch regions of DNA (Durandy, 2003; Casali and Zan, 2004). The free ends of the DNA are rejoined by non-homologous end-joining processes to link the variable domain exon to the desired downstream constant domain exon of IgH. The intervening DNA between the switch regions is deleted from the chromosome and forms an excision circle (Lieber *et al.,* 2006). With the exception of the μ and δ genes, only one antibody class is expressed by a B cell at any one point in time.
(taken from http://en.wikipedia.org/wiki/Immunoglobulin_class_switching).

1.1.5. Termination of the germinal centre transcriptional programme and post germinal centre B cell differentiation

The key event which terminates the GC transcriptional programme is the down-regulation of BCL6 (Shaffer *et al.,* 2000; Fearon *et al.,* 2002; Tunyaplin *et al.,* 2004). BCL6 down-regulation is mediated by several mechanisms; these include the transcriptional repression of the *BCL6* gene through NF-κB/IRF4 activation in response to CD40 triggering (Saito *et al.,* 2007); proteasomal degradation of BCL6 following phosphorylation by mitogen-activated protein kinases (MAPKs) in response to BCR engagement (Niu *et al.*, 1998), and p300-mediated acetylation of BCL6 (Bereshchenko *et al.*, 2002). BCR signalling also activates the PI3K/AKT pathway which in turn silences FOXO3A-dependent induction of BCL6 (Okkenhaug *et al.*, 2007; Fernandez de Mattos *et al.*, 2004; Omori *et al.*, 2006). BCL6 can also be down-regulated by IL-2-dependent activation of STAT5 (Cattoretti *et al.*, 2005a), OBF-1, PAX5 (Corcoran *et al.*, 2005), and MAD1; an E box protein which is highly expressed in plasma cells (Lee *et al.*, 2006).

Following the silencing of BCL6, GC B cells ($CD10^{+}$, $CD20^{+}$, $CD38^{low}$) differentiate into either precursors of antibody-producing plasma cells ($CD20^{-}$, $CD38^{high}$, $CD138^{+}$) or memory cells ($CD20^{+}$, $CD27^{+}$, $CD138^{-}$). The signals which determine if a GC B cell will differentiate into to a plasma cell or into a memory cell are only poorly understood, but are governed by the activity of several transcription factors (described below), as well as the strength of the BCR signal (Phan *et. al*, 2006; Paus *et. al*, 2006).

Plasma cell differentiation is regulated by at least three transcription factors: BLIMP1, IRF4 and XBP1 (Shapiro-Shelef *et al.*, 2003; Klein *et al.*, 2006; Sciammas *et al.*, 2006;

Reimold *et al.*, 2001). The contribution of these transcription factors to plasma cell differentiation is described in detail below (section 1.2.).

The processes which regulate memory cell differentiation are not fully understood and compared to plasma cell differentiation appear to be more stochastic, with a fraction of GC B cells constantly selected to enter the memory B cell pool (Blink *et. al*, 2005). Memory B cells retain high-level expression of B220, BCR, CD19, CD20, CD86, and MHC class II and can recirculate to peripheral lymphoid organs (Calame, 2006). There are two well described subsets of memory B cells: the more highly proliferative $B220^{+}$ memory cells, and $B220^{-}$ pre-plasma memory cells which are capable of rapid differentiation into plasma cells in response to antigen re-challenge (McHeyzer-Williams *et al.,* 2000; Driver *et al.,* 2001). It has been suggested that these cells represent distinct stages along a linear path of differentiation (Shapiro-Shelef *et al.,* 2003). The structure of GC is summarized in Figure 1.1.4.

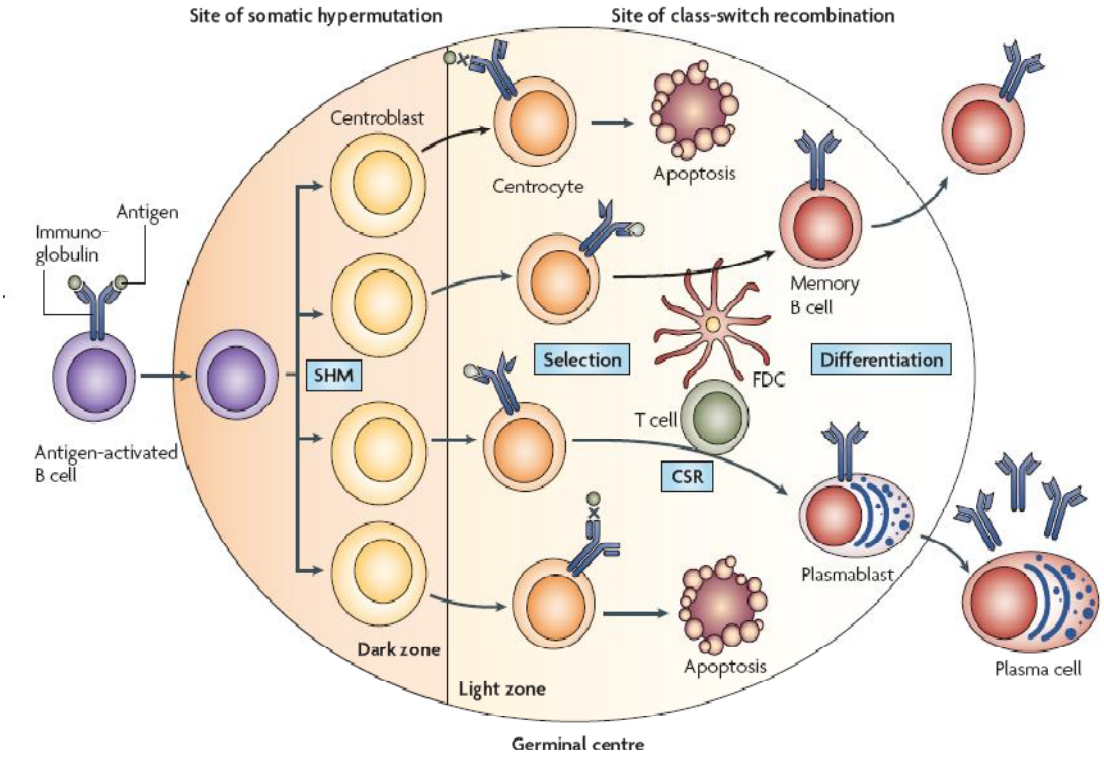


Figure 1.1.4. The GC reaction. GCs are highly organized anatomic structures essential for the clonal expansion of GC B cells. Naïve B cells activated by antigen differentiate to form centroblasts that proliferate in the dark zone and undergo SHM. Centroblasts develop into centrocytes in the light zone. Here, B cells are selected for their ability to bind antigen. Cells with high affinity BCR emerge as either long-lived memory cells or precursors of plasma cells. (de Vinuesa *et al.*, 2000; Endres *et al.*, 1999; Cyster *et al.*, 2000) (taken from Klein and Dalla-Favera, 2008).

1.2. B LYMPHOCYTE INDUCED MATURATION PROTEIN 1 (BLIMP1)

1.2.1. General introduction

BLIMP1, encoded by the *PRDM1* gene, is often described as the 'master regulator' of plasma cell differentiation. BLIMP1 was originally identified as a silencer of the human β-interferon gene (Keller and Maniatis, 1991) that bound to the positive regulatory domain I (PRDI) of the β-interferon promoter and was therefore designated 'positive regulatory domain I-binding factor 1' (PRDIBF1). Davis and colleagues isolated a murine cDNA that was induced following the cytokine-dependent differentiation of the mouse lymphoma cell line, BCL1, and named this B lymphocyte induced maturation protein 1 (BLIMP1) (Turner *et al.*, 1994; Blackman *et al.*, 1986). The same group also demonstrated that ectopically expressed BLIMP1 was sufficient to drive plasma cell differentiation in BCL1 cells (Turner *et al.*, 1994). Later, Huang recognized BLIMP1 as the mouse homolog of human PRDIBF1 (Huang *et al.*, 1994). Although the murine protein differs from the human protein by an additional 67 amino acids at the N terminus (Huang *et al.*, 1994), both proteins are highly homologous and interchangeable in functional assays. Definitive proof of the indispensable role of BLIMP1 in plasma cell differentiation and Ig secretion was provided by the Calame group using a conditional knock-out of BLIMP1 in mice (Shapiro-Shelef *et al.*, 2003).

1.2.2. Gene organization, protein domains, and biochemical function of BLIMP1

Gene structure

The human *PRDM1* gene is located on chromosome 6q21-q22.1 (Mock *et al.,* 1996) and encodes two major isoforms, designated BLIMP1α and BLIMP1β, which arise from alternate promoters (György *et al.,* 2003). The full-length BLIMP1α protein is responsible for plasma cell differentiation (Shapiro-Shelef *et al.,* 2003; Calame *et al.,* 2003). In contrast, BLIMP1β *is* transcribed from a novel promoter and new exon, 1β, located upstream of exon 4 of the full-length gene (György *et al.,* 2003) (Figure 1.2.1). The BLIMP1β protein lacks the first 101 amino acids of BLIMP1α and instead contains 3 novel amino acids fused to amino acids 102-789 of BLIMP1α. BLIMP1β, which lacks most of the PR domain, has a diminished capacity to repress target genes (György *et al.,* 2003). Since BLIMP1β contains the DNA-binding domain but bears a disrupted regulatory domain, it has been suggested that it might inhibit BLIMP1α (György *et al.,* 2003). In normal B cells, BLIMP1β mRNA levels are substantially lower relative to the full-length form (György *et al.,* 2003).

In mice, alternative splicing of exon 7 of *PRDM1* leads to production of the BLIMP1Δ7 protein which lacks the first 3 zinc fingers and is therefore predicted to be non-functional (Tunyaplin *et al.,* 2000). Despite having impaired DNA binding activity, the BLIMP1Δ7 form was shown to negatively regulate proliferation and cell survival when expressed in an immature B cell line and to interfere with the activity of full-length BLIMP1α, presumably by forming non-functional heterodimers (Schmidt *et al.,* 2008). The BLIMP1Δ7 isoform is preferentially expressed in naïve B cells where it might regulate the levels of BLIMP1α

(Schmidt *et al.,* 2008). A similar alternatively spliced form of the human protein was described (BLIMP1Δ6) (Smith *et al.,* 2010).

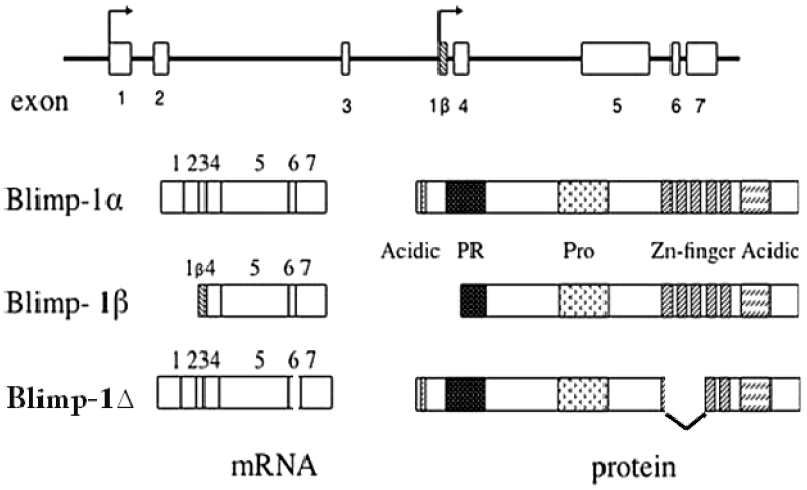


Figure 1.2.1. Schematic of the genomic structure and α, β and Δ mRNAs of human BLIMP1. BLIMP1β lacks the first 3 exons and the amino-terminal 101 amino acids of BLIMP1α and has a new exon, 1β. BLIMP1β transcripts initiate from an alternative promoter between exons 3 and 4. Numbered open boxes represent the exons. BLIMP1Δ6 lacks exon 6 and the first 3 zinc fingers (modified from Hangaishi and Kurokawa, 2010).

Protein domains

BLIMP1 is a member of PRDM gene family and is characterized by the presence of a PR domain so-called after it was identified in both the BLIMP1 (**P**RDIBF1) and the **R**b-binding protein RIZ1 proteins (Keller and Maniatis, 1991; Huang *et al.*, 1998). The PR domain is a subclass of the SET domain of histone methyl transferases (HMT) (Dillon *et al.*, 2005). Human BLIMP1 contains 789 amino acids with a predicted molecular weight of 87.9 kDa. Murine BLIMP1 consists of 856 amino acids and is predicted to be 95.8 kDa. BLIMP1

localises exclusively to the nucleus in both mouse and human cells (Angelin-Duclos *et al.*, 2000; Cattoretti *et al.*, 2005b). BLIMP1 contains five Krüppel-type zinc finger DNA-binding domains located near its C-terminus; however, only the first two finger motifs appear to be required for binding to target loci (Keller and Maniatis, 1992). The consensus-binding site for BLIMP1, the PRDI site, is an 11-bp sequence (A/C)AG(T/C)**GAAAG**(T/C)(G/T) and is similar to the binding sites for IFN regulatory factor (IRF)1 and IRF2 (Kuo and Calame, 2004). BLIMP1 also contains a proline-rich region and two acidic regions (one each at the N and C termini) (Figure 1.2.2). The proline-rich region and the first two zinc fingers of BLIMP1 are required for transcriptional repression.

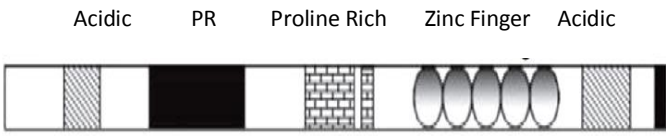


Figure 1.2.2. Domain structures of BLIMP1. The BLIMP1 protein harbors five zinc fingers involved in DNA binding and protein-protein interactions, two acidic regions, a PR domain and a proline rich region (taken from John and Garrett-Sinha, 2009).

Mechanisms of BLIMP1-mediated repression of target genes

BLIMP1 is a transcriptional repressor that uses different mechanisms to silence its target genes in a context-dependent manner. Individual domains of the BLIMP1 protein recruit specific co-repressor complexes or chromatin modifying enzymes to mediate transcriptional repression.

The proline-rich region and the zinc finger domains are involved in the recruitment of transcriptional co-repressors of the Groucho family (Ren *et al.*, 1999). BLIMP1 complexes with the G9a histone methyltransferase through the first 2 zinc fingers resulting in methylation of lysine 9 on histone H3 (H3K9) and repression of the interferon-β promoter (György *et al.*, 2004). The proline-rich region and the zinc finger domains also interact with histone deacetylases (HDAC)s 1/2 to deacetylate histone H3 (Yu *et al.*, 2000) as well as with a lysine-specific demethylase, LSD1 (Su *et al.*, 2009), that demethylates mono- or di-methyl groups on H3K4 (Shi *et al.*, 2004). It has been proposed that recruitment of HDACs 1/2 and LSD1 is a prerequisite for H3K9 methylation and for the silencing of mature B cell gene expression program during plasma cell differentiation (Su *et al.*, 2009). In addition, in primordial germ cells, BLIMP1 recruits an arginine-specific histone methyl transferase (Prmt5) that catalyzes the dimethylation of arginine 3 on histone H2A and H4 (Ancelin *et al.,* 2006). Thus, BLIMP1 serves as a scaffold to recruit proteins or co-repressor complexes that modify histones (by deacetylation, H3K9 methylation, and arginine methylation) and is doing so assembles silent chromatin over the target loci. However, it remains to be determined how exactly chromatin is modified at specific BLIMP1 target genes and whether chromatin modification is the only mechanism by which BLIMP1 represses transcription. Figure 1.2.3. summarises the mechanisms of repression by BLIMP1.

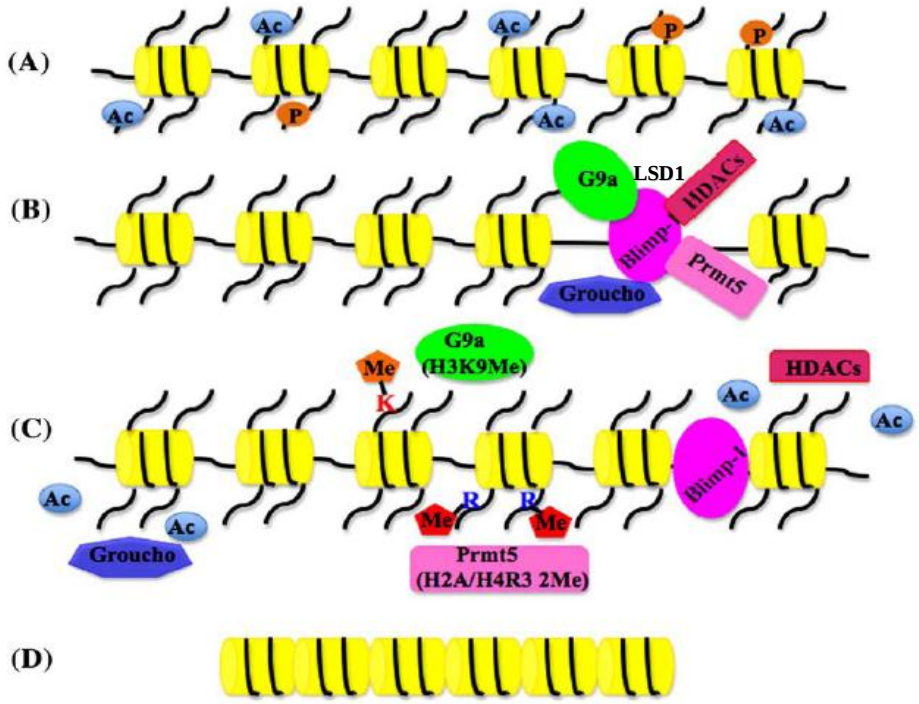


Figure 1.2.3. Mechanisms of BLIMP1-mediated gene repression. A) BLIMP1 target gene prior to BLIMP1 binding is transcriptionally active with open chromatin structure and acetylated histone tails. B) BLIMP1 recruits co-repressors Groucho, HDAC1/2, LSD1 and HMTs (G9a and Prmt5) to the target promoters. C) This results in histone modifications including the deacetylation and methylation of histone tails. D) The histone changes promote the adoption of a closed chromatin conformation to prevent gene transcription (modified from John and Garrett-Sinha, 2009).

1.2.3. BLIMP1 functions in B cells

1.2.3.1. Expression of BLIMP1 in different B cell subsets

BLIMP1 expression increases as plasma cell differentiation proceeds, so that levels are highest in long-lived plasma cells in the bone marrow (Kallies *et al.*, 2004). Plasma cells generated from secondary responses express higher levels of BLIMP1 than plasma cells generated during the primary response (Gonzalez-Garcia *et al.*, 2006). In GCs, BLIMP1 protein is expressed by a subset of GC B cells (up to 15%) with a phenotype intermediate between GC B cells and plasma cells ($BCL6^-$, $CD20^-$, $CD10^+$, $CD138^+$, $IRF4^+$). BLIMP1-positive GC B cells are non-apoptotic, some are proliferating and are probably centrocytes destined to leave the GC and become plasmablasts (Angelin-Duclos *et al.*, 2000). In contrast, BLIMP1 protein is not found in memory cells of either human (Kuo *et al.*, 2007) or murine (Blink *et al.*, 2005) origin.

1.2.3.2. BLIMP1 is required for plasma cell differentiation

BLIMP1 is not required for B cell development in the bone marrow or for the maturation of peripheral naïve B cells. However, BLIMP1 expression is essential and sufficient for terminal differentiation of all types of naïve B cells (Shapiro-Shelef *et al.*, 2003; Savitsky and Calame, 2006). The first indication of the importance of BLIMP1 in plasma cell differentiation was provided by the demonstration that ectopic expression of BLIMP1 in mouse B cell lines or in mouse primary splenocytes promoted a plasma cell-like phenotype (Turner *et al.*, 1994; Schliephake and Schimpl, 1996). Knock-out mice with a

conditional deletion in mature B cells of all five zinc finger domains of BLIMP1, showed normal B cell development and number (Shapiro-Shelef *et al.*, 2003). However, following challenge with both T-independent (TI) and T-dependent (TD) antigens, these mice showed a virtual absence of plasma cells and diminished Ig secretion. Moreover, mice lacking BLIMP1 in the B cell lineage, generated normal numbers of peripheral B cell subsets which were capable of self-renewal, but in which the secretion of all Ig isotypes was severely reduced. GCs in these mice were enlarged, suggesting a developmental block at the late/post-GC stages (Savitsky and Calame, 2006).

1.2.3.3. Plasma cell differentiation

Naïve, mature B cells can be subdivided into four subsets that belong to either the B-1 lineage- comprising B-1a ($B220^{low}IgM^{high}CD11b^{+}CD5^{+}$) and B-1b cells ($B220^{low}$ $IgM^{high}CD11b^{+}CD5^{-}$) or the B-2 lineage which can be further subdivided into follicular B cells ($B220^{+}CD23^{high}CD21^{low}$) and marginal zone B cells ($B220^{+}CD23^{low}CD21^{high}$) (Martin *et al.*, 2001). In response to diverse stimuli, each of these subsets can differentiate into plasma cells of distinct size, function, surface marker expression and location in lymphoid organs (Martin and Kearney, 2002).

Follicular plasma cell differentiation

Plasma cell differentiation of follicular B cells is a multi-step process, which occurs in GCs and critically depends on the interaction of B cells with $CD4^{+}$ T cells (Hasbold *et al.*, 2004).

The initial step of plasma cell differentiation is BLIMP1-independent and occurs as a result of the functional inactivation of PAX5 in mature B cells by a so far unidentified stimulus (Kallies *et al.*, 2007). PAX5 is a transcription factor that is essential for the commitment of lymphoid progenitors to the B cell lineage and for the maintenance of 'B-cell identity' (Cobaleda *et al.*, 2007). Because memory B cells continue to express PAX5, it has been suggested that the silencing of PAX5 is the critical step which diverts terminal differentiation towards plasma cells. The experimentally induced down-regulation of PAX5 in mature B cells results in the establishment of a 'pre-plasmablast' stage characterised by the secretion of low amounts of antibodies (Kallies *et al.*, 2007). The production of antibodies is mediated by the induction and splicing of XBP1, a transcription factor essential for plasma cell formation. XBP1 activates multiple genes which regulate antibody production, the ER stress response (the unfolded protein response), changes in cell size and protein synthesis (Reimold *et al.*, 2001; Calfon *et al.*, 2002; Shaffer *et al.*, 2004).

Pre-plasmablast are primed for BLIMP1 expression. The induction of BLIMP1 in these cells in necessary to stabilize and maintain the plasma cell differentiation program (Shapiro-Shelef *et al.*, 2003; Kallies *et al.*, 2007). Pre-plasmablasts are precursors of immature short-lived plasma cells, known as plasmablasts which have intermediate levels of BLIMP1, express Ig, and have a high rate of proliferation (Kallies *et al.*, 2004) and apoptosis (Messika *et al.*, 1998). Expression of IRF4 is induced in plasmablasts in response to NF-κB-mediated CD40 signalling or calcium-dependent activation of nuclear factor of activated T cells (NFAT) transcription factors (Grumont and Gerondakis, 2000; Lalmanach-

Girard *et al.,* 1993; Berberich *et al.,* 1994; Winslow *et al.* 2006). IRF4 expression is essential for plasma cell differentiation (Klein *et al.*, 2006; Sciammas *et al.*, 2006).

The differentiation of plasmablasts to long-lived, post-mitotic plasma cells producing large amounts of antibodies is associated with a further increase in BLIMP1 expression (Kallies *et al.*, 2004). Terminally differentiated plasma cells can migrate to survival niches in the bone marrow and spleen following chemotactic stimuli mediated by CXCR12 and CXCR4 (Kabashima *et al.*, 2006). Here, plasma cells receive survival signals from IL-6, BAFF or APRIL (Minges Wols *et al.*, 2002; O'Connor *et al.*, 2004) allowing them to survive, independently of antigen or cell division, for periods up to the lifetime of the organism (Slifka *et al.*, 1995; Manz *et al.*, 1997; Manz *et al.*, 1998; Hyland *et al.*, 1994). The continued expression of BLIMP1 is required for the maintenance of plasma cells in survival niches (Shapiro-Shelef *et al.*, 2005). BLIMP1 is not required for memory B cell formation (Shapiro-Shelef *et al.*, 2003), but is required for the development of pre-plasma memory B cells (Blink *et al.*, 2005; Driver *et al.*, 2001; Bell and Gray, 2003). The individual steps of follicular plasma cell differentiation are shown in Figure 1.2.4.

Extrafollicular plasma cell differentiation

B-1 B cells and marginal zone B cells are located in extrafollicular sites; marginal zone B cells are found predominantly in the spleen, and B-1 B cells in the peritoneal and pleural cavities (Herzenberg, 2000; Hardy and Hayakawa, 2001). Extrafollicular B cells express increased levels of BLIMP1 mRNA and decreased levels of transcriptional repressors of BLIMP1 (compared to their follicular counterparts) and can therefore rapidly differentiate

into plasma cells, which usually occurs independently of T cell help (Kuo *et al.,* 2007; Fairfax *et al.*, 2007). Extrafollicular B cells differentiate into short-lived plasma cells producing low affinity antibodies of predominantly IgM phenotype, which contribute mainly to the initial response against viral and bacterial infections (Manz *et al.*, 2002; Martin *et al.*, 2001).

1.2.3.4. Regulation of BLIMP1 and plasma cell differentiation

Transcriptional regulation of BLIMP1

The induction of BLIMP1 in B cells triggers irreversible plasma cell differentiation. Therefore, the repression of BLIMP1 in the earlier stages of B cell differentiation is essential to prevent inappropriate differentiation. At the same time, the rapid induction of BLIMP1 must occur to ensure a rapid response to antigenic challenge. The induction of BLIMP1 in B cells simultaneously requires both the repression of BLIMP1 inhibitors and the induction of BLIMP1 activators (Martins and Calame, 2008).

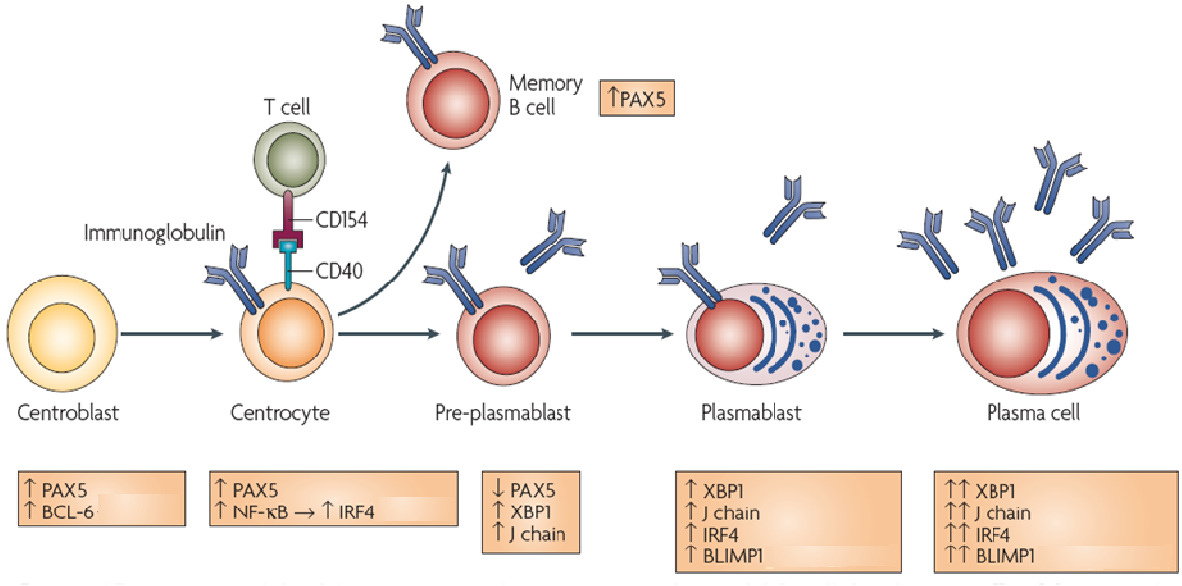


Figure 1.2.4. Termination of the GC transcriptional programme and post-GC B cell development. The stages of B cell differentiation from centroblasts to centrocytes and to terminally differentiated plasma and memory B cells following the stimulation of CD40 and antigen are indicated. Centrocytes appear to be a common precursor for both plasma cells and memory B cells. The down-regulation of PAX5 activity represents the crucial step in the initiation of plasma cell differentiation. Pre-plasmablasts form independently of BLIMP1 and secrete low levels of antibodies. Plasmablasts are immature plasma cells that express intermediate levels of BLIMP1 and antibodies, rapidly proliferate and have migratory potential. The further increase (approximately 5 fold) in BLIMP1 expression generates plasma cells which are long-lived, post-mitotic and produce large amounts of antibodies. The levels of BLIMP1, IRF4, XBP1 and J chain (a down-stream target of XBP1) and Ig secretion gradually increase with the progress of plasma cell differentiation (modified from Klein and Dalla-Favera, 2008).

Repression of BLIMP1

Three major proteins are known to repress BLIMP1 in naïve B cells, these are, BACH2, PAX5 and MITF. De-repression of BLIMP1 is required for plasma cell differentiation (Kuo *et al.*, 2007).

BACH2 delays BLIMP1 expression and plasma cell differentiation in mouse lymphocytes, and mice lacking BACH2 display a hyper-IgM syndrome and spontaneous plasma cell differentiation (Muto *et al.*, 2004; Muto *et al.*, 2010). BACH2 has been shown to interact with MAFK, which can bind to the *PRDM1* promoter and repress *BLIMP1* transcription (Muto *et al.*, 2004; Ochiai *et al.*, 2006). PAX5 represses BLIMP1 expression following its binding to a site in exon 1 of the *PRDM1* gene (Mora-Lopez *et al.*, 2007) and the loss of PAX5 has been shown to promote plasma cell differentiation (Nera *et al.*, 2006). PAX5 can also down-regulate BLIMP1 expression by up-regulating BACH2 (Schebesta *et al.,* 2007). MITF indirectly represses BLIMP1 by down-regulating IRF4. Consistent with this the loss of MIFT in mice induces spontaneous plasma cell differentiation (Lin *et al.*, 2004). BCL6 is the main repressor of BLIMP1 and plasma cell differentiation in GC B cells (Shaffer *et al.*, 2000; Reljic *et al.*, 2000). Thus, BCL6 knock-out mice show elevated levels of BLIMP1 mRNA and accelerated plasma cell differentiation (Tunyaplin *et al.*, 2004). MTA3, a cell type-specific subunit of the Mi-2-NuRD, acts as a co-repressor with BCL6 (Fujita *et al.*, 2004) and directly represses *BLIMP1* by binding to sites in intron 3 (Parekh *et al.*, 2007) and in intron 5 (Tunyaplin *et al.*, 2004). BCL6 represses the activity of the AP-1 transcription factor complex which has been shown to activate BLIMP1 (Vasanwala *et al.*, 2002). The transcription factors known to repress *PRDM1* in GC B cells are summarised in Figure 1.2.5.

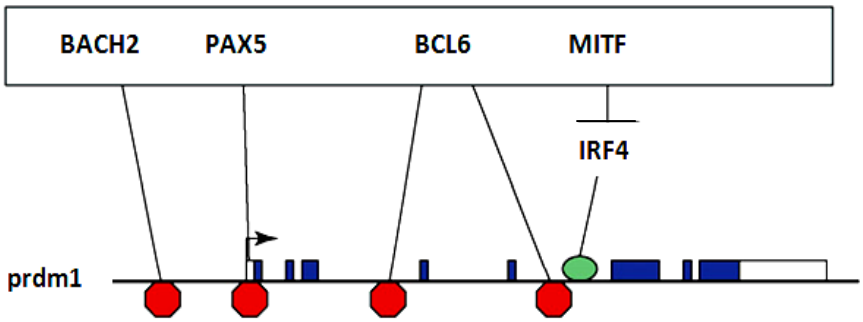


Figure 1.2.5. Repression of *PRDM1* transcription in B cells. The approximate binding sites of the direct repressors are shown in red and that of the activator, IRF4, in green. Blue boxes indicate individual *PRDM1* exons. The white box shows untranslated sequences (taken from Calame, 2008).

Activation of BLIMP1 expression

The removal of BLIMP1 repressors is not sufficient for plasma cell differentiation, but induces instead a memory-like phenotype suggesting a critical role in plasma cell differentiation, for those proteins which induce BLIMP1 expression (Kuo *et al.*, 2007). The main inducers of BLIMP1 include cytokines, toll-like receptors and NF-κB signalling.

Cytokines which induce BLIMP1

Several cytokines, including IL-2, IL-5, IL-6, IL-10 and IL-21, can induce BLIMP1 mRNA. For example, IL-5 alone, or in combination with IL-2 has been shown to induce BLIMP1 mRNA in BCL1 cells (Horikawa and Takatsu*,* 2006; Turner *et al.,* 1994). The addition of IL-2 and IL-10 to purified human GC B cells generates plasma cells (Arpin *et al.,* 1995). In contrast, the combination of IL-2 and IL-4 promotes B cell proliferation rather than differentiation (Arpin *et al.,* 1995). Furthermore, if human memory B cells are cultivated with IL-10, rapid plasmacytic differentiation occurs (Choe and Choi, 1998), accompanied by induction of BLIMP1 (Kuo *et al.,* 2007). IL-6 is known to be essential for the maintenance of long-lived plasma cells in the bone marrow (Minges Wols *et al.,* 2002) and has been shown to induce plasma cell differentiation in transformed human B cell lines (Chen-Kiang, 1995). IL-21 induces the expression of BLIMP1 mRNA as well as the differentiation of murine B cells to plasma cells (Ozaki *et al.,* 2004).

Many of these cytokines signal through JAK-STAT pathways (Shuai and Liu, 2003). For example, IL-2 activates STAT3 and STAT5 (Frank *et al.,* 1995), IL-5 activates STAT5 (de Groot *et al.;* 1998), IL-6 activates STAT3 (Heinrich *et al.,* 1998) and IL-10 activates STAT3

and STAT1 (Riley *et al.,* 1999). IL-21 mainly activates STAT3, but can activate STAT5 and STAT1 (Leonard *et al.,* 2005). An IL-21 response element downstream of the *PRDM1* promoter which recruits STAT3 and IRF4 has recently been identified (Kwon *et al.,* 2009). STAT3 is a strong inducer of BLIMP1 transcription. For example, the ectopic expression of a dominant negative form of STAT3 in BCL1 cells blocks BLIMP1 induction (Reljic *et al.,* 2000). Furthermore, STAT3-deficient mice lack IgG-expressing plasma cells (Fornek *et al.*, 2006). There is also evidence that STAT5 may induce BLIMP1 in an IL-2 and IL-5-dependent manner (Moriggl *et al.,* 1999; Horikawa and Takatsu, 2006). However, the activation of STAT5 in human B cells has been recently shown to block plasma cell differentiation (Scheeren *et al.,* 2005).

Toll-like receptors which induce BLIMP1

Plasma cell differentiation can also be efficiently induced by stimuli that trigger the activation of certain Toll-like receptors (TLR) (Ruprecht and Lanzavecchia, 2006). Engagement of TLR4 by lipopolysacharide (LPS) (Hoshino *et al.*, 1999) has been shown to induce plasma cell differentiation and BLIMP1 mRNA in murine B cells (Schliephake and Schimpl, 1996; Savitsky and Calame, 2006). The polysaccharides of *Ganoderma lucidum* (Reishi), which activate TLR4/TLR2, induced antibody production in purified murine splenic B cells as well as in human peripheral B cells; in both cases this was associated with the induction of BLIMP1 (Lin *et al.*, 2006). Furthermore, unmethylated CpG containing DNA, which triggers TLR9 activation (Hemmi *et al.*, 2001), has also been reported to induce plasma cell differentiation *in vitro* (Pasare and Medzhitov, 2005; Ruprecht and Lanzavecchia*,* 2006); an effect which could be mediated by PPARγ which

enhances B cell differentiation and antibody production in response to TLR9 and BCR-mediated stimulation of B cells (Garcia-Bates *et al.*, 2009). PPARγ signalling also promotes CpG-induced expression of COX-2 and BLIMP1. The activity of COX-2 in B cells has been shown to be important for optimal antibody production (Ryan *et al.,* 2005; Garcia-Bates *et al.*, 2009; Bernard and Phipp, 2010). In addition, the VAV family of Rho guanine nucleotide exchange factors, which mediate signalling via TLR4 and TLR9, were shown to act up-stream of BLIMP1 in mouse marginal zone cells (Stephenson *et al.,* 2006).

Regulation of BLIMP1 by NF-κB

NF-κB, which can be activated by various receptors on B cells including the BCR, TLRs, CD40, BCMA and BAFFR, can also induce BLIMP1 expression. In M12 and CH12 lymphoma lines, the induction of BLIMP1 mRNA which follows the activation of TLR4 by LPS, can be blocked by Helenalin, an inhibitor of the p65 subunit of NF-κB (Lyss *et al.,* 1998; Johnson *et al.,* 2005; Morgan *et al.*, 2009). Helenalin also inhibited the NF-κB-dependent up-regulation of BLIMP1 in response to stress stimuli in macrophages and in B cell lines (Doody *et al.*, 2006). In the activated B cell-like form of diffuse large B cell lymphoma (DLBCL), the constitutive activation of NF-κB in GC B cells is associated with loss of BLIMP1 expression, disruption of plasma cell differentiation and tumour development (Calado *et al.,* 2010).

Regulation of BLIMP1 by IRFs

After LPS stimulation, B cells from IRF4-/- mice fail to induce BLIMP1 mRNA and are prevented from undergoing plasma cell differentiation. IRF4 appears to induce BLIMP1

directly following its binding to a site situated between exons 5 and 6 of the *PRDM1* gene (Sciammas *et al.*, 2006). However, although mice with a conditional deletion of IRF4 in GC B cells also fail to undergo plasma cell differentiation, it was observed in another study that BLIMP1 mRNA induction in these IRF4-/- cells was similar to that observed in wild-type cells (Klein *et al.*, 2006). The discrepancy between these two studies remains unresolved, but could be because of minor differences in the activation conditions between experiments or to the stage of B cell differentiation targeted by the knockout. IRF5 has also been shown to bind to the IRF site in the *PRDM1* gene in mice and stimulate its expression (Lien *et al.,* 2010). The transcription factors known to activate *PRDM1* in B cells are summarised in Figure 1.2.6.

Regulation of BLIMP1 by miRNA

Although the regulation of BLIMP1 in B cells occurs primarily at the transcriptional level, a recent study showed that FDCs can induce BLIMP1 mRNA in B cells by down-regulating the expression of the miR-9 and let-7 families of miRNAs (Tunyaplin *et al.*, 2000; Lin *et al.*, 2011). At the same time, FDCs were shown to mediate the down-regulation of BCL6 by up-regulating expression of the miR-30 family. The down-regulation of BLIMP1 by miR-9 and let-7 has also been reported in Hodgkin's lymphoma (Nie *et al.,* 2008) and by let-7 in DLBCL (Nie *et al.,* 2010). BLIMP1 is also a target of let-7 in mouse embryonic stem (ES) cells and the inhibition of let-7 maturation by LIN28 and consequent induction of BLIMP1 is required for the specification of ES cells into primordial germ cell (West *et al.,* 2009).

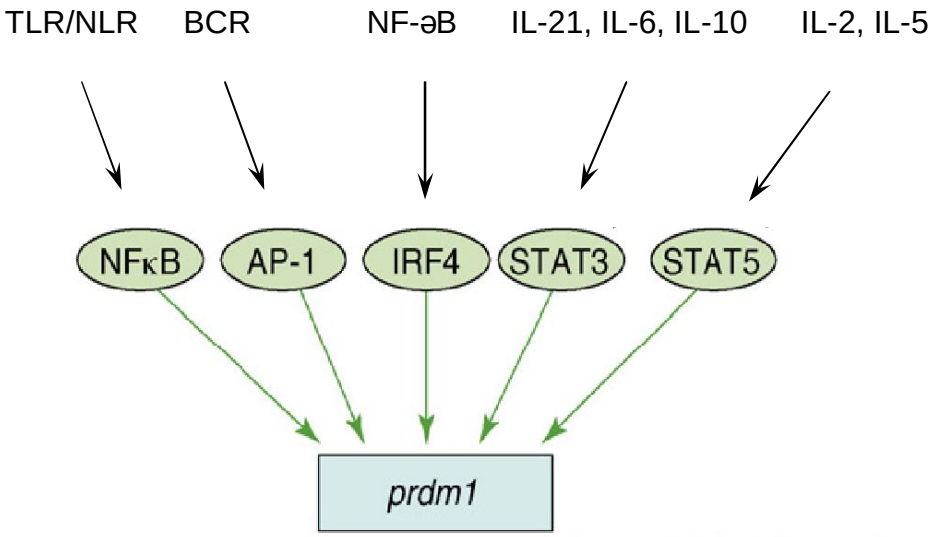


Figure 1.2.6. Activation of *PRDM1* transcription in B cells. Transcriptional activators of *PRDM1* are shown as green ovals, with mechanisms known to cause their induction or activation indicated above. The role for STAT5 is extrapolated from the effects of IL-2 and IL-5 (taken from Calame, 2008).

Induction of plasma cell differentiation

The developmental stage of B cells and the nature, strength and duration of signals that B cells receive combine to regulate BLIMP1 expression and plasma cell differentiation. MZ B cells and B-1 B cells can differentiate into antibody secreting cells if stimulated only by TLR ligands (Genestier *et al.,* 2007). In contrast, the T cell-dependent differentiation of follicular B cells requires synergistic signals delivered via antigen, T cells, TLRs and/or cytokines (Ruprecht and Lanzavecchia*,* 2006) (Figure 1.2.7.).

Although CD40 activates NF-κB, it does not favour plasma cell differentiation (Callard, *et al.*, 1995) and can inhibit BLIMP1 expression *in vitro* (Randall *et al.,* 1998). For example, when purified human GC cells were cultured with IL-2, IL-10, and with cells expressing CD40 ligand, cells with the characteristics of memory B cells were generated. Removal of CD40 ligand resulted in cells with the characteristics of plasma cells (Arpin *et al.,* 1995). Furthermore, the administration of CD40 to primary splenic B cells stimulated with LPS inhibited plasma cell differentiation in a dose-dependent fashion and was accompanied by reduction in the levels of intracellular BLIMP1 (Satpathy *et al.,* 2010).

Signals delivered following BCR ligation have been shown to either suppress or induce BLIMP1 expression and plasma cell differentiation (Kearney *et al.,* 1976). For example, continuous BCR stimulation activated the RAS/MEK/ERK pathway and inhibited the LPS-mediated induction of BLIMP1 and plasma cell dnifferentiation. Furthermore, in these experiments inactivation of ERK by the dual-specificity phosphatase 5 (DUSP5) restored BLIMP1 expression (Rui *et al.,* 2006). The combination of CD40, BCR and IL-4 signals blocked the LPS-dependent induction of BLIMP1 in mouse B cells (Knödel *et al.*, 2001).

Furthermore, the cultivation of human B cells with CD40, IL-4 and IL-21 strongly promoted plasma cell differentiation; the effect was dramatically reduced in the presence of BCR signalling (Caven *et al.,* 2007).

In contrast, Desai et al. (2009) reported that the treatment of CA46 lymphoma cells with anti-IgM up-regulated BLIMP1 mRNA and protein levels. Furthermore, synergistic signals delivered by CD40, BCR and IL-10 were shown to induce plasma cell differentiation in human B cell lymphocytes (Rousset *et al.,* 1992; Avery *et al.,* 2005; Choe *et al.,* 1996). Plasma cell differentiation was also observed when the BCR was stimulated with anti-IgM in human B cell cultures activated with anti-CD40 and IL-21 (Ettinger *et al.,* 2005).

1.2.3.5. BLIMP1 downstream targets

BLIMP1 target genes have been identified in transformed human B cell lines (Shaffer *et al.*, 2002) and in murine M12 cells (Sciammas and Davis, 2004) following their transfection with BLIMP1; and in BCL1 cells following their cytokine-induced differentiation (Sciammas and Davis, 2004).

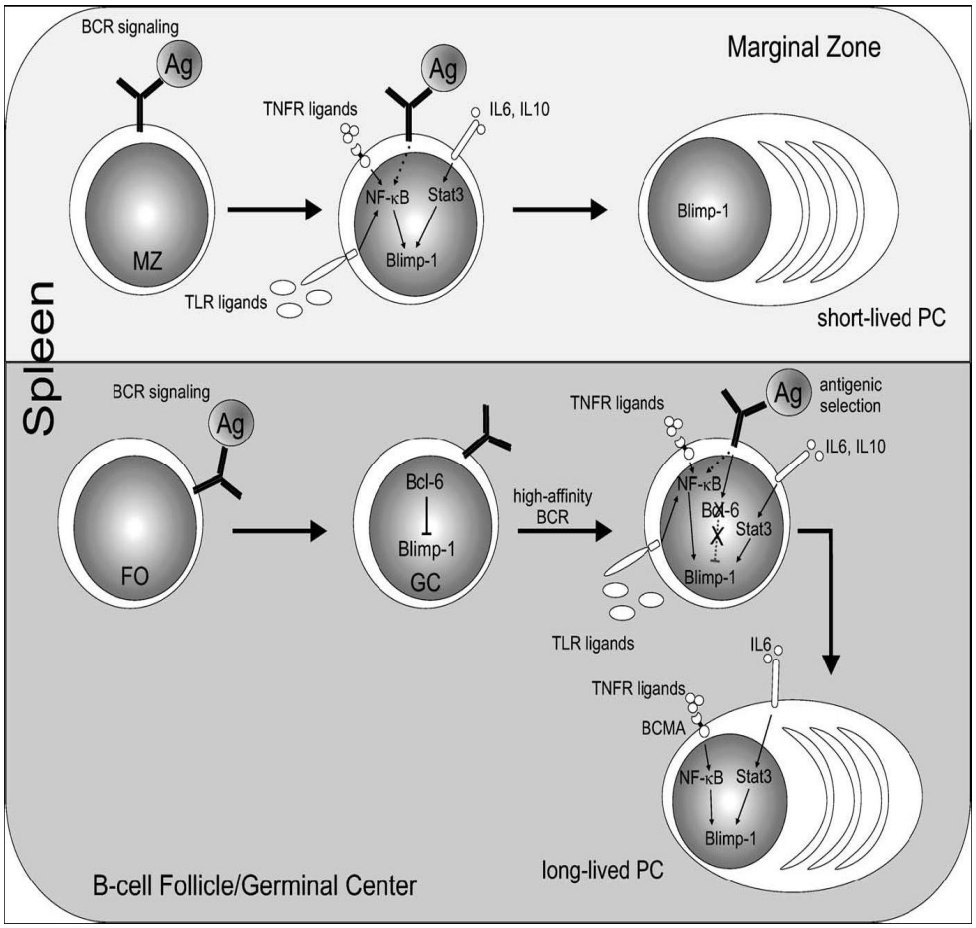


Figure 1.2.7. Model for the regulation of BLIMP1 expression during plasma cell differentiation. In comparison to extrafollicular B cells, GC B cells undergo prolonged ontogeny in order to create plasma cells secreting high-afinity antibodies. GC B cells express high levels of BCL6 which must be down-regulated prior to the terminal differentiation of the cells (taken from Johnson *et al.*, 2005).

BLIMP1 target genes controlling mature B cell functions and affinity maturation

BLIMP1 silences genes which specify B cell identity, these include: surface B cell markers (*CD19, CD20, CD22, CD45*); B cell activation markers (*CD69, MIP-1β*, *A1*); B cell-associated transcription factors (*BCL6, PAX5*, *SPIB, OCT-2, STAT6, EBF*); BCR signalling components (*BLNK, CD79A, SYK, BTK, PKCβ, LYN*); and, genes induced by BCR signalling (*A1, MIP-1β, CD69, CD83*, and *SPI-1/PU.1*) (Shaffer *et al.,* 2002). BLIMP1 also inhibits CSR by silencing genes essential for CSR and/or SHM (e.g. *KU70, KU86, DNA-PKCs and AID*) (Manis *et al.,* 1998; Muramatsu *et al.,* 2000; Zelazowski *et al.,* 1997; Shaffer *et al.,* 2002). BLIMP1 also silences *STAT6* expression (Shaffer *et al.,* 2002), which can modulate the switch to the IgG1 phenotype (Linehan *et al.*, 1998). *CIITA* is also repressed by BLIMP1 (Piskurich *et al.,* 2000; Shaffer *et al.,* 2002), which leads to the down-regulation of MHC class II genes and antigen presentation in plasma cells (Silacci *et al.,* 1994).

BLIMP1 participates in several reciprocal regulatory loops with other transcription factors, including BCL6, PAX5 and IR4 (Shaffer *et al.,* 2002; Lin *et al.,* 2002; Sciammas and Davis, 2004) (Figure 1.2.8.).

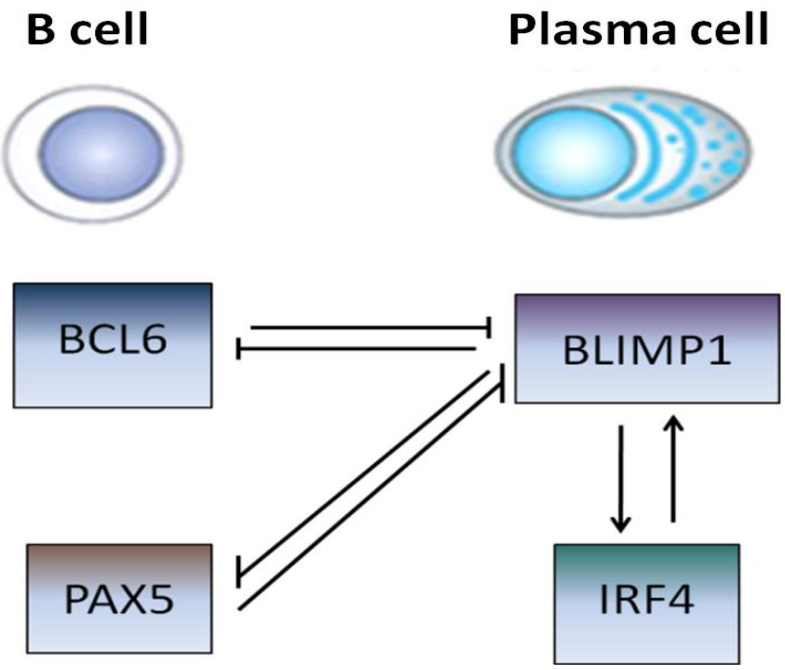


Figure 1.2.8. BLIMP1 participates in reciprocal regulatory loops. GC B cells express BCL6 and PAX5 which down-regulate BLIMP1. Upon plasma differentiation, BCL6 and PAX5 are silenced (as discussed earlier) and further down-regulated by BLIMP1 which makes the process of plasma cell differentiation irreversible.

BLIMP1 target genes controlling cell cycle and apoptosis

BLIMP1 has been shown to down-regulate genes involved in cell proliferation, partly by directly repressing *C-MYC* expression (Lin *et al.,* 1997; Shaffer *et al.,* 2002). Accordingly, many C-MYC target genes are also regulated by BLIMP1 (e.g. *RCL, ODC*, *LDH-A*, and *DHFR)* (Coller *et al.,* 2000; Eilers, 1999; Shaffer *et al.,* 2002). BLIMP1 also down-regulates genes involved in DNA synthesis and repair (*PCNA, PMS4, KU70, KU86, MCM2, primase*) and cell cycle progression (*PLK, aurora kinase, CKSHS1, CKSHS2, CDC2, CDK2, E2F-1*), perhaps as an indirect consequence of the cell cycle arrest due to the loss of C-MYC (Shaffer *et al.,* 2002). Although the repression of *C-MYC* transcription is necessary for the exit of B cells

from the cell cycle and terminal differentiation of B cells, the removal of C-MYC activity is not sufficient to trigger plasma cell differentiation (Lin *et al.,* 2000).

The expression of BLIMP1 in B cell lymphomas representative of immature or partially activated B cells induces cell cycle arrest and apoptosis, in part by repressing the expression of C-MYC and of the anti-apoptotic BCL2 family member A1 (BCL2A1). In contrast, in fully activated B cells BLIMP1 expression promotes differentiation (Messika *et al.,* 1998; Knödel *et al.,* 1999). This has led to suggestion that BLIMP1 expression induces growth arrest and cell death at earlier stages of B cell development, but promotes maturation and Ig secretion at later stages of B cell differentiation.

BLIMP1 target genes controlling immunoglobulin secretion and plasma cell functions

BLIMP1 activates genes involved in antibody production and the stress response (Shaffer *et al.,* 2002). This induction is in part is mediated by the inactivation of PAX5 and de-repression of down-stream targets of PAX5 including *XBP1* (Reimold *et al.*, 1996; Lin *et al.,* 2002; Sciammas and Davis*,* 2004), *J chain* (Rinkenberger *et al.*, 1996) and *IgH c*hain gene (Singh and Birshtein, 1993). Accordingly, BLIMP1-expressing cells exhibit a dramatic increase of both total *Ig* mRNA as well as individual *Ig* mRNA isoforms (Sciammas and Davis*,* 2004). BLIMP1 also up-regulates the expression of *CXCR4* and the integrin receptor *VLA4* (Sciammas and Davis*,* 2004), both of which have been shown to participate in the migration of plasma cells to specialized niches in the bone marrow (Kabashima *et al.*, 2006).

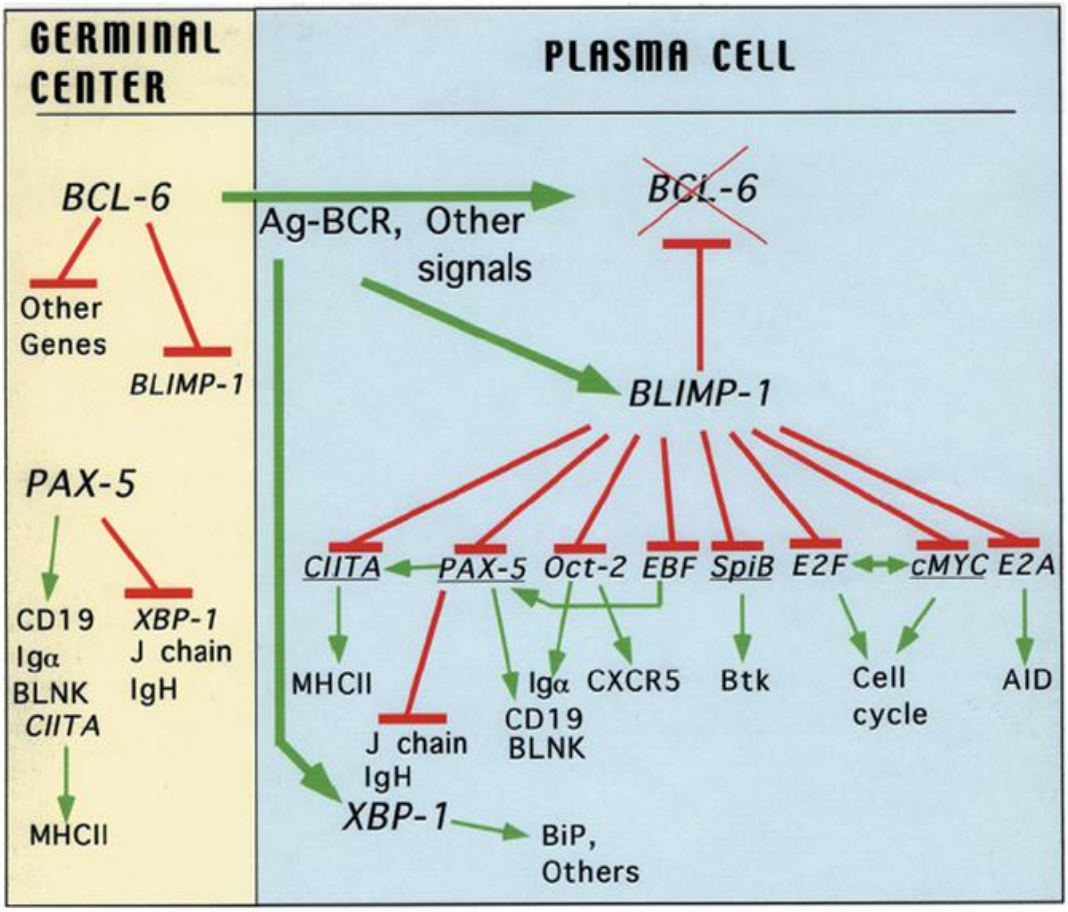


Figure 1.2.9. Transcriptional regulation that inhibits terminal B cell differentiation in the GCs and promotes it in plasma cells. Green arrows represent activation and red bars indicate repression (taken from Lin *et al.*, 2003).

1.2.4. BLIMP1 is a tumour suppressor gene

The region encompassing the *PRDM1* gene (6q21–q22.1) is frequently deleted in B cell lymphomas (Jackson *et al.,* 2000; Thelander *et al.,* 2008). Inactivation of the *PRDM1* gene was found in a subset of diffuse large B cell lymphoma (DLBCL) of the activated B-cell type (ABC) and is believed to contribute to lymphomagenesis by blocking post-GC B cell differentiation (Tam *et al.,* 2006; Pasqualucci *et al.,* 2006; Mandelbaum *et al.,* 2010). Although *PRDM1* mutations occur in only 25% of ABC-DLBCL biopsies; the majority of other cases of this subtype lack BLIMP1 protein, suggesting that additional mechanisms may inhibit BLIMP1 translation or stability (Pasqualucci *et al.,* 2006). For example, a role for the microRNA let-7 family in mediating the translational down-regulation of BLIMP1 in DLBCL has been proposed (Nie *et al.,* 2010). Alternatively, reciprocal translocations resulting in aberrant expression of BCL6 could contribute to the pathogenesis of DLBCL (Pasqualucci *et al.,* 2006). The malignant Hodgkin/Reed–Sternberg (HRS) cells of classical Hodgkin lymphoma (cHL) have BLIMP1 mRNA (Garcia *et al.,* 2006). However, several studies show that in most cases BLIMP1 protein is only weakly expressed by HRS cells (Buettner *et al.,* 2005; Cattoretti *et al.,* 2005b; Garcia *et al.,* 2006; Natkunam *et al.,* 2007). This could indicate that plasma cell differentiation is initiated in a fraction of HRS cells but remains abortive (Buettner *et al.,* 2005). The absence of BLIMP1 protein in HRS cells could be due to the overexpression of miR-9 and let-7a miRNA (Nie *et al.,* 2008). The malignant lymphoplasmacytic cells found in patients with Waldenstrom's macroglobulinemia also express lower levels of BLIMP1 compared to normal plasma cells (Gutierrez *et al.,* 2007). Deleterious mutations of *PRDM1* associated with loss of BLIMP1 protein have also been reported in primary central nervous system lymphoma (Courts *et al.,* 2008). Finally, ETS-1,

the transcription factor, which is amplified in certain leukemias, physically interacts with BLIMP1 leading to a block in BLIMP1 DNA binding activity and a reduction in the ability of BLIMP1 to repress target genes (Rovigatti *et al.,* 1986; Crossen *et al.,* 1999; Yoshida *et al.,* 1999; Sait *et al.,* 2002; John *et al.,* 2007).

In contrast, the over-expression of the BLIMP1β isoform has been reported in multiple myeloma, DLBCL and in some T cell lymphomas (György *et al.,* 2003; Ocana *et al.,* 2006; Liu *et al.,* 2007; Zhao *et al.,* 2008). BLIMP1β over-expression is associated with advanced Ann Arbor stage and a high-risk International Prognostic Index in T cell lymphomas and with a shorter patient survival in both DLBCL and T cell lymphoma patients (Liu *et al.,* 2007; Zhao *et al.,* 2008). In both B and T cell lymphomas, BLIMP1β expression is also associated with *in vitro* resistance to chemotherapeutic agents (Liu *et al.,* 2007; Zhao *et al.,* 2008). Interestingly, the up-regulation of the BLIMP1β isoform in T cell lymphomas was associated with high C-MYC levels (Zhao *et al.,* 2008). A recent study by Zhang et al. (2010) demonstrated that the up-regulation of the PRDM1β isoform was associated with hypomethylation of the PRDM1β specific promoter in a subset of DLBCL with aggressive behaviour.

1.3. THE EPSTEIN-BARR VIRUS

1.3.1. General introduction

Epstein-Barr virus (EBV) infects more than 90% of humans and in most individuals is carried life-long as a silent passenger (Rickinson and Kieff, 2001). Primary infection usually occurs in early childhood and is often asymptomatic (Fleisher *et al.*, 1979). EBV is orally transmitted and is believed to replicate in epithelial cells of the oropharynx (Allday and Crawford, 1988), leading to the release of high titres of infectious virions into the throat (Sixbey *et al.*, 1984). In order to achieve long-term persistence *in vivo*, EBV establishes a latent infection with approximately 1 in 10^6 B cells carrying the virus in a latent state, where the viral genome is maintained (Thorley-Lawson, 2001). During its life cycle, the virus also periodically enters the replicative (lytic) cycle when the viral DNA is amplified and numerous viral lytic cycle genes are expressed to generate infectious virus for transmission to other susceptible hosts (Kieff, 1996).

When primary infection with EBV is delayed until adulthood, it can result in acute infectious mononucleosis (IM) (Henle and Henle, 1979) with clinical features of fever, sore throat, headache, malaise, fatigue, weakness, enlarged tonsils, lymphadenopathy, hepato-splenomegaly and mononuclear leukocytosis (Ebell, 2004).

EBV is also associated with a number of cancers arising in both lymphoid and epithelial tissues. EBV-positive tumours have characteristic phenotypes and patterns of EBV gene expression, suggesting that EBV might contribute to the pathogenesis of each tumour type in different ways (Landais *et al.*, 2005). Some diseases, such as endemic Burkitt's

lymphoma (BL), are almost always EBV-positive, indicating an essential role for EBV. Others, for example, Hodgkin´s lymphoma (HL) and gastric carcinoma, have smaller fractions associated with EBV.

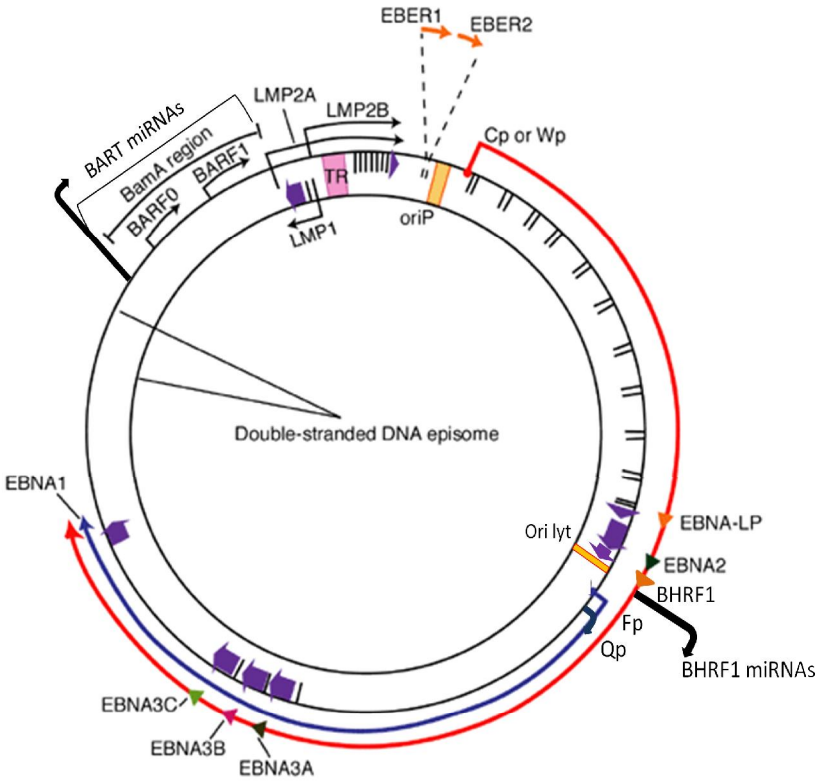
1.3.2. Classification and structure

EBV is a lymphotropic virus of the γ-herpesvirinae subfamily of the *Lymphocryptovirus* (LCV) genus. EBV has been found exclusively in humans and like other LCV, EBV has the capacity to drive autonomous B cell growth (Davison *et al.*, 2002; Marr-Belvin *et al.*, 2008).

The viral genome consists of 172 kilobase (kb) of linear double-stranded DNA, which enfolds an icosahedral nucleocaspid composed of 162 capsomeres surrounded by an inner and outer envelope and a toroid-shaped protein core. A 152 kDa protein tegument is found between the nucleocaspid and an inner envelope as well as glycoprotein spikes on the surface of the outer membrane structure (Johannsen *et al.*, 2004).

The EBV genome has been cloned and sequenced (Baer *et al.*, 1984). Because the genomic viral DNA was digested with the *Bam* HI restriction enzyme; open reading frames, genes and sites for transcription or RNA processing are referenced to specific *Bam* HI fragments, in descending order of fragment size, from A to Z (Figure 1.3.1b). EBV genome consists of 0.5 kb unique segments composed of 60% guanine or cytosine with relatively short terminal direct repeats (TR) sequences (Hayward and Kieff, 1977) and connecting internal repeat (IR) sequences dividing the genome into short or long sequence domains (Given and Kieff, 1979).

A



B

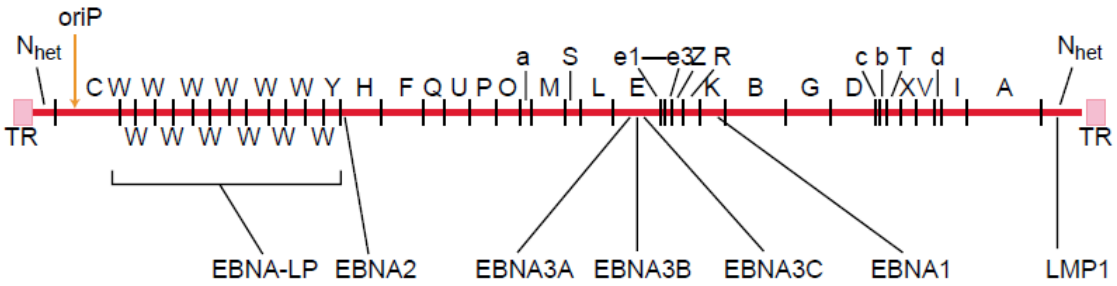


Figure 1.3.1. Schematic representation of the EBV genome. A) Transcription map of the EBV latent genes on the double-stranded viral DNA episome. The origin of plasmid replication (oriP) and lytic replication (ori lyt) are shown in orange. The large solid blocks (in purple) represent coding exons for each of the latent proteins and the arrows indicate the direction in which they are transcribed. The TR region is formed as the linear DNA joins at each end to produce the circular viral episome (indicated in pink). B) The location of open reading frames for each of the EBV latent proteins on a *Bam* HI restriction endonuclease map of the original B95.8 EBV genome (modified from Murray and Young, 2001).

1.3.3. Epstein-Barr virus latency and growth transformation *in vitro*

EBV can infect resting B lymphocytes *in vitro* through binding of viral glycoproteins gp350 and gp42 to the CD21 receptor and human leukocyte antigen (HLA) class II molecules on surface of B cells (Nemerow *et al.*, 1987; Borza and Hutt-Fletcher, 2002). This results in the establishment of permanently growing immortalised lymphoblastoid cell lines (LCLs), which express a number of viral genes including the Epstein-Barr nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and -LP), latent membrane proteins (LMPs 1, 2A and 2B), the *Bam*HI A rightward transcripts (BARTs) and the small non-polyadenylated RNAs (EBERs 1 and 2) (Kieff and Rickinson, 2001). The relative positions and orientation of the latent EBV genes are shown in Figure 1.3.1a.

In LCL, all EBNA mRNAs are generated by differential splicing of the same primary transcript expressed from either the Cp or Wp promoter (Speck and Strominger, 1985). The LMP1 gene can be transcribed from two separate promoters; the proximal ED-L1 and the distal TR-L1. The LMP1 promoters are regulated by different viral and cellular factors (Chang *et al.*, 2004, Chen *et al.*, 2001a; Goormachtigh *et al.*, 2006). For example, EBNA1 can stimulate LMP1 transcription from both ED-L1 and TR-L1 through a distal enhancer (Gahn and Sugden, 1995). In contrast, EBNA2 can only activate ED-L1 (Johannsen *et al.*, 1995). ED-L1 can also be activated by cellular proteins including NOTCH1, IRF7, STATs, upstream stimulatory factor (USF), and ATF-1/cAMP responsive element binding protein (CREB)-1 (Chang *et al.*, 2004). TR-L1 is activated by cellular transcription factors which include SP1/SP3 and STATs (Tsai *et al.*, 1999).

LMP2A and LMP2B are transcribed under the control of two separate promoters separated by 3 kb and differ only in their first exons; exon 1 of LMP2A encodes a 119-amino-acid N-terminal tail, while exon 1 of LMP2B is non-coding (Laux *et al.*, 1988; Laux *et al.*, 1989; Longnecker and Kieff, 1990; Sample *et al.*, 1989). The promoter of LMP2A lies directly upstream of the first exon, while LMP2B shares a bidirectional promoter with LMP1 (Laux *et al.*, 1989; Sample *et al.*, 1989). Both are transcribed across the fused terminal repeats of the EBV episome. More recently, a novel LMP2 transcript originating in the TRs and expressed in NK/T cell lymphomas has been described (Fox *et al.*, 2010).

LCLs express high levels of the B cell activation markers CD23, CD30, CD39 and CD70 and the cellular adhesion molecules LFA1 (CD11a/18), LFA3 (CD58) and ICAM1 (CD54) (Rowe *et al.*, 1987; Gregory *et al.*, 1988). These markers are usually found at such high levels only on activated B cells (Young *et al.*, 2000) suggesting that EBV-induced immortalisation can be achieved through the constitutive activation of cellular pathways that normally drive physiological B cell proliferation.

The viral growth program described in LCLs is also found in post-transplant lymphoproliferative disease and is referred as latency III. Two other major forms of EBV latency, latency I and II, are described. Latency I, observed in EBV-positive BL tumours, is typified by expression of EBNA1, EBERs and BARTs (Gregory *et al.*, 1990; Rickinson and Kieff, 2001; Rowe *et al.*, 1987; Nonkwelo *et al.*, 1996). In contrast, latency II, found in EBV-positive HL and in a proportion of NPCs (Brooks *et al.*, 1992; Deacon *et al.*, 1993; Pallesen *et al.*, 1991; Young *et al.*, 1988), is characterized by expression of the EBERs, EBNA1 and BARTs transcripts along with LMP1 and LMP2A/B. Some studies have defined a fourth

form of latency in peripheral B cells referred to as 'latency 0', here only the EBERs and possibly LMP2 are expressed (Chen *et al.*, 1995).

Following B cell infection, the first viral genes to be expressed are EBNA-LP and EBNA2 (Alfieri *et al.*, 1991); followed by EBNA2-driven expression of LMP1, LMP2 and other viral genes (Wang *et al.*, 1990). The EBNAs are transcribed either from Cp or Wp promoters located in the *Bam* HI C and W fragment of EBV genome (Woisetschlaeger *et al.*, 1991). The Wp promoter is used during the initial phase of EBV infection and is subsequently replaced by the Cp promoter which is transactivated by EBNA2 (Schlager *et al.*, 1996). In latency I and II, EBNA1 is transcribed from the Qp promoter located in the *Bam* HI Q region.

1.3.4. Epstein-Barr virus infection and persistence *in vivo*

Although EBV persists in B cells, it has been suggested that epithelial cells of the oropharynx are the initial target of primary infection and that these cells replicate the virus which then spreads to B cells (Morgan *et al.*, 1979). CD21 is not expressed on most epithelial cells suggesting that infection of epithelial cells involves mechanisms distinct from those required for B cell infection (Shannon-Lowe *et al.*, 2009).

Newly-infected B cells expressing the growth programme can be recognized by NK cells and latent-antigen-specific CTLs and CD4 T helper cells (Williams *et al.*, 2005; Hislop *et al.*, 2007). However, a fraction of these cells can down-regulate expression of most viral antigens to evade immune recognition, and establish a stable pool of resting EBV-positive

memory B cells (Young and Rickinson, 2004) (Figure 1.3.2). Two models of how the virus achieves this have been suggested. In the first model, EBV directly infects already established memory B cells. In the second model, EBV infects naïve B cells and activates them through the coordinated expression of all nine latent proteins of the growth latency III programme. The EBV-activated B blasts then enter GCs of secondary follicles and switch into the viral latency II programme. Through surrogate T cell help (LMP1) and BCR engagement (LMP2A), the EBV infected cells receive the signals necessary for antigen-independent proliferation in the GC and subsequently for memory B cell differentiation (Caldwell *et al.,* 1998; Caldwell *et al.,* 2000; Roughan and Thorley-Lawson*,* 2009). In memory B cells viral antigen expression is down-regulated (latency 0). Alternatively, GC B cells can undergo plasma cell differentiation which is associated with the induction of viral lytic cycle (Thorley-Lawson, 2001).

It is proposed that EBV-positive peripheral memory B cells remain under the control of the normal physiological mechanisms that regulate memory B cell migration and differentiation (Laichalk *et al.*, 2002), and occasionally enter GC reaction to differentiate into plasma B cells or replenish the reservoir of memory cells (Young and Rickinson, 2004).

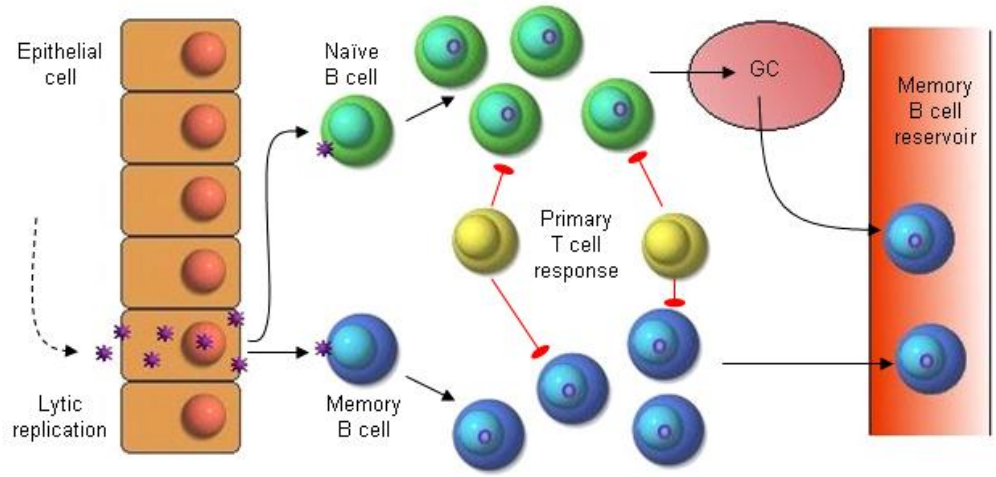


Figure 1.3.2. Primary infection of EBV *in vivo.* EBV establishes initial lytic replication in the oropharynx and subsequently spreads to B cells. Two possible mechanisms of B cell infection are shown: EBV infects naïve B cells and drives them through GC reaction to produce memory B cells or EBV might directly infect already established memory B cells. Numbers of virus infected cells are controlled by the host immune response, where a fraction of the cells down-regulate antigen expression to escape immune recognition and establish a stable reservoir of resting EBV-positive memory B (modified from Young and Rickinson, 2004).

1.3.5. Epstein-Barr virus latent proteins

Epstein-Barr nuclear antigen 1 (EBNA1)

EBNA1 binding to OriP, the plasmid origin of viral replication, is essential for virus replication during latency (Lupton and Levine 1985; Yates et al 1985). EBNA1 also tethers viral episomes to host chromosomes ensuring the partitioning of viral genomes during cell division (Marechal et al. 1999; Sears et al., 2004). EBNA1 transactivates the Cp and LMP1 promoters and can bind Qp to negatively regulate its own expression (Kieff, 1996; Nonkwelo *et al.*, 1996). More recently, EBNA1 was shown to regulate EBERs expression by increasing pol III transcription following its binding to and activation of ATF2, C-MYC and TFIIIC genes (Owen *et al.*, 2010).

Although originally thought to be indispensable for EBV-induced transformation of B cells, more recently it has been shown that LCLs can be generated from an EBNA1-deficient EBV mutant, albeit with an efficiency several thousand-fold less than with wild-type EBV (Humme *et al.,* 2003).

There are conflicting reports of the role of EBNA1 in oncogenesis. Although, it was originally reported that EBNA1 expression in B cells could induce lymphomas in transgenic mice, other studies have not been able to reproduce this effect (Wilson *et al.*, 1996; Kang *et al.*, 2005; Kang *et al.*, 2008; Tsimbouri *et al.*, 2002). Expression of EBNA1 in EBV negative Akata BL cells demonstrated that EBNA1 alone is not sufficient to confer tumourigenic potential (Komano *et al.*, 1998; Ruf *et al.*, 2000). Furthermore, a dnEBNA1 did not affect the growth of a LCL carrying an integrated EBV genome (Kang *et al.*, 2001).

In contrast, EBNA1 expression in HL cells was shown to enhance tumour formation in NOD-SCID mice (Kube *et al.*, 1999), whereas its down-regulation either by introduction of a dnEBNA1 or by RNAi reduced the proliferation and survival of EBV-positive BL cells (Kennedy *et al.*, 2003; Hong *et al.*, 2006). EBNA1 expression was also shown to increase tumour formation and metastasis of NPC and breast carcinoma cells following their transplantation into nude mice (Sheu *et al.*, 1996; Kaul *et al.*, 2007). Some of the apparent tumour promoting effects of EBNA1 are likely to be mediated by the ability of EBNA1 to bind to cellular DNA and to modulate cellular gene expression through transcription factors which include AP-1 and NF-κB (Wood *et al.*, 2007; Canaan *et al.*, 2009; O'Neil *et al.*, 2008; Valentine *et al.*, 2010). EBNA1 was also shown to disrupt PML nuclear bodies which impaired DNA repair and apoptosis in NPC cells (Sivachandran *et al.*, 2008).

The EBNA1 protein contains a repetitive sequence of glycine-alanine amino acids which makes it relatively resistant to ubiquitin-proteosome mediated degradation and therefore epitopes from EBNA1 cannot be efficiently processed through the MHC class I pathway (Levitskaya *et al.*, 1995). In contrast, EBNA1 generally elicits strong CD4-positive T cell responses and contains several well-defined CD4-positive epitopes. However, endogenous presentation of some CD4 T cell epitopes from EBNA1 requires autophagy, a process restricted to the cytoplasm (Leung *et al.*, 2010).

Epstein-Barr nuclear antigen 2 (EBNA2)

EBNA2 is required for EBV induced transformation as demonstrated by the inability of the P3HR-1 virus strain, in which the EBNA2 gene is deleted, to transform B cells *in vitro* (King *et al.*, 1982; Kieff and Rickinson, 2001). The restoration of the EBNA2 gene into P3HR-1 by homologous recombination retrieves the transformation ability of EBV (Hammerschmidt and Sugden, 1989; Cohen *et al.*, 1989).

EBNA2 functions as a transcriptional activator of both viral and cellular genes (Kieff and Rickinson, 2001). For example, EBNA2 up-regulates LMP1 (Tsang *et al.*, 1991), LMP2 (Zimber-Strobl *et al.*, 1991), CD21 (Wang *et al.*, 1990) and CD23 (Cordier *et al.*, 1990) and also initiates the switch from Wp to Cp promoter early in B cell infection (Rooney *et al.*, 1992). EBNA2 interacts with a sequence-specific DNA-binding protein, RBP-Jκ (Grossman *et al.*, 1994). In Drosphilia, the RBP-Jκ homologue is involved in signal transduction from the NOTCH receptor, a pathway that is important in determining cell fate, and which has also been linked to the development of T cell tumours in humans (Artavanis-Tsakonas *et al.*, 1995). EBNA2 can functionally replace the intracellular region of NOTCH (Sakai and Honjo, 1997; Strobl *et al.*, 2000; Hsieh *et al.*, 1997) leading to the transactivation of its viral and cellular down-stream targets. The cellular C-MYC, cyclin D2 and cyclin-dependent kinase (cdk)-4 are also transcriptional targets of EBNA2 and are likely to be important for EBV-induced B cell proliferation (Jayachandra *et al.*, 1999; Kaiser *et al.*, 1999).

Epstein-Barr nuclear antigen 3 (EBNA3) family

The EBNA3 family members are the least abundant EBNA mRNAs in latently infected cells. Their distribution is similar to that of EBNA2, with large clumps localised in the nucleus, nuclear matrix and chromatin (Petti *et al.*, 1990). All three proteins interact with RBP-Jκ which can lead to disruption of its binding to DNA and to EBNA2, thus repressing EBNA2-mediated transactivation (Robertson and Ambinder, 1997). *In vitro* studies show that both EBNA3A and EBNA3C (Tomkinson *et al.*, 1993), but not EBNA3B (Robertson, 1997) are essential for B cell transformation, although more recently EBNA3A deleted EBV has been used to transform B cells albeit with reduced efficiency (Hertle *et al.*, 2009). All the EBNA3 proteins have repressive activities when targeted to DNA which can be explained by their interaction with several cellular factors involved in transcriptional repression or silencing, including histone deacetylases and C-terminal binding protein (CtBP) (Hickabottom *et al.*, 2002; Radkov *et al.*, 1999). EBNA3A and EBNA3C can co-operate with oncogenic ras to transform embryonic rat fibroblasts (Parker *et al.*, 1996). EBNA3C is required for cell cycle progression in LCLs, can mediate the degradation of the retinoblastoma protein pRb and can stimulate cyclin A dependent kinase activity (Knight *et al.*, 2004; Knight *et al.*, 2005; Maruo *et al.*, 2006). Consistent with its ability to override cell cycle check points, EBNA3C is thought to contribute to increased genomic instability in infected cells (Gruhne *et al.*, 2009). EBNA3C has been shown to activate expression of some cellular (e.g. CD21) and viral genes (e.g. LMP1) (Wang *et al.*, 1990; Allday and Farrell, 1994). Although it has been shown that EBNA3A and EBNA3C can contribute to apoptosis protection in BL cells, another study showed no effect of the EBNA3 family on BL cell survival (Kelly *et al.*, 2009; Anderton *et al.*, 2008).

Epstein-Barr nuclear antigen leader protein (EBNA-LP)

EBNA-LP is not absolutely essential for *in vitro* transformation of B cells, but is required for the efficient outgrowth of LCLs (Allan *et al.*, 1992). EBNA-LP is diffusely localised in cell nuclei with some concentrated areas assembling small nuclear granules (Petti *et al.*, 1990) which are assumed to contribute to EBV RNA processing (Jiang *et al.*, 1991). EBNA-LP is encoded by the leader of each of the EBNA mRNAs generating proteins of variable size depending on the number of *Bam* HI W repeats present in each specific EBV isolate (Bodescot *et al*., 1984). EBNA-LP in cooperation with EBNA2 induces cyclin D2 expression resulting in G0 to G1 transition and cell cycle progression in B cells (Sinclair *et al.*, 1994). EBNA-LP also enhances the ability of EBNA2 to transactivate its down-stream targets including LMP1 (Harada and Kieff, 1997; Nitsche *et al.*, 1997).

Latent membrane protein 1 (LMP1)

LMP1 is essential for the EBV-induced transformation of B cells, and can transform rodent fibroblasts and induce tumours in transgenic mice (Kaye *et al.*, 1993; Wang *et al.,* 1985; Moorthy and Thorley-Lawson, 1993; Wang *et al.,* 1985; Kulwichit *et al.,* 1998; Stevenson *et al.,* 2005). In B cells, LMP1 induces expression of the cell-surface adhesion molecules, ICAM1, LFA1 and LFA3 and genes associated with B cell activation, including CD23, CD30 and CD40 (Wang *et al.*, 1990). LMP1 also induces IL-6, IL-8 and IL-10 expression (Eliopoulos *et al.*, 1999; Eliopoulos *et al.*, 1997; Nakagomi *et al.*, 1994), and up-regulates several anti-apoptotic proteins, including MCL-1, A20 and BCL2 (Wang *et al.*, 1996; Laherty *et al.*, 1992; Henderson *et al.*, 1991).

LMP1 is a membrane protein that has a long cytoplasmic C-terminal domain, six hydrophobic membrane-spanning domains and a cytoplasmic N-terminal domain (Liebowitz *et al.*, 1986). The N-terminal domain orientates LMP1 in the cell membrane; the six transmembrane loops promote self aggregation and oligomerisation; and the C-terminal domain possesses most of LMP1´s signalling activity (Wang *et al.*, 1988). The C-terminus contains at least two distinct functional regions: the membrane-proximal C-terminal activating region 1 (CTAR1) and the distal CTAR2. The CTAR1 domain contains an essential motif that recruits several members of the TNFR associated factor (TRAF) family, including TRAF1, TRAF2, TRAF3 AND TRAF5 (Mosialos *et al.*, 1995; Brodeur *et al.*, 1997). The CTAR2 domain associates with the TNF receptor associated death domain (TRADD) protein and the receptor-interacting protein (RIP), which are involved in TNFR-I signalling (Izumi *et al.*, 1999). Whereas CTAR1 has been shown to be essential for EBV-mediated transformation, CTAR2 is required for long-term growth of EBV-infected cells (Izumi and Kieff, 1997).

Functionally, LMP1 resembles an activated CD40 receptor (Bishop *et al.*, 2001; Lam and Sugden, 2003; Panagopoulos *et al.*, 2004) (Figure 1.3.3). However, in contrast to CD40, LMP1 provides a constitutive signal in the absence of ligand binding, which is mediated by the oligomerization of the transmembrane domains (Gires *et al.*, 1997; Kilger *et al.*, 1998; Kaykas *et al.*, 2001).

LMP1 activates several signalling pathways, which include NF-κB, p38/MAPK, JAK/STAT, PI3K/AKT and JNK/AP-1 pathways (Kieser *et al.*, 1997; Dawson *et al.*, 2003; Huen *et al.*, 1995; Gires *et al.*, 1999; Roberts and Cooper, 1998). Activation of the canonical (classical)

NF-κB pathway follows the degradation of IκBα by IκB kinase (IKK)-β, and results in the release and subsequent translocation of free p50/p65 to the nucleus (Wu *et al.*, 2006). The non-canonical (alternative) NF-κB pathway is activated by the phosphorylation of p100 by IKKα and results in the generation of free p52/RelB heterodimers (Hayden and Ghosh, 2004). Both CTAR1 and CTAR2 can activate NF-κB; CTAR1 stimulates mainly the non-canonical pathway and CTAR2 the canonical pathway (Atkinson *et al.*, 2003; Wu *et al.*, 2006). Both CTAR1 and CTAR2 activate STAT proteins directly (Brennan *et al.*, 2001), although the region between CTAR1 and CTAR2 might enhance the tyrosine phosphorylation of JAK3 which in turn activates STAT1 and STAT3 (Gires *et al.*, 1999). LMP1 also engages the p38 pathway through both CTAR1 and CTAR2 (Eliopoulos *et al.*, 1999) and the AP-1 transcription factor complex through CTAR2 (Blake *et al.*, 2001). LMP1 can also activate the PI3K/AKT pathway in epithelial cells resulting in cell survival, actin polymerisation and cell motility (Dawson *et al.*, 2003).

Several studies have compared CD40 and LMP1 signalling *in vivo*. For example, when transgenic mice expressing LMP1 under the control of the Ig promoter and enhancer were crossed with CD40-/- mice, it was shown that although LMP1 could rescue some of the humoral defects in these mice, it failed to restore the production of high affinity IgG1 and also blocked GC formation (Uchida et al., 1999). However, in two separate studies LMP1 transgenic mice expressing a CD40/LMP1 chimera consisting of the extracellular and transmembrane domains of CD40 and the cytoplasmic tail of LMP1, showed remarkable similarities to normal CD40 signalling (Stunz *et al.,* 2004; Rastelli *et al.*, 2008). For example, these mice had normal numbers of B cell subsets, GC formation, responses against TD antigen, isotype switching and affinity maturation. In contrast, the B cells of

transgenic mice expressing an LMP1/CD40 chimera, in which the C-terminus of LMP1 is replaced by the C-terminus of CD40, display an activated phenotype, prolonged survival and increased proliferation (Hömig-Hölzel *et al.*, 2008). Furthermore these mice develop B cell lymphomas after 12 months of age, a finding that is consistent with the late onset of lymphomas arising in LMP1 transgenic mice (Kulwichit *et al.,* 1998). These data demonstrate that it is likely to be the constitutive nature of the LMP1 signal which promotes oncogenesis.

Latent membrane protein 2 (LMP2)

The LMP2 gene encodes two separate proteins, LMP2A and LMP2B (Longnecker and Miller, 1996). The N-terminus of LMP2A contains two tyrosine residues (Tyr74 and Tyr85) that form an immunoreceptor tyrosine-based activation motif (ITAM) that is homologous to the ITAM motif in the BCR (Fruehling and Longnecker, 1997) (Figure 1.3.4.).

In un-infected primary B cells, cross-linking of the BCR by antigen aggregates the BCR into glycosphingolipid-rich microdomains (lipid rafts) in the plasma membrane (Cheng et al., 2001b). These lipid rafts contain an increased concentration of the protein kinases, Lyn and Syk which can phosphorylate the ITAM motif found in the Igα and Igβ signaling subunits of the BCR (Johnson et al., 1995; Kurosaki, 1999; Pleiman et al., 1994).

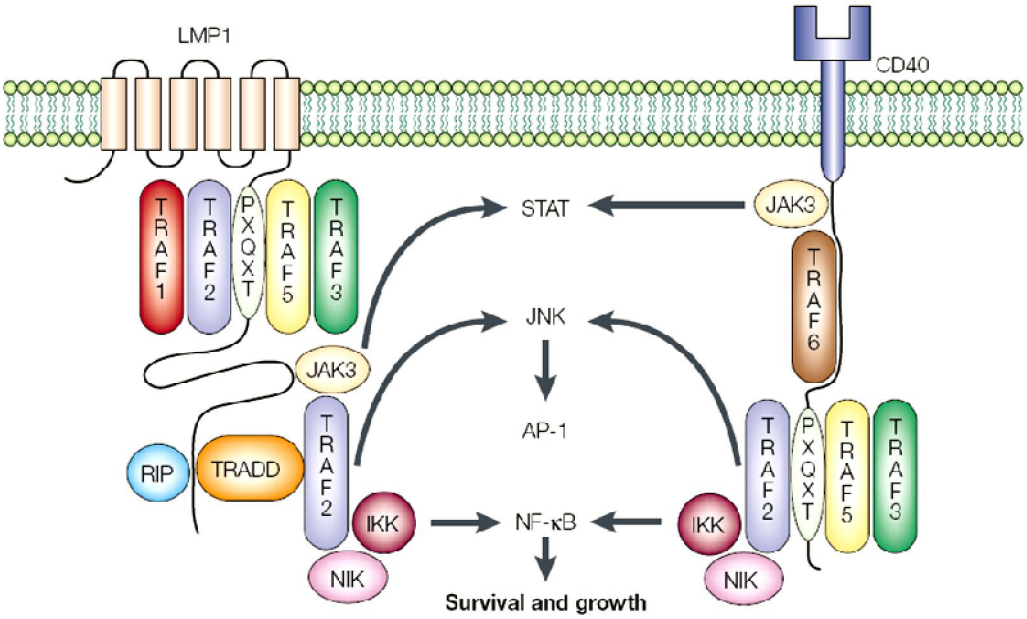


Figure 1.3.3. Signalling relationship between LMP1 and CD40. LMP1 contains cytoplasmic N-terminal and C-terminal domains joined by six transmembrane sequences. The C-terminal domain has extensive functional homology with CD40. Both LMP1 and CD40 contain consensus (TRAF)-binding domains and interact with multiple members of that family. The downstream signalling from LMP1 and CD40 causes activation of NF-κB, STATs, p38/MAPK, and AP-1 (taken from Thorley-Lawson, 2001).

When phosphorylated, the LMP2A ITAM competes with the BCR for the recruitment of the Src family of protein tyrosine kinases (PTK) and the Syk PTK. LMP2A also recruits Nedd4 ubiquitin ligases resulting in the degradation of Lyn (Fruehling and Longnecker, 1997; Ikeda et al., 2000; Ikeda et al., 2001; Winberg et al., 2000). As a result, LMP2A can inhibit BCR signal transduction and block calcium mobilisation and tyrosine phosphorylation (Miller *et al.*, 1995). The LMP2A-mediated block in BCR-induced signalling appears to be important for the maintenance of virus latency since it suppresses induction of the viral lytic cycle (Miller et al., 1994). However, in the absence of BCR signalling, LMP2A can induce the EBV lytic cycle, albeit less efficiently (Schaadt *et al.*, 2005).

Expression of LMP2A in B lymphocytes of transgenic mice causes the appearance of Ig-negative B cells in lymphoid organs, suggesting that LMP2A drives their proliferation in the absence of BCR signalling (Caldwell *et al.*, 1998). LMP2A has also been shown to be essential for the EBV-induced growth transformation of GC B cells which do not express a functional BCR because of deleterious somatic hypermutations in their *Ig* genes (Mancao and Hammerschmidt, 2007). Interestingly, in this study it was also shown that the survival and continued proliferation of both EBV-transformed BCR-negative and BCR-positive B cells was also dependent upon LMP2A (Mancao and Hammerschmidt, 2007).

LMP2B, the other isoform of the LMP2 gene, has not been well studied, and as a result the function of LMP2B in B cells is less well understood. Recently, it was shown that LMP2B could bind to LMP2A, preventing its phosphorylation and restoring normal signal transduction after BCR cross-linking (Rovedo and Longnecker, 2007). Consistent with a

role for LMP2B in inhibiting LMP2A signalling, it was also shown that LMP2B increases the susceptibility of BL cells to induction of the EBV lytic cycle following BCR cross-linking (Rechsteiner *et al.*, 2007).

In epithelial cells, both LMP2A and LMP2B were shown to promote cell spreading and migration on extracellular matrix (Allen *et al.*, 2005). Furthermore, LMP2A- and LMP2B-expressing epithelial cells also show decreased responsiveness to interferon IFN-α and IFN-γ; an effect mediated by the accelerated turnover of IFN receptors and resulting in global inhibition of IFN-stimulated gene expression (Shah *et al.*, 2009).

1.3.6. Epstein-Barr virus lytic cycle

The cascade of events in the EBV lytic cycle is divided into three phases: immediate-early, early and late. The immediate-early phase is characterized by the activation of transactivator proteins which subsequently stimulate expression of early lytic genes including enzymes required for viral DNA replication. During the late phase of the lytic cycle, the viral structural proteins are produced and assembled into virus particles enclosing newly synthesised DNA; prior to their release as infectious virions. *In vitro*, EBV replication can be triggered by treatment with phorbol esters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Hudewentz *et al.*, 1980); the demethylating agent, 5-Azacytidine (Ben-Sasson and Klein, 1981) or by crosslinking of surface Ig (Miller *et al.*, 1994).

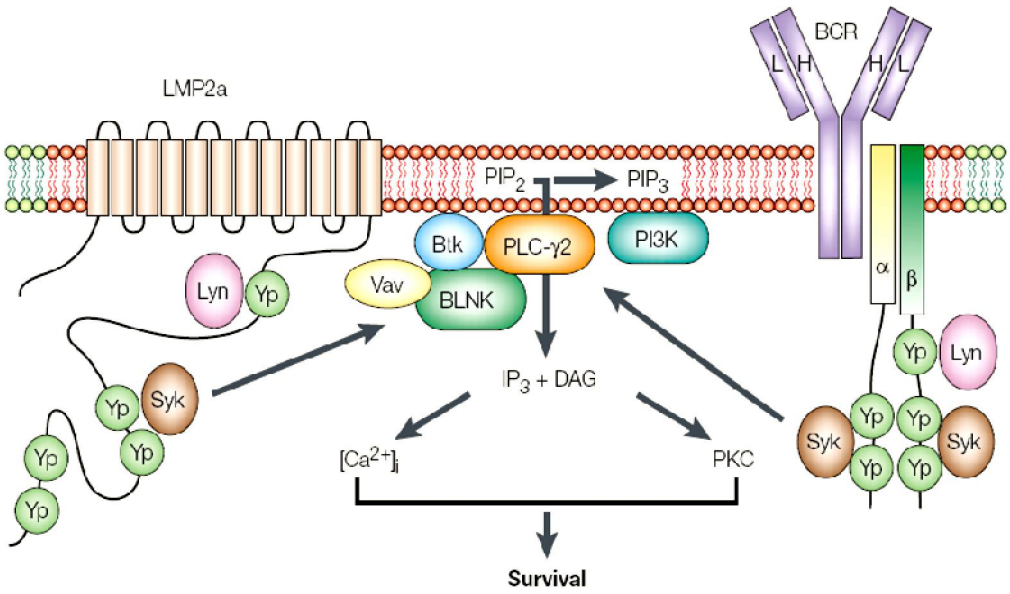


Figure 1.3.4. Comparison of LMP2A and BCR signalling. LMP2A consists of cytoplasmic N-terminal and C-terminal domains linked by 12 transmembrane sequences. The N-terminal tail contains two tyrosine residues which form an ITAM motif similar to that found in the α- and β-chains of the BCR. Both LMP2A and BCR bind the Lyn tyrosine kinase that mediates the phosphorylation of other tyrosine residues. The phosphorylated ITAM recruits members of the Src family of PTKs and the Syk tyrosine kinase resulting in downstream BCR signalling events including entry into lipid rafts (shown in red), the production of lipid second messengers by PI3K, and the PLC-γ2-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to yield diacylglycerol (DAG) and 1,4,5-inositoltrisphosphate (IP3). DAG activates the protein kinase C (PKC), whereas IP3 promotes Ca^{2+} signalling (taken from Thorley-Lawson, 2001).

Immediate-early lytic genes

The activation of two immediate-early lytic genes BZLF1 (ZEBRA) and BRLF1 (Rta), triggers the switch from latency to lytic cycle (Biggin *et al.*, 1987; Countryman and Miller *et al.*, 1985; Hardwick *et al.*, 1988; Rooney *et al.*, 1989). BZLF1 and BRLF1 are induced simultaneously (Sinclair *et al.*, 1991) from the Z-promoter (Zp) and the R-promoter (Rp) of bicistronic R-Z RNA. These two proteins transactivate many viral promoters of early, and subsequently, late genes (Kudoh *et al.*, 2003; Gradoville *et al.*, 1990; Tsurumi *et al.*, 2005). While some genes are activated either by BZLF1 or by BRLF1, others are induced by the synergistic effects of both BZLF1 and BRLF1 (Feederle *et al.*, 2000; Ragoczy and Miller *et al.*, 1999).

The BZLF1 protein is a homolog of the AP-1 transcription factors (Kieff and Rickinson, 2007) and binds into the lytic origin of replication (ori-lyt) (Hammerschmidt and Sugden, 1988) which alone is sufficient to trigger the entire lytic cascade (Rooney *et al.*, 1989). Recently, it has been shown that BZLF1 is expressed early after EBV infection of B cells. In this initial phase during which time viral DNA is unmethylated, BZLF1 drives the proliferation of newly infected cells, but does not induce the virus lytic cycle. Following the methylation of the viral genome, BZLF1 preferentially binds to methylated viral target genes and activates their transcription (Kalla *et al.*, 2010).

Early lytic genes

The products of early lytic genes constitute the serologically defined early antigen (EA) complex which has been used as a diagnostic marker for nasopharyngeal carcinoma (Henle *et al.*, 1970; Zeng *et al.*, 1983). At least 30 mRNAs encoding early lytic genes, independent of cellular protein and viral DNA synthesis, have been identified (Biggin *et al.*, 1987).

For example, BMRF1 is a transcriptional activator which has activities of a viral DNA polymerase (Holley-Guthrie *et al.*, 2005). The BMLF1 protein acts as a mRNA export factor and shuttles unspliced EBV lytic mRNAs. It is also required for the assembly of infectious virions (Hiriart *et al.*, 2003). EBV encodes two BCL2 homologues, BHRF1 and BALF1. BHRF1 is expressed in the first few days following B cell infection and is important for optimal transformation efficiency (Altmann *et al.*, 2005). BALF1 is a 182 amino-acid polypeptide that seems to counteract the anti-apoptotic function of BHRF1 (Bellows *et al.*, 2002). The single-stranded DNA-binding protein encoded by BALF2 gene is believed to be essential for EBV DNA replication as suggested by *in vitro* experiments in Raji cells (Tsurumi *et al.*, 1996; Polack *et al.*, 1984; Decaussin *et al.*, 1995). The BGLF5 protein enhances mRNA turnover leading to the reduced synthesis of host proteins including HLA class I and II molecules, thereby providing a possible mechanism of immune recognition escape (Rowe *et al.,* 2007). BNLF2a inhibits HLA class I antigen presentation by blocking TAP-mediated peptide transport (Hislop *et al.*, 2007).

Late lytic genes

The late lytic genes are expressed 48-72 hours after induction of the lytic cycle and encode two major classes of protein: glycoproteins and non-glycoproteins.

The glycoproteins include BLLF1, BILF1, BILF2, BDLF3, BALF4, BMRF2 and BXLF2. Some of them are present in the classical membrane antigen (MA) complex (Edson and Thorley-Lawson, 1981).

The BLLF1 gene encodes the most abundant viral glycoprotein gp350/220, which mediates the binding of EBV to its B cell receptor, CD21 (Torrisi *et al.*, 1989; Fingeroth *et al.*, 1984; Nemerow *et al.*, 1989). This interaction induces the penetration of B lymphocytes by EBV (Tanner *et al.*, 1987) as well as activation of the Wp promoter (Sinclair and Farell, 1995; Sugano *et al.*, 1997). The gp350/220 protein is a major target of host neutralising antibodies (Thorley-Lawson and Poodry 1982). BXLF2 (gH) forms a heterotrimeric complex with two more glycoproteins, BKRF2 (gL) and BZLF2 (gp42), which participate in the endocytosis of EBV into B cells using HLA class II as co-receptor. In epithelial cells, EBV infection does not involve BZLF2 since these cells do not generally express HLA II molecules (Borza and Hutt-Fletcher, 2002). The highly conserved BALF4 is thought to be involved in the egress of virions from infected cells (Herrold *et al.*, 1996).

The non-glycoprotein group is composed of structural proteins, such as BcLF1, BNRF1 and BXRF1 that make up the viral nucleocaspid (Kieff, 1996). Some of these are part of the immunologically defined viral capsid antigen (VCA) complex.

1.4. EPSTEIN–BARR VIRUS ASSOCIATED DISEASES

In this section, I describe two EBV-associated cancers, Hodgkin's lymphoma and Burkitt's lymphoma, as these are subject of this thesis.

1.4.1. Hodgkin's lymphoma

General introduction

Hodgkin's lymphoma (HL) was first described by Thomas Hodgkin in 1832 (Hodgkin, 1832) and is characterised by the disruption of normal lymph node architecture and the presence of malignant mononuclear 'Hodgkin' and multinuclear 'Reed/Sternberg' cells with bilobed nuclei (resembling an "owl's eye" appearance) (Sternberg, 1898; Reed, 1902). Hodgkin-Reed Sternberg (HRS) cells represent only 1-2% of the total tumour mass and are surrounded by a non-malignant reactive infiltrate including T and B cells, eosinophils, neutrophils, plasma cells, histiocytes and fibroblasts (Harris *et al.,* 1994) (Figure 1.4.1). HRS cells can communicate with the surrounding infiltrate via a complex of cytokines and cell contact dependent interactions; these are likely to include proliferative and anti-apoptotic signals favouring the expansion and survival of HRS cells (Pinto *et al.*, 1998).

Histological classification

About 95% of HL cases belong to the classical form of disease (cHL), while the remaining 5% represent nodular lymphocyte predominance HL (NLPHL) (Harris *et al.*, 1994). cHL and

NLPHL are immunophenotypically distinct; HRS cells of cHL are typically CD30 and CD15 positive, but usually lack expression of B cell lineage genes such as CD20 (Drexler, 1992). In contrast, HRS cells of the NLPHL form so-called lymphocytic and histiocytic (L&H) cells, regularly express B cell markers, but are usually negative for CD30 and CD15 (Mason *et al.*, 1994). Based on the nature of the surrounding infiltrate and the morphology of HRS cells, cHL is further subdivided into four subcategories: nodular sclerosis, mixed cellularity, lymphocyte depleted and lymphocyte-rich forms (Jaffe *et al.*, 2001).

Epidemiology

With an incidence of about 3 new cases per 100,000 persons per year in the Western World, HL is one of the most frequent lymphomas (Kuppers, 2009). Epidemiological studies of HL in the 1960s identified an unusual bimodal age distribution in the USA with one peak in incidence in young adults between 15-34 years and a second peak occurring after 50 years of age (MacMahon, 1966). In 1971, three epidemiological patterns of HL were characterised. The type I pattern prevails in developing countries and is characterised by two peaks in incidence, one in male children and the other in the elderly with only low incidence in young adults. The type III pattern is the opposite of the type I, being most common in developed countries with a significant peak in incidence in young adults with only low rates in children and the elderly. The type II pattern is observed mainly in rural parts of some developed countries and has features which are intermediate between the type I and III patterns (Correa and O'Conor, 1971).

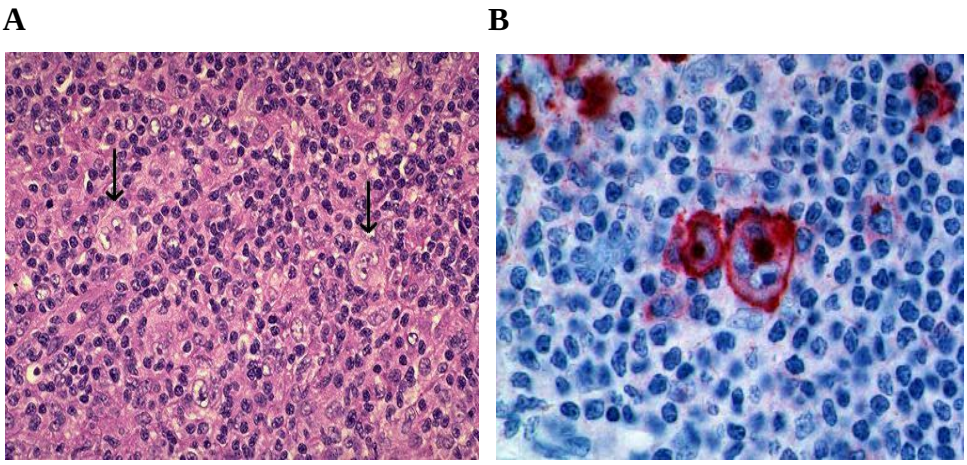


Figure 1.4.1. Histological features of HL. A) Haematoxylin and eosin (H&E) stain of HL with HRS cells (arrowed) typically constituting less than 1-2% of the total tumour mass (x 400 magnification). B) Immunostaining of a HRS cell using CD30 antibody (x 600 magnification).

The cellular origin of the HRS cell

Because HRS cells aberrantly express markers of several hematopoietic lineages, early studies using immunohistochemistry incorrectly identified the cell origin as histiocytes (Kadin *et al.*, 1978), dendritic cells (Curran and Jones, 1978) and granulocytes (Stein *et al.*, 1982). However, sequence analysis of *Ig* genes of single HRS cells revealed rearranged *IgV* and *IgL* chain genes; demonstrating that these cells are of B cell origin (Kuppers *et al.*, 1994). Furthermore HRS cells show evidence of SHM suggesting they are derived from GC or post-GC cells (Kuppers, 2009). Furthermore HRS cells from the same case carry identical *IgV* gene rearrangements which demonstrate their monoclonal nature (Kanzler *et al.*, 1996).

In about one quarter of cHL, HRS cells carry non-functional (crippled) *IgV* genes (for example as a consequence of nonsense mutations) and are incapable of expressing a functional BCR. Since Fas-induced apoptosis is the normal fate of such cells, HRS cells are presumed to derive from pre-apoptotic GC B cells (Kanzler *et al.*, 1996). The proximal negative regulator of CD95-induced apoptosis, c-FLIP, is highly expressed in HRS cells and might contribute to escape from Fas-induced death (Re *et al.*, 2000; Thomas *et al.*, 2002; Mathas *et al.*, 2004; Dutton *et al.*, 2004). Nearly all cases carrying these destructive *Ig* gene mutations are EBV positive, suggesting that EBV is required to prevent the apoptosis of BCR negative HRS-cell precursors (Bräuninger *et al.*, 2006). This has been substantiated by the demonstration that LCLs can be derived from human GC B cells harbouring crippled *Ig* genes or BCR-negative B cells can be rescued by EBV from cell death, giving rise to LCLs (Bechtel *et al.*, 2005; Chaganti *et al.*, 2005; Mancao *et al.*, 2005). The proposed origin of HRS cells is illustrated in Figure 1.4.2.

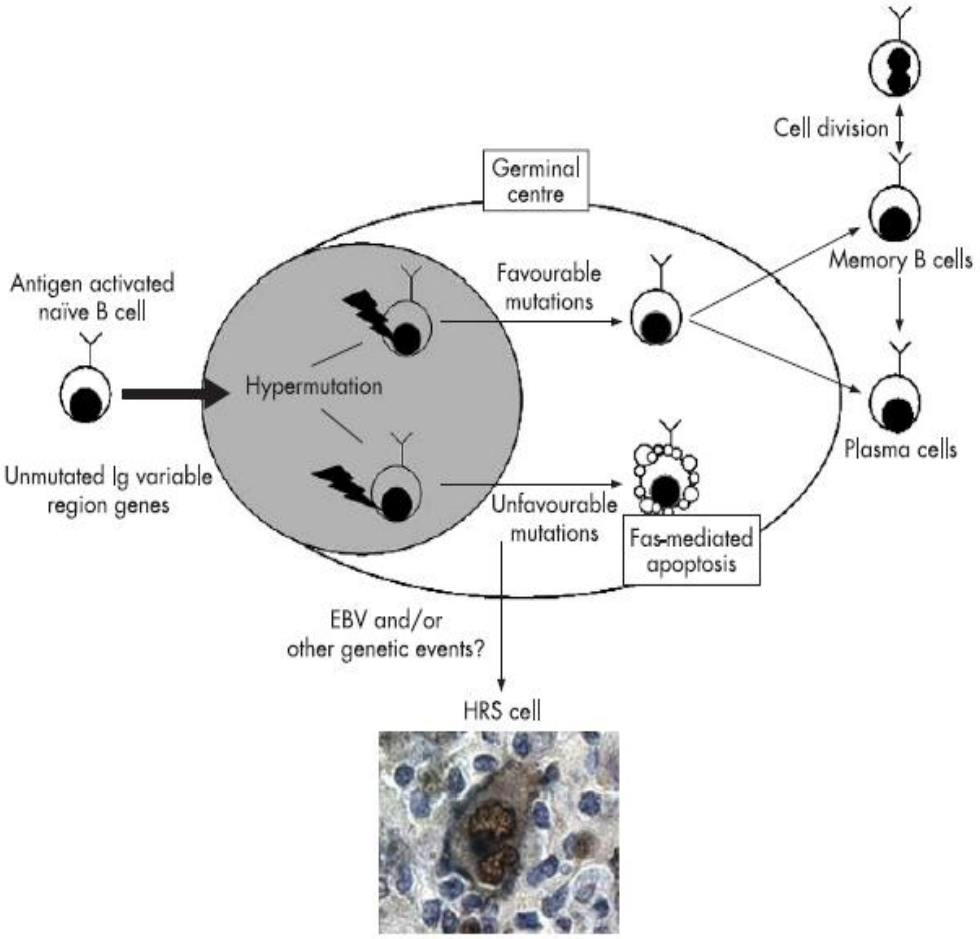


Figure 1.4.2. HRS cells may originate from pre-apoptotic GC B cells. Naive B cells enter GCs and undergo somatic hypermutation of the V region genes. Cells which acquire favourable BCR mutations leave the GC as memory or plasma cells. Cells which acquire unfavourable BCR mutations undergo apoptosis. EBV-infected GC B cells with non-functional BCR might be rescued from apoptosis. Such cells may be the progenitors of HRS cells (taken from Kapatai and Murray, 2007).

Other mechanisms could account for the loss of surface BCR expression on HRS cells. These include loss of Ig-specific transcription factors such as BOB-1, OCT-2 and PU.1 (Re *et al.,* 2002; Jundt *et al.,* 2001; Torlakovic *et al.,* 2001; Marafioti *et al.,* 2000; García-Cosío *et al.,* 2004), SHM in the octamer region of the *Ig* gene promoter or epigenetic silencing (Re *et al.*, 2001; Ushmorov *et al.*, 2004).

HRS cells also show a striking loss of their B cell phenotype (Schwering *et al.*, 2003). This may result from the down-regulation or functional impairment of several B cell specific transcription factors and from the aberrant induction of non-B cell lineage genes. For example, the key transcription factor for the B cell differentiation programme, EBF1, is expressed in HRS cells only at low levels (Mathas *et al.*, 2006; Hertel *et al.*, 2002). The helix-loop-helix transcription factors, E12 and E47, which are generated by splicing of the E2A gene, are expressed in HRS cells, but their function is impaired by two inhibitors: ABF1 and ID2 (Mathas, *et al.*, 2006; Kuppers *et al.*, 2003; Renné *et al.*, 2006). ID2 also negatively regulates PAX5 (Renné *et al.*, 2006) which explains why PAX5 is expressed, but apparently not functional, in HRS cells (Cobaleda *et al.*, 2007). Other factors implicated in the down-regulation of B cell genes include STAT5A and STAT5B (Scheeren *et al.*, 2008).

In contrast, the main T cell transcription factor NOTCH1, is highly expressed in HRS cells (Jundt *et al.*, 2002). NOTCH1 can inhibit the B cell programme by down-regulating E2A, EBF1, PAX5 and by inducing ABF1 (Jundt *et al.*, 2008). Aberrant expression of key transcription factors of haematopoietic stem cells, for example GATA2 or Polycomb group genes may further contribute to the reprogramming of HRS cells (Schneider *et al.*, 2004; Dukers *et al.*, 2004; Sanchez-Beato *et al.*, 2004). It has been speculated that the down-

regulation of B cell identity might allow HRS cells or their progenitors to escape the apoptosis that should occur in the absence of functional BCR (Kuppers and Hansmann, 2005).

In contrast to cHL, L&H cells of NLPHL frequently express *Ig* genes (Stoler *et al.*, 1995) with intraclonal *IgV* gene diversity due to ongoing mutations, indicating that they originate from antigen-selected GC B cells (Schmitz *et al.*, 2009).

Deregulated transcription factor networks and signalling in Hodgkin's lymphoma

A number of cell signalling pathways that are normally only transiently activated in B cells, such as NF-κB, JAK-STAT and PI3K-AKT, have been shown to be aberrantly activated in HRS cells.

Constitutive activation of the NF-κB pathway in HRS cells can result either from genetic alterations in several members of the NF-κB family or from increased CD40, CD30, TACI, BCMA and RANK receptor signalling on HRS cells, mediated by ligands expressed on cells in the microenvironment (Bargou *et al.*, 1997; Cabannes *et al.*, 1999; Emmerich *et al.*, 1999; Jungnickel *et al.*, 2000; Carbone *et al.*, 1995; Chiu *et al.*, 2007; Fiumara *et al.*, 2001). Similarly, signals from the micro-environment can activate the PI3K-AKT and ERK pathways (Dutton *et al.*, 2005; Georgakis *et al.*, 2006; Zheng *et al.*, 2003). The aberrant activation of NOTCH1 in HRS cells is most likely mediated by its binding to JAGGED1, expressed by cells in the microenvironment (Jundt *et al.*, 2008). STAT3, STAT5A, STAT5B and STAT6 have also been reported to be constitutively active in HRS cells, in some cases this is a consequence of JAK2 amplification (Scheeren *et al.*, 2008; Baus and Pfitzner,

2006; Kube *et al.*, 2001; Skinnider *et al.*, 2002; Joos *et al.*, 2003). HRS cells also show deregulated signalling of AP-1 dimers, c-Jun and JunB (Mathas *et al.*, 2002) and the aberrant activation of multiple RTKs, including PDGFRA, DDR2, MSPR, TRKA and TRKB (Renné *et al.*, 2005).

The Hodgkin's lymphoma microenvironment

cHL is characterized by the presence of various cell types such as T cells, B cells and plasma cells that infiltrate the lymphoma microenvironment and which are believed to support the growth and survival of HRS cells (Figure 1.4.3.). These cells are attracted to the tumour by multiple cytokines and chemokines secreted by HRS cells. The importance of the microenvironment for HRS cell survival is underscored by the difficulty in growing primary HRS cells (in culture and in immunodeficient mice), by the rare occurrence of HRS cells in the peripheral blood of patients with HL, and by the observation that the microenvironment is maintained in non-lymphoid metastases of HL (Kapp *et al.*, 1994).

$CD4^+$ T cells are the most frequent cell type in HL. HRS cells secrete CCL5 (RANTES), CCL17 (TARC), CCL20 and CCL22 which can attract helper and regulatory CD4+ T cells (Skinnider and Mak, 2002; Aldinucci *et al.*, 2008; Fischer *et al.*, 2003; Baumforth *et al.*, 2008). CCL5 also attracts eosinophils and mast cells. The secretion of IL-8 by HRS cells presumably recruits neutrophils (Skinnider and Mak, 2002). HRS cells can also activate fibroblasts which in turn produce eotaxin and CCL5, thus further contributing to the attraction of eosinophils and T regulatory cells (Aldinucci, *et al.*, 2004; Jundt *et al.*, 1999). As described above, the microenvironment can provide survival signals to HRS cells mediated by

various ligand-receptors interactions which include CD40-CD40L, CD30-CD30L, APRIL-BCMA and NGF-TRKA (Carbone *et al.*, 1995; Chiu *et al.*, 2007).

HRS cells produce various immunosuppressive factors including IL-10 (Marshall *et al.*, 2004), galectin 1 (Juszczynski *et al.*, 2007; Gandhi *et al.*, 2007), TGFβ and PD1L, all of which can potentially inhibit cytotoxic T cell responses directed to HRS cells (Newcom and Gu, 1995; Chemnitz *et al.*, 2007; Yamamoto *et al.*, 2008).

Contribution of EBV to the pathogenesis of Hodgkin's lymphoma

EBV can be detected in approximately 40% of cases of cHL in the Western world and more frequently in tumours occurring in developing populations and in up to 100% of patients with AIDS (Glaser *et al.*, 1997; Dolcetti *et al.*, 2001). In EBV-associated cHL, the virus episome is monoclonal suggesting that all HRS cells arise from a single EBV-infected progenitor (Anagnostopoulos *et al.*, 1989). EBV positive HRS cells exhibit a type II pattern of virus gene expression. In some EBV positive tumours, the virus has also been detected at relapse suggesting that EBV is required for tumour maintenance (Meyer *et al.*, 2004). In contrast to cHL, NLPHL is considered an EBV negative disease (Chan, 1999). It has been suggested that the expression of LMP1 and LMP2A might contribute to the survival of HRS progenitors in the GC (Eliopoulos *et al.*, 1999; Kilger *et al.*, 1998; Mancao and Hammerschmidt, 2007). LMP2A induces a global down-regulation of B cell lineage genes when expressed in mouse B cells (Portis et al., 2003). LMP1 induces a similar loss of B cell identity when expressed in primary human GC B cells (Vockerodt et al., 2008). Thus, LMP2A and LMP1 when expressed in normal B cells induce global alterations in gene transcription which are similar to those seen in HRS cells.

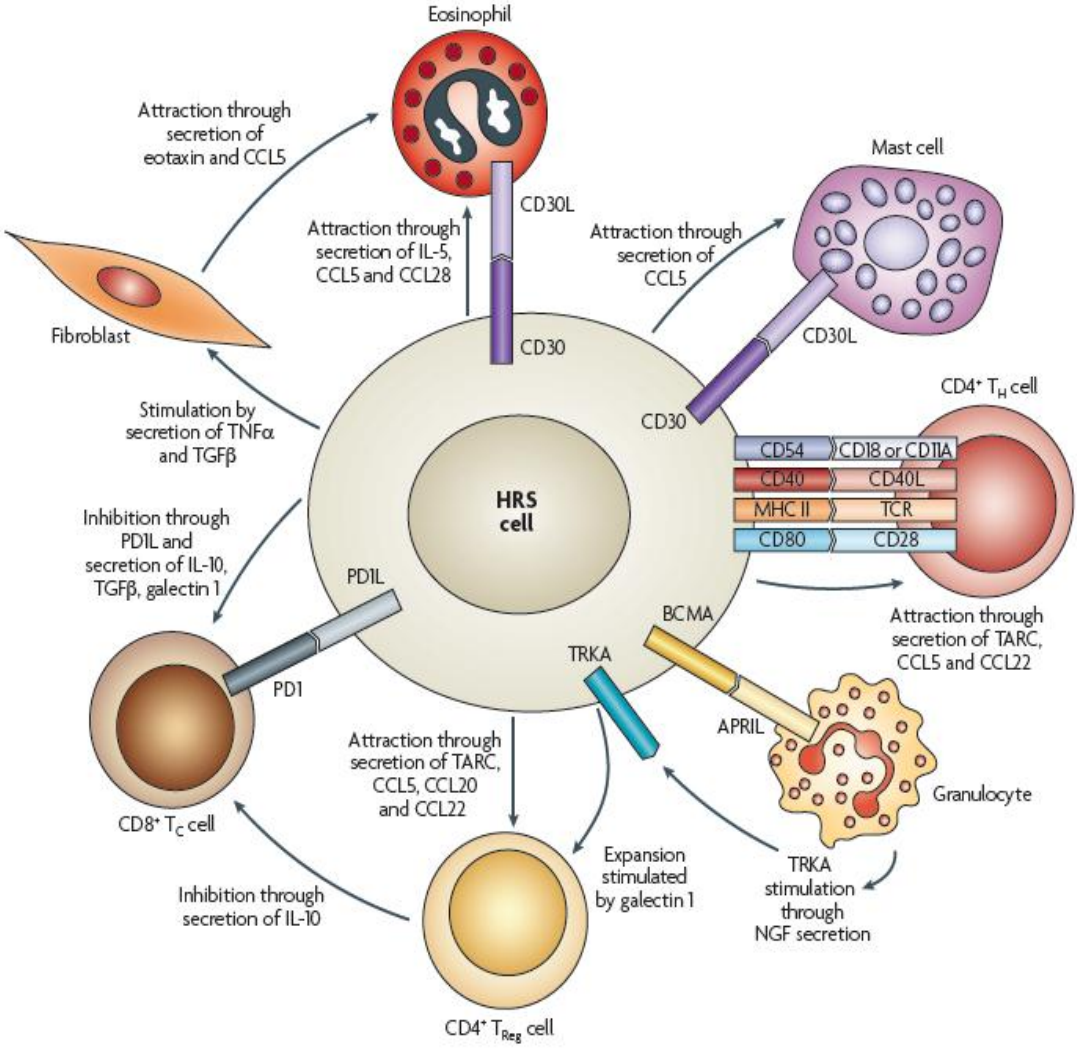


Figure 1.4.3. Cellular interactions in the HL microenvironment.

(taken from Kuppers, 2009).

1.4.2 Burkitt's lymphoma

General introduction

Burkitt's lymphoma (BL) was first described by Denis Burkitt in 1958 (Burkitt, 1958). All forms of BL display a similar histological appearance, with a malignant population of medium to large monomorphic B cells with large vacuolated basophilic cytoplasm and multiple small central nucleoli. BL is characterized by unusually high rates of proliferation and apoptosis with solid sheets of interspersed debris containing macrophages, giving the tumor histology a "starry sky" pattern. The detection of ongoing *Ig* gene mutations and the cellular phenotype ($CD10^+$, $CD38^+$, $CD77^+$, $BCL6^+$) suggests that BL cells are derived from GC B cells (Kieff and Rickinson, 2007).

Epidemiology of Burkitt's lymphoma

Three epidemiologically distinct forms of BL are recognized, these are the endemic, sporadic and AIDS-associated forms. The high-incidence endemic form is restricted to areas of equatorial Africa and Papua New Guinea (Burkitt *et al.,* 1961; Booth *et al.,* 1967), where *Plasmodium falciparum* malaria infection is holoendemic. This form of BL is almost always EBV positive, typically presents in young children and affects extranodal sites including the jaw, abdomen and endocrine organs. Sporadic BL is less common, also occurs mainly in children, but only 15% of tumours are EBV positive. AIDS-associated BL often presents as the first AIDS-associated illness in relatively immunocompetent patients; 30-40% of these cases are EBV-positive (Kieff and Rickinson, 2001).

Genetic changes in pathogenesis of Burkitt's lymphoma

A consistent feature of all forms of BL is the presence of characteristic chromosome translocations. These always involve a reciprocal translocation of the long arm of chromosome 8 (8q24) in the region of the *C-MYC* oncogene and either chromosome 14 in the region of the *IgH* gene or, less frequently, chromosomes 2 or 22 in the region of the *IgL* genes (Magrath, 1990). These translocations result in high levels of C-MYC expression, ultimately affecting cell proliferation, growth and apoptosis.

In normal B cells, the pro-proliferative effects of C-MYC are counterbalanced by several checkpoints including the ARF-MDM2-p53 pathway and the pro-apoptotic protein, BIM, which combine to deliver a cumulative apoptotic signal. In contrast, in BL these apoptotic signals are inhibited for example by p53 mutations, MDM2 over-expression and ARF or BIM inactivation (Li *et al.,* 2003; Lindstrom and Wiman*,* 2002; Hemann *et al.,* 2005). Two mutant C-MYC alleles, P57S and T58A have been described, which can in an adoptive transfer mouse model, accelerate tumour onset compared with wild-type C-MYC (Chang *et al.,* 2000; Hemann *et al.,* 2005). In this model, both p53 and p19ARF were wild-type in the mutant MYC tumors, but the MYC mutants were unable to activate the pro-apoptotic BH3-only protein, BIM (Hemann *et al.,* 2005).

Contribution of EBV to the pathogenesis of Burkitt's lymphoma

Serological studies indicate that EBV infection occurs before the clinical onset of disease and children with high titres of EBV have increased susceptibility to BL (Henle *et al.*, 1969; de-The *et al.*, 1978; Geser *et al.*, 1982). Monoclonal episomes are present in BL tumours

and express a latency I pattern of virus gene expression (Neri *et al.*, 1991). The use of Qp in most BL cells might result from selection of a Wp/Cp-driven LCL-like progenitor (Kelly *et al.*, 2002), where the absence of the EBNA2 and the LMPs comes from T cell surveillance or from a presumed incompatibility with the high levels of C-MYC (Pajic *et al.*, 2001). An alternative form of latency, known as Wp-restricted latency, has been described is some BL cells. This form of latency is characterised by Wp-driven EBNA expression in absence of EBNA2 and the EBNA2-induced LMP proteins (Kelly *et al.*, 2006). A variant has also been described in which EBNA2 is expressed, but the LMPs are not. This is consistent with previous studies which suggest that the expression of LMP1 is incompatible with the C-MYC-driven growth program (Floettmann, *et al.*, 1996; Polack *et al.,* 1996; Pajic *et al.,* 2001).

Experimental evidence suggested that EBV might contribute to the pathogenesis of BL by complementing for the activity of C-MYC, presumably by ablating the apoptotic activity of this oncoprotein. This could be achieved by the down-regulation of BIM which occurs in normal B cells following their transformation with EBV *in vitro.* A role for viral mediators, BHRF1 and EBNA3A/EBNA3C, in the down-regulation of BIM has been suggested (Clybouw *et al.*, 2005; Anderton *et al.*, 2008; Rowe *et al.*, 2009).

It has also been suggested that EBV might contribute to the pathogenesis of EBV-negative sporadic BLs by initially infecting and transforming the progenitor cells followed by loss of the virus during the later stages of tumour progression ('hit and run' mechanism) (Razzouk *et al.*, 1996).

1.5. PROJECT AIMS

The Epstein-Barr virus (EBV) is associated with several types of lymphoma originating from germinal centre B cells. An important pathogenic event in these lymphomas is the suppression of virus replication which would otherwise result in tumour cell death. Because the induction of virus replication in EBV-infected B cells appears to be intimately linked to their differentiation towards plasma cells this thesis examines if the physiological signals which drive normal B cell differentiation are altered in EBV-transformed B cells and in EBV-associated lymphoma cells. I have focussed on BLIMP1α, a transcription factor that is required for plasma cell differentiation.

The specific objectives are to:

1) Investigate if the EBV-encoded LMP1 can regulate BLIMP1α expression in primary human germinal centre B cells and in doing so disrupt the BLIMP1α transcriptional programme.
2) Study the effects of the ectopic expression of BLIMP1α on the regulation of the viral lytic cycle in EBV positive B cells.
3) Investigate the mechanisms responsible for the regulation of BLIMP1α by LMP1 in B cells.
4) Determine if EBV can also regulate expression of the BLIMP1β isoform in EBV transformed germinal centre B cells and in Hodgkin's lymphoma cells.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1. Collection and preparation of tonsil specimens and cell lines

2.1.1. Tonsil specimens

Fresh tonsils were obtained with informed consent from pediatric patients under local ethics committee approval (Ref No.06/Q2702/50) and transported in cold phosphate buffered saline (PBS) on ice.

2.1.2. Cell lines

Cell lines were maintained at 37°C in 5% carbon dioxide (CO_2) in RPMI 1640 (Sigma-Aldrich Ltd., Gillingham, UK) supplemented with 10% fetal calf serum, 2mM L-glutamine (all Invitrogen Ltd., Paisley, UK) and 1% penicillin-streptomycin solution (Sigma-Aldrich Ltd., Gillingham, UK).

KMH2 was originally established from the pleural effusion of a 37-year-old man with mixed cellularity Hodgkin lymphoma (HL) (Kamesaki *et al.,* 1986). KMH2 EBV was derived from EBV-negative KMH2 cells infected with Akata-derived recombinant virus and maintained under geneticin selection (Invitrogen Ltd., Paisley, UK; 1 mg/mL) (Baumforth *et al.,* 2005). L591 was established from the pleural effusion of a 31-year-old woman with histologically confirmed HL (nodular sclerosis; stage IVB) (Diehl *et al.,* 1982). L428 was derived from the pleural effusion of a 37-year-old woman with HL (stage IVB, nodular sclerosis) (Schaadt *et al.,* 1980). L1236 was established from the peripheral blood of a 34-year-old man with HL (mixed cellularity, stage IV) (Wolf *et al.,* 1996). Akata is an EBV

positive cell line established from a Japanese patient with Burkitt's lymphoma (BL) with typical BL-type chromosome translocation (t8q-; 14q+) (Rowe *et al.,* 1992). BL2 cells were established from the bone marrow of a 7-year-old Caucasian boy with non endemic BL (stage III) (Cohen *et al.,* 1987). DG75 was derived from the pleural effusion of a 10-year-old boy with sporadic BL (Ben-Bassat *et al.,* 1977). MUTU I and MUTU III are EBV positive BL cell lines derived from the same patient, but are phenotypically distinct: MUTU I has retained a latency I form of infection and expresses only EBNA1 but not the other EBV-encoded EBNAs; whereas MUTU III is a subclone of the same line that has drifted to the latency III type of infection in which all six EBNAs and three latent membrane proteins are expressed (Gregory *et al.,* 1990). Rael is a latency I EBV-positive cell line which was used as a negative control of BLIMP1 expression (Klein *et al.,* 1972). U266 was established from the peripheral blood of a 53-year-old man with IgE-secreting myeloma. This cell line served as a positive control for BLIMP1 (Nilsson *et al.,* 1970). X50-7 is a lymphoblastoid cell line generated by EBV-immortalization of cord blood B cells (Miller *et al.,* 1984). B95-8 was established from peripheral blood lymphocytes of a cotton-top tamarin (cotton-top marmoset) monkey *(Saguinus oedipus)*; cells were described to release high titres of EBV (Klein *et al.,* 1974). SL1-LCL, SL2-LCL, SL3-LCL cell lines (kindly provided by Dr. Sarah Leonard, University of Birmingham, Birmingham, UK) were established by infecting GC B cells isolated from three separate donors with 2089 wild-type EBV (based on the genome of the EBV strain B95.8). The cells were maintained in culture for six weeks. OKU-LCL and SAL-LCL (kindly provided by Dr. Gemma Kelly, University of Birmingham, Birmingham, UK) were generated *in vitro* by transforming normal peripheral B cells from an adult EBV seronegative donor with virus rescued from atypical group I BL lines OKU and SAL lines.

PER213 and HK-LCL (kindly provided by Dr. Heather Long, University of Birmingham, Birmingham, UK) were established from normal peripheral B cells using the reference EBV strain B95.8.

A tetracycline regulatable expression system in DG75 BL cells (kindly provided by Professor Martin Rowe, University of Birmingham, Birmingham, UK) (Floettmann *et al.,* 1996) was used to study the effects of LMP1 expression on the regulation of BLIMP1. In this system LMP1 was cloned into the pUHD10-3 plasmid downstream of a promoter containing binding sites for a hybrid tetracycline-regulated transactivator (tTA) that is constitutively expressed from a second, co-transfected plasmid, pUHD15-1 (Gossen and Bujard, 1992). Tetracycline binds to the tTA and prevents binding to the promoter which remains silent, but upon removal of tetracycline the tTA binds to the LMP1 promoter and activates its transcription. Stock cultures of DG75-LMP1 cells were maintained under drug selection with 2 mg/ml of geneticin (Invitrogen Ltd., Paisley, UK, 1 mg/mL); 500 µg/ml of hygromycin B (Roche Diagnostics Ltd., West Sussex, UK) and 1 µg/ml of tetracycline (Sigma-Aldrich Ltd., Gillingham, UK) until required. To induce LMP1 expression, cells were centrifuged, washed three times in PBS, incubated in growth media for 15 minutes at 37°C, washed again with PBS and re-cultured in the presence or absence of 2 mg/ml of tetracycline for a period of 24, 48 or 96 hours.

Cell counts were determined using a haemocytometer (Marienfeld, Harsewinkel, Germany). Cell suspensions were diluted, mixed with Trypan blue 1:1 (Sigma-Aldrich Ltd., Gillingham, UK) and pipetted into a counting chamber of a haemocytometer. Using an inverted light microscope, only living cells (determined by uptake of Trypan blue)

contained within 16 zones of the chamber were counted. This number was then divided by four to obtain an average count of cell number per zone. This average value was then multiplied by 10^4 and a dilution factor to obtain the number of cells per ml of culture medium.

2.2. Plasmid preparation

2.2.1. Agar preparation

L-broth agar (LBA) was prepared using 2 g of L-broth powder and 1.5 g selected agar (both Invitrogen Ltd., Paisley, UK) in 100 ml distilled water and autoclaved. Ampicillin (Sigma-Aldrich Company Ltd., Gillingham, UK) was added at a final concentration of 100 µg/ml once the LBA cooled down. LBA was then poured into Petri dishes (Bibby Sterilin Ltd., Stone, UK) and left to set at room temperature. Sealed plates were stored at 4°C until required.

L-broth buffer (LB) was prepared using 2 g L-Broth powder in 100 ml distilled water, autoclaved and stored at room temperature. Ampicillin was added prior to use at a final concentration 100 µg/ml.

2.2.2. Transformation

TOP10 Competent *E.Coli* cells (Invitrogen Ltd., Paisley, UK) were thawed on ice and plasmid DNA was added to each tube at a ratio of 1:10. The bacteria were then incubated on ice for 30 minutes, heat shocked at 42°C in a water bath for 60 seconds and immediately returned on ice for 2 minutes. 250 µl of broth medium without antibiotics

was added to each reaction tube and gently mixed and incubated at 37°C for one hour. The bacteria were then transferred onto pre-warmed LBA plates and spread across the whole of the agar surface. The LBA plates were placed upside down in the 37°C incubator overnight.

2.2.3. EndoFree plasmid purification

To bulk up plasmids from bacterial cells, EndoFree Plasmid Maxi Kit (Qiagen Ltd, Crawley, UK) was used according to manufacturer's instructions. Briefly, a single colony from a freshly streaked LBA plate was incubated in a starter culture of 2 ml of LB buffer with ampicillin for 8 hours at 37°C with vigorous shaking. The starter culture was then diluted in 100 ml of LB and further incubated at 37°C for 16 hours (overnight). Following the incubation, bacteria were harvested by centrifugation at 6000 g for 15 minutes at 4°C. Cell pellets were then resuspended and lysed under alkaline conditions. Cell lysates were further neutralised and genomic DNA, proteins and cell debris were retained on a QIAfilter Maxi Cartridge. At this stage, the Endotoxin Removal Buffer was added to the filtered lysate in order to remove endotoxins which could significantly reduce transfection efficiencies or induce nonspecific activation of immune cells. Following incubation on ice for 30 minutes, the cleared lysate was loaded onto the anion-exchange tips to selectively bind plasmid DNA under appropriate low-salt and pH conditions. RNA, proteins, metabolites, and other low-molecular-weight impurities were removed by a medium-salt wash, and ultrapure plasmid DNA was eluted in high-salt buffer. DNA was then concentrated and desalted by isopropanol precipitation and collected by centrifugation at 15000 g for 30 minutes. Pelleted DNA was washed in 70% ethanol in order to remove

precipitated salt and further centrifuged at 15000 g for 10 minutes. Air dried pellets were resuspended in TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA); DNA was measured using a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE, USA) and stored at 4°C. Details of plasmids used the study are given in Appendix 1.

2.3. RNA detection and analysis

2.3.1. RNA extraction from cultured cells

Culture media with cells were pelleted at 900 rpm for 5 minutes. Total RNA was isolated from cells using the QIAGEN RNeasy™ MINI or MICRO (<$5x10^5$ cells) kit according to manufacturer's instructions (Qiagen Ltd, Crawley, UK). Briefly, cells were disrupted in RLT lysis buffer containing a denaturant guanidinium isothiocyanate. β-mercaptoethanol was added to inactivate RNAse enzymes by reducing disulfide bonds in their native conformation. Cell lysates were then homogenized on a QIAshredder spin column (Qiagen Ltd, Crawley, UK) or by vigorous vortexing for 1 minute. Samples were thoroughly mixed with 1 volume of 70% ethanol to provide ideal binding conditions and loaded onto the RNeasy silica membrane of MINI/MICRO spin columns. This was followed by centrifugation at 10000 rpm and washing of the spin column membrane with RWI buffer. If required, samples were incubated for 15 minutes with DNAse solution (RNase-Free DNase Set; QIAGEN Ltd., West Sussex, UK) at room temperature to remove contaminating bound DNA. Salts, metabolites and cellular components were washed off in several washing steps with RWI, RPE and RPE (or 80% ethanol) buffers. Finally, the spin columns

were centrifuged with their lids open in order to completely dry column membranes and RNA was eluted with DNase/RNase-free. To obtain RNA concentration and quality (ratio 260/280) for each sample, eluted RNA was measured on a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE, USA) and stored at -80°C.

2.3.2. Reverse transcription (RT) reaction

Complementary DNA was synthesized from 400 ng (or less) of total RNA extracted from each sample. RNA was transferred into sterile thin walled 0.2 ml PCR tubes and the volume was made up to 11 µl using DNase/RNase-free water. A negative (no RNA) control was set up in parallel, consisting of 11 µl DNase/RNase-free water alone. 1 µl of 250 ng of random hexamer primers (Promega UK Ltd, Hampshire, UK) and 1 µl of 10 mM dNTP Mix (dATP, deoxyadenosine triphosphate; dCTP, deoxcytosine triphosphate; dGTP, deoxyguanosine triphosphate and dTTP, deoxythymidine triphosphate; all Roche Diagnostics Ltd., West Sussex, UK) were added to RNA. The mixture was heated to 65°C for 5 minutes followed by incubation on ice for at least 1 minute. Tubes were briefly centrifuged to collect all liquid and 1 µl of 0.1 M DTT, 4 µl of 5x First-Strand buffer, 1 µl of DNase/RNase-free water and 1 µl of SuperScript® III Reverse Transcriptase (all Invitrogen Ltd., Paisley, UK) were added to each sample. Tubes were further incubated in an Eppendorf Thermal Cycler for 5 minutes at 25°C; followed by 60 minutes at 50°C. The reaction was terminated by heating to 70°C for 15 minutes. c-DNA was stored at 4°C for short term or at -20°C. If required, cDNA was cleaned-up using GenElute™ PCR Clean-Up kit following the protocol supplied by the manufacturer (Sigma-Aldrich Company Ltd., Gillingham, UK).

2.3.3. Polymerase chain reaction (PCR) of cDNA

Primers used in the studies were supplied by Alta Biosciences (University of Birmingham, Birmingham, UK) and prepared according to the data sheet supplied. Appendix 2 shows details of the primers used in these studies.

For each PCR reaction sample the following master mix was prepared in thin walled 0.2 ml PCR tubes: 12.5 µl of premixed 2x PCR Master Mix (Promega UK Ltd, Hampshire, UK) containing 50 units/ml of Taq DNA polymerase, 400 µM dNTPs (dATP, dGTP, dCTP and dTTP), 3 mM $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR; 2.5 µl of each 3' and 5' primers (2.5 µM) and DNase/RNase-free water - up to a total volume of 25 µl per reaction after the addition of cDNA. PCR amplification was then performed in an Eppendorf Thermal Cycler using 'hot start' whereby all samples were denaturated for 2 minutes at 94°C; followed by 40 cycles consisting of a denaturation step for 30 seconds at 94°C, an annealing step for 1 minute at temperatures specific to individual primers used; and an extension step for 1 minute at 72°C. PCR products were stored at 4°C (short term) or -20°C until required.

Agarose gel electrophoresis of PCR products

Amplified samples were analysed by electrophoresis through agarose gels. 2% agarose gels were made by melting 4 g of agarose (Eurogentec Ltd., Southampton, UK) in 200 ml of 1 x tris-borate buffer solution (TBE; 45 mM tris, 1 mM EDTA pH 8.3 and 45 mM boric acid; Fisher Scientific UK Ltd., Loughborough, UK). Solution was microwaved on full power until the liquid became transparent. Once the solution cooled, ethidium bromide was

added at a final concentration of 1 µg/ml (Sigma-Aldrich Ltd., Gillingham, UK). The solution was then poured into a standard gel casting tray (Fisher Scientific UK Ltd., Loughborough, UK) sealed with masking tape. The gel was allowed to stand at room temperature for 1 hour to harden. The comb and tray barriers were then removed and the gel was submerged into 1 x TBE in a mini-gel electrophoresis unit (Fisher Scientific UK Ltd., Loughborough, UK).

6x Blue/Orange Loading Dye (Promega UK Ltd, Hampshire, UK) containing 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10 mM Tris-HCl pH 7.5 and 50 mM EDTA pH 8.0) was added to each 15 µl of PCR product samples at 1:5 dilution. Samples were then loaded onto the gel and electrophoresed at 100 V for an appropriate length of time in parallel with 100 bp DNA Ladder (Promega UK Ltd, Hampshire, UK). Amplified products were visualized on a UV transilluminator and the gel was photographed using Polaroid 667 film (Fisher Scientific UK Ltd., Loughborough, UK).

2.3.4. Real-time PCR

Primer and probe design

Primer/TaqMan probe combinations to detect expression of EBV genes were designed using the Primer Express package (Applied Biosystems, Warrington, UK) and were chosen to hybridize across exon-exon junction sequences. Probes were labeled with 6-carboxyfluorescein phosphoramidite (FAM) fluorophore at the 5’ end and 6-carboxytetramethylrhodamine (TAMRA) quencher at the 3’ end. All primers were

purchased from Alta Bioscience (University of Birmingham, Birmingham, UK); TaqMan probes were obtained from Eurogentec (Southampton, UK). BRLF1, BMLF1, BNLF2A and BALF4 primers and probes were also kindly provided by Dr. Andrew Bell (University of Birmingham, Birmingham, UK), their sequences are listed in Appendix 3.

The probe targeting BLIMP1β isoform was published elsewhere (Ocaña *et al.*, 2006), but for our study it was re-labeled with MGB™ (minor groove binder) reporter dye at the 5' end and non-fluorescent quencher (NFQ) at the 3' end and purchased from Applied Biosystems.

Target gene expression assays containing FAM(6-carboxyfluorescein)/NFQ-labeled probes were all designed and generated by Applied Biosystems and are listed in Appendix 4.

Expression of endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and β-2-microglobulin (β2m) was quantified using pre-developed house-keeping assays containing VIC/TAMRA(Carboxytetramethylrhodamine)-labeled probes (both Applied Biosystems).

Real-time PCR conditions

All real-time PCR assays were performed using an ABI Prism 7700 sequence detection system (Applied Biosystems, Warrington, UK). The reactions were set up in MicroAmp Fast optical 96-well reaction plates covered with MicroAmp Optical Adhesive Film (Applied biosystems). A final reaction volume of 25 µl contained 1× TaqMan universal PCR mastermix (Applied Biosystems) or FastStart Universal Probe Master Mix (Roche

Diagnostics Ltd., West Sussex, UK), 2.5-25.0 pmol primers, 5 pmol probe, 0.5 µl of 20x house-keeping assay and 5 µl cDNA (equivalent to required ng input RNA).

Thermal-cycling conditions were: initial uracil/N-glycosylase incubation (2 minutes at 50°C), AmpliTaq Gold activation step (12 minutes at 95°C) and 40 rounds of amplification (denaturation for 15 seconds at 95°C, annealing and extension for 1 minute at 60°C). All test samples were run in triplicate and template-negative reactions served as controls.

Real-time PCR data analysis

Relative quantification of gene expression was performed as recommended by the manufacturer. Briefly, amplification of target genes and the endogenous controls were monitored continuously by changes in FAM, MGB or VIC fluorescent intensities, using the ABI 7700 software. The resulting amplification plots were used to determine the threshold cycle (Ct) value, defined as the number of PCR cycles taken for fluorescent intensity to reach a fixed threshold for each signal. The Ct values were inversely proportional to the amount of gene present. Finally, transcript levels of target gene were normalized to amount of endogenous control in the same samples. The normalized values were then expressed relative to the appropriate reference sample, which was assigned an arbitrary value of 1.

2.4. Protein detection and analysis

2.4.1. Western blotting

Cell lysis

Culture media with cells were pelleted at 900 rpm for 5 minutes and supernatant was decanted. Cell pellets were washed in cold PBS to ensure complete removal of culture media and centrifuged again. Pellets were then lysed in appropriate volume of lysis buffer which consisted of 50 mM Tris HCL (pH 8.0); 150 mM NaCl; 1 mM EDTA; 1% Nonidet P40 (NP40; Roche) and supplemented with protease inhibitors (complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics Ltd., West Sussex, UK). Lysates were incubated on ice for 30 minutes and then centrifuged at 13000 rpm at 4°C for 15 minutes; the protein supernatants were stores at -20°C.

Determination of protein concentration

Protein concentration was quantified using the BioRad DC Protein Assay Kit (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Five standards composing different concentrations of bovine serum albumin (BSA; Sigma-Aldrich Company Ltd., Gillingham, UK): 0, 0.1, 0.2, 0.4, 0.8 and 1.5 mg/ml were used. An aliquot of each sample was diluted 1:20 with sterile distilled water. 100 µl of BioRad DC Protein Assay Reagent A was added to each standard and sample, vortexed, followed by addition of 800 µl BioRad DC Protein Assay Reagent B, vortexed again and incubated at room temperature for 15 minutes. Absorbance of standards were read on a Thermo Spectronic BioMate 3 spectrophotometer (Thermo Electron Corporation, Basingstoke, UK) at 750 nm and were

used to plot a calibration curve from which the protein content of the samples was read. Sample concentrations were calculated by multiplying by dilution factor; typically 30 µg of protein lysates were used.

Gel electrophoresis

Protein lysate samples were mixed 1:1 with 2x Laemmli sample buffer, containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris/HCl (pH 6.8). Samples were then boiled for 2 minutes at 97°C in a heat-block, centrifuged and loaded on 10% SDS-polyacrylamide gels which were made fresh and left to harden prior each electrophoresis (Appendix 5).

Gels were submerged in running buffer consisting of 0.25 M TRIS, 1.92 M glycine (Fisher Scientific UK Ltd., Loughborough, UK) and 1% SDS in distilled water. Pre-stained standards were prepared and run alongside samples (Kalidoscope Marker, Bio-Rad, UK; SeeBlue® Plus2 Pre-Stained Standard, Invitrogen, Paisley, UK). Samples were separated by electrophoresis at 100 V for 1.5 hours in an XCell Surelock Mini-Cell (Invitrogen Ltd., Paisley, UK).

Protein transfer

Proteins were transferred from the gel to a 0.45 µm nitrocellulose transfer membrane (Protran BA85 membrane; Schleicher & Schuell UK Ltd., London, UK). Membranes, sponges and filter papers (Whatman® chromatography paper; Sigma-Aldrich Ltd., Gillingham, UK) were cut to the gel size and soaked in transfer buffer (30 g tris, 144 g glycine, 2 L methanol in 8 L distilled water) for at least 5 minutes. The 'transfer sandwich'

was then set up in a XCell II Blot Module (Invitrogen Ltd., Paisley, UK), in the following order: 2 sponges, 3 pieces of filter paper, first gel, membrane, 2 sponges, 3 pieces of filter paper, second gel, membrane, 3 pieces of filter paper and 2 sponges. The transfer module was placed into the XCell Surelock Mini-Cell, filled with transfer buffer and surrounded with ice to avoid overheating during the transfer. Proteins were transferred at 30 V for 2 hours.

Labeling of specific protein

After blotting, membranes were washed in PBS-Tween-20 (0.1%) and transferred protein visualized with Ponceau S (Sigma-Aldrich Ltd., Gillingham, UK). Blots were then incubated for 1 hour at room temperature on a shaker in blocking solution (5% non-fat milk powder in PBS-Tween-20) to prevent unspecific binding. Primary antibodies were diluted to the appropriate concentration with blocking solution (see Appendix 6 for details on primary antibodies) and applied overnight at 4°C. Following day, membranes were rinsed in PBS-Tween-20 (0.1%) for 1 hour at room temperature with washes changed every 10 minutes. HRP-conjugated secondary IgG antibodies (DakoCytomation Ltd., Cambridgeshire, UK) were diluted in blocking solution and applied for 1 hour at room temperature. Finally, the membranes were again rinsed in PBS-Tween-20 (0.1%) for 1 hour.

Visualisation of proteins

Proteins were visualized using the enhanced chemiluminescence (ECL) technique (all; Amersham Biosciences UK Ltd., Buckinghamshire, UK). Membranes were incubated with ECL mixture for 1 minute, covered with clean film and placed in a Hypercassette™

autoradiography cassette in a dark room. A sheet of Hyperfilm™ was placed on top of membranes for appropriate length of time. Hyperfilm™ was then removed from the Hypercassette™ and developed in a Kodak X-OMAT 1000 processor (Kodak Limited, Hemel Hempstead, Herts, UK).

Stripping membranes for re-probing with primary antibody

To facilitate re-probing of membranes with different primary antibodies after western blotting, previous blotting reagents were removed using Re-Blot Plus Mild Antibody Stripping Solution (Chemicon International, Hampshire, UK). Firstly, membranes were washed in PBS-Tween-20 (0.1%) for 10 minutes followed by gentle mixing in stripping solution for 15 minutes at room temperature. The membranes were further washed in PBS-Tween-20 (0.1%) and incubated for 1 hour in blocking solution (5% non-fat milk powder in PBS-Tween-20).

2.4.2. Immunohistochemistry

Cytospin preparation for immunohistochemistry

2 x 10^6 cells were centrifuged, washed in PBS (pH 7.6), resuspended in 1 ml of PBS with 10% formal-saline and stored at 4°C. Cells were attached on X-tra Adhesive micro slides (Surgipath Europe, Peterborough, UK) in Cytospin3 cytocentrifuge (Shandon, Runcorn, UK) using Cytofunnel® disposable sample chambers, filter cards and Cytoclips™ (all Thermo Electron Corporation, Basingstoke, UK). Following centrifugation at 1000 rpm for 5 minutes, monolayers of cells were air dried, fixed in 10% formal-saline solution (Genta Medical, York, UK) for 10 minutes and then air dried again. Cytospin preparations were stored in aluminum foil at -20°C. When required, slides were thawed and washed in running tap water.

Blocking of endogenous peroxidase activity and antigen retrieval

Endogenous peroxidase activity was blocked by incubating slides in 3% hydrogen peroxide (Sigma-Aldrich Company Ltd., Gillingham, UK) in distilled water for 15 minutes. Following this, the slides were rinsed thoroughly in running tap water.

Low temperature antigen retrieval

Low temperature antigen retrieval was performed by incubating slides in 1 mM EDTA (pH 8.0), Tween-20 (0.1%) on a hotplate stirrer at 65°C for 16 hours (overnight). Agitation was achieved by using a magnetic bar with the stirrer set to 600 rpm. Sections were washed in tap water and then in PBS-Tween-20 (0.1%) (pH 7.6).

Detection of a single antigen

All slides were mounted onto a Sequenzer (Shandon, Runcorn, UK) or placed in a metal microscope slide staining tray (Richardsons of Leicester Ltd., Leicester, UK) and covered with a lid to minimize evaporation. Samples were washed with PBS-Tween-20 (0.1%) for 5 minutes and incubated with 100 µl of primary antibody appropriately diluted in PBS-Tween-20 (0.1%) for 1 hour at room temperature (see Appendix 6 for details on primary antibodies). Following incubation, samples were washed with PBS-Tween-20 (0.1%) for 5 minutes and incubated with 2 drops of DAKO Envision secondary antibody (Dako UK Ltd., Cambridgeshire, UK) for 30 minutes at room temperature. Visualization was carried out using the ImmPact diaminobenzidine (DAB) substrate system (Vector Laboratories Ltd., Peterborough, UK) for 1 minute which generated a brown product that is insoluble in water and organic solvents. Slides were further rinsed with distilled water and counterstained with Harris' haematoxylin (Sigma-Aldrich Ltd., Gillingham, UK) for 10 seconds and washed under running tap water for 5 minutes. Slides were again dehydrated in IMS and xylene and mounted under cover slips with DPX mounting medium (Sigma-Aldrich Ltd., Gillingham, UK) for microscopic examination.

Detection of two antigens (double immunohistochemistry)

All steps were performed as described above (page 92- 93); except that first visualization was carried out using Vector® NovaRED™ Substrate (Vector Laboratories Ltd., Peterborough, UK) which developed a red product. To achieve detection of the second antigen, slides were immediately washed with PBS-Tween-20 (0.1%) for 5 minutes and the second primary antibody was applied for 1 hour. Following further washing with PBS-

Tween-20 (0.1%) and incubation with Dako secondary antibodies, visualization of the second antigen was carried out using Vector® SG Substrate (Vector Laboratories Ltd., Peterborough, UK) which developed grey/black product. Slides were again dehydrated in IMS and xylene and mounted under cover slips with DPX mounting medium (Sigma-Aldrich Ltd., Gillingham, UK) for microscopic examination.

2.5. Primary B cell studies

2.5.1. Purification of tonsillar mononuclear cells (TMCs)

Tonsils were minced with a scalpel in cold RPMI 1640 medium (Sigma-Aldrich Ltd., Gillingham, UK) without foetal calf serum, supplemented with 2mM L-glutamine (Invitrogen Ltd., Paisley, UK), 0.5% ciprofloxacin (Bayer, Newbury, UK) and 1% penicillin-streptomycin solution (Sigma-Aldrich Ltd., Gillingham, UK). Mononuclear cells were then isolated by Ficoll-Isopaque centrifugation. 15 ml of Lymphoprep® solution (Axis-Shield Diagnostics Ltd. UK, Dundee, UK) was pipetted into universal tubes and carefully overlaid with media containing a mixture of tonsillar cells. Tubes were then centrifuged at 2200 rpm at room temperature for 30 minutes creating the following visible layers: plasma and other constituents (top), mononuclear cells (middle), Ficoll-Paque, and erythrocytes and granulocytes debris (pellet). The layer of mononuclear cells was transferred to a fresh tube, washed twice with cold media and once with cold autoMACS™ Rinsing Solution (autoMACS; Miltenyi Biotec Ltd, Surrey, UK) supplemented with 1% penicillin-streptomycin solution (Sigma-Aldrich Company Ltd., Gillingham, UK), 0.5% ciprofloxacin

(Bayer, Newbury, UK) and 5% MACS® BSA Stock Solution (Miltenyi Biotec Ltd, Surrey, UK); each time centrifuged at 900 rpm at 4°C for 10 minutes.

2.5.2. Purification of GC B cells

GC B cells ($CD10^+$) were isolated from TMCs by positive enrichement of $CD10^+$ cells using magnetic separation with anti-CD10-Phycoerythrin (PE) (eBioscience, San Diego, CA, USA), anti-PE microbeads and LS columns (both Miltenyi Biotec Ltd, Surrey, UK). Magnet and autoMACS buffer were pre-cooled at 4°C and all purification steps performed on ice to prevent rapid apoptosis of GC B cells and unspecific antibody binding. First, 10^7 TMCs were resuspended in 100 µl of autoMACS and CD10-PE antibody was added at 1:50 dilution. Following incubation at 4°C for 15 minutes, cells were washed with 10 volumes of autoMACS and centrifuged at 900 rpm at 4°C for 10 minutes. 10^7 CD10-PE-labelled TMCs were then resuspended in 80 µl of autoMACS and 20 µl of anti-PE beads were added. Following incubation at 4°C for 15 minutes, cells were washed with 10 volumes of autoMACS and centrifuged at 900 rpm at 4°C for 10 minutes. Finally, 10^8 CD10-PE-anti-PE-beads-labelled TMCs were resuspended in 500 µl of autoMACS and transferred on pre-washed filters (Partec UK Limited, Canterbury, UK) placed on LS columns. When the cells passed through; columns and filters were washed three times with 3 ml of autoMACS. Finally, columns were removed from the magnet and retained population of $CD10^+$ positive GC B cells was eluted with 5ml of autoMACS.

2.5.3. Purification of naïve and memory B cells

Naïve B cells used for PCR to assess the levels of BLIMP1 isoforms were isolated from TMCs indirectly by depletion of all non-naïve cells using a cocktail of antibodies to CD10, CD2, CD16, CD27, CD36, CD43 and CD235a (Miltenyi Biotec Ltd, Surrey, UK) following the protocol of the manufacturer. Memory B cells were purified from TMCs first by negative depletion using antibodies against CD2, CD14, CD16, CD36, CD43 and CD235a, and then by positive selection with CD27 microbeads (Miltenyi Biotec Ltd, Surrey, UK) following manufacturer's instructions.

2.5.4. Transfection of GC B cells by nucleofection

LMP1, BLIMP1α, C-MYC or control plasmid (pcDNA3.1-LMP1 or pSG5-LMP1; pcDNA3.1-BLIMP1α; pcDNA3.1-MYC; pcDNA3.1 or pSG5) were transiently transfected into GC B cells using non-viral Nucleofector technology with the Nucleofector II device (amaxa GmbH, Cologne, Germany). For each reaction, 10^7 cells were pelleted by centrifugation in universal tubes at 636 rpm at room temperature for 10 minutes. Supernatant was completely removed and the cell pellets were resuspended in 100 µl of Nucleofector reaction mix consisting of Cell Line Nucleofector Solution B mixed with Supplement I (both components of Cell Line Nucleofector Kit B; amaxa GmbH, Cologne, Germany).

7 µg of each plasmid were mixed with 3 µg of pMACS LNGFR. The pMACS LNGFR vector encodes the truncated human low-affinity nerve growth factor receptor surface molecule that served as a marker to track transfected cells. Both plasmids were combined with the cell suspensions and transferred to a 0.1cm cuvette (component of Cell Line Nucleofector

Kit B; amaxa GmbH, Cologne, Germany). The cuvette was placed in to the Nucleofector II device and U-15 program was selected. Following the pulse, the entire contents of the cuvette were immediately transferred into 24-well plates (Nalge Europe Ltd., Hereford, UK) using plastic pipettes and a small amount of pre-warmed RPMI. The 24-well plates were prepared in advance and filled with 2 ml of RPMI containing supplemented with 20% of foetal calf serum. Cells were then incubated at 37°C in 5% CO_2 for 16 hours (overnight) prior to MoFlo enrichment of transfected cells.

2.5.5. MoFlo enrichment of transfected GC B cells

Following incubation, transfected GC B cells were pooled together, washed twice with cold autoMACS and pelleted at 636 rpm at 4°C for 10 minutes. The cell pellets were resuspended in 250 µl of autoMACS and 25 µl anti-LNGFR-Allophycocyanin (APC) antibody (Miltenyi Biotec Ltd, Surrey, UK) was added. Following 10-minute incubation at 4°C in the dark, cells were washed with 6 ml of autoMACS and centrifuged again at 636 rpm at 4°C for 10 minutes. Cells were passed through pre-washed filters into sorting tubes and kept on ice in the dark. The APC-labelled cells were collected by FACS on a MoFlo sorter (Dako Cytomation, Colorado, USA) using propidium iodide (Sigma-Aldrich Ltd., Gillingham, UK) to separate living cells. In some cases, CD10+ cells were further separated into CD77+ and CD77- subpopulations using anti-CD77-fluorescein isothiocyanate (FITC) antibody (BD Biosciences PharMingen). The transfection efficiency of the living cells was generally between 10%-20% and purity of the collected cells was >95%. Western blotting or real-time PCR were performed to validate successful gene expression in transfected cells.

2.5.6. Immunofluorescence analysis

To detect cell-surface marker expression, 10^5cells/ml were twice washed with autoMACS, pelleted and resuspended in 50 µl of autoMACS and 5 µl of antibody of interest. Following 10-minute incubation on ice and two more washes, cells were resuspended in 200 µl of autoMACS and kept on ice in the dark. Propidium iodide was added to all stained samples immediately before measurement. Cells were analysed by flow cytometry using a FACS-Calibur (Becton Dickinson, Franklin Lakes, NJ). The CellQuest software was used for acquisition and Flow Jo software was used for analysis of the samples. Only the viable cells were considered for analysis based on their light scatter (FSC/SSC) characteristics. Data are presented within individual result chapters as the mean of linear fluorescent intensity after subtraction of background staining with isotype-matched control.

2.5.7. Gene expression array of GC B cells

2.5.7.1. RNA amplification

RNA was extracted using RNeasy Mini Kit (QIAGEN Ltd., Crawley, UK) following manufacturer's instructions (see section 2.3.1. for details). RNase-Free DNase Set (QIAGEN Ltd., Crawley, UK) was included to remove any genomic DNA which could interfere with amplification. Two carriers, N-Carrier and P-Carrier, were also combined with the RNA isolation and served as precipitation carriers to protect against RNA degradation and loss due to unspecific surface adsorption. Extracted RNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol.

Following 10-minute incubation on ice, RNA was recovered by spinning at 14000 g at 4°C for 20 minutes. Supernatant was then removed, RNA pellet was washed with 80% ethanol, centrifuged again, air dried for 5 minutes and dissolved in DNase/RNase-free water. The quality of the isolated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE, USA) and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). Only those samples with a 260/280 nm ratio between 1.8-2.1 and a 28S/18S ratio within 1.5-2.0 were processed further. The RNA amplification was carried out with ExpressArt® mRNA Amplification Kits Nano version & Nano *plus* Version (AmpTec GmbH, Germany).

First Round Amplification

First strand cDNA Synthesis

First strand cDNA was synthesised by combining 5 µl of RNA (input of total RNA: 1-700ng) with First strand cDNA Mix 1 (2.5 µl of DEPC-H_2O, 1 µl dNTP-Mix and 1.5 µl Primer A). Samples were incubated for 4 minutes at 65°C in a thermocycler and cooled to 37°C. The First Strand cDNA Synthesis Mix 2 (4 µl of DEPC- H_2O, 4 µl 5x RT Buffer, 1 µl RNase Inhibitor and 1 µl RT Enzyme) was added to each sample and mixed well by gently flicking the tubes. Samples were then incubated in a thermocycler, using the following incubation conditions: 37°C for 5 minutes, 42°C for 50 minutes, 45°C for 10 minutes, 50°C for 10 minutes and 70°C for 15 minutes; and immediately placed on ice.

RNA removal

RNA was removed by adding 5 µl of RNase Mix 3 (3 µl of DEPC-H2O, 1 µl of 5x Extender Buffer and 1 µl of RNase) and incubating at 37°C for 20 minutes.

Second strand cDNA Synthesis

Second strand cDNA was synthesised by combining the Second strand cDNA Mix 4 (14 µl of DEPC-H_2O, 4 µl of 5x Extender Buffer, 1 µl of Primer B and 1 µl of dNTP-Mix) with the First Strand cDNA Reaction and placing in a thermocycler for 1 minute at 96°C and then 1 minute at 37°C. 5 µl of Extender Enzyme A Mix 5 (3 µl of DEPC-H_2O, 1 µl of 5x Extender Buffer and 1 µl of Extender Enzyme A) was added to the reaction, followed by 30 minutes at 37°C, adding Primer Erase Mix 6 (3 µl of DEPC-H_2O, 10 µl of 5x Extender Buffer and 1 µl of Primer Erase) and placing samples at 37°C for 5 minutes and 96°C for 6 minutes. All samples were then immediately placed on ice, 5 µl of Primer C was added and samples were incubated again for 1 minute at 96°C and then 1 minute at 37 °C. Finally, the Extender Enzyme B Mix 7 (2 µl of DEPC-H_2O, 2 µl of 5x Extender Buffer and 1 µl of Extender Enzyme B) was added and samples were incubated for 30 minutes at 37°C, 15 minutes at 65°C and placed on ice.

cDNA purification and ethanol precipitation of the purified cDNA

cDNA purification was achieved by adding 350 µl of Binding buffer and 3 µl of Carrier DNA to the Second Strand cDNA Reaction. The samples were transferred into cDNA Purification Spin Columns in Collection Tubes and centrifuged for 1 min at maximum speed and washed twice with 500 µl and with 200 µl of Washing Buffer. Samples were

eluted into fresh 1.5 ml reaction tubes twice with 50 µl of Elution Buffer. Purified cDNA was precipitated with Precipitation Mix 9 (2 µl of Precipitation Carrier and 10 µl of Sodium Acetate) and 220 µl of absolute ethanol and centrifuged at maximum speed for 10 minutes. The pink pellet was washed with 200 µl of 70% ethanol, centrifuged again and air dried for about 5 minutes.

Amplification by in vitro Transcription

cDNA for each sample was dissolved in 8 µl of Solubilisation Buffer and amplified by *in vitro* Transcription. In 0.5ml RNase-free PCR tubes, *in vitro*-Transcription Mix 10 (8 µl of NTP-Mix, 2 µl of 10x Buffer and 2 µl of RNA Polymerase) was combined with 8 µl cDNA and incubated overnight at 37°C. 1 µl of DNase I was then added to each reaction and incubated further at 37°C for 15 minutes.

RNA-Purification using RNeasy Mini Kit

Amplified RNA was purified using the RNeasy Mini Kit (QIAGEN Ltd., Crawley, UK) as follows: 80 µl of RNase-free water, 350 µl of RLT (Lysis Buffer) were added to each in vitro-Transcription Reaction and mixed thoroughly with 250 µl of absolute ethanol. The mixture was transferred onto spin columns, centrifuged for 15 seconds at 10000 rpm, washed twice with 500 µl of RPE and eluted into new 1.5 ml RNase-free reaction tubes twice with 50 µl of RNase-free water.

Ethanol precipitation of purified antisense RNA

The eluted purified antisense RNA was precipitated with 10 µl of sodium acetate, 2 µl of Precipitation Carrier and 220 µl of absolute ethanol. Following 2-minute incubation at room temperature, RNA was recovered by centrifugation at maximum speed for 10 minutes. After supernatant was discarded, pink-coloured pellet was washed with 70% ethanol, centrifuged again, air dried for 5 minutes and dissolved in 6 µl of DEPC-water. Quality and quantification of purified antisense RNA were assessed using spectrophotometric analysis of samples.

Second Round Amplification

During this step, amplified RNA was again reverse transcribed into cDNA to produce high yields of antisense RNA via a second round of amplification.

First strand cDNA Synthesis

To synthesise the First strand cDNA, 500-800 ng of RNA for each sample was combined with the First Strand Mix 12 (1 µl of NTP-Mix, 2 µl of Primer D and 2 µl of Reaction Additive) and incubated for 4 minutes at 65°C in a thermocycler. First Strand cDNA Synthesis Mix 2 (4 µl of DEPC-H_2O, 4 µl of 5x RT Buffer, 1 µl of RNase Inhibitor and 1 µl of RT Enzyme) was added following incubation at 45°C for 30 min, 70°C for 15 minutes and placing the samples immediately on ice.

RNA removal

RNA removal was achieved by adding RNase Mix 3 (3 µl of DEPC-H_2O, 1 µl of 5x RT Buffer and 1 µl of RNase) to the First Strand cDNA Reaction followed by incubation at 37°C for 20 minutes.

Second strand cDNA Synthesis

Second strand cDNA was synthesised by adding the Second Strand cDNA Synthesis Mix 13 (10 µl of DEPC-H_2O, 5 µl of Primer C, 4 µl of 5x Extender Buffer and 1 µl of dNTP-Mix) to the First Strand cDNA Reaction and incubating as follows: 1 minute at 96°C and 1 minute at 37°C. Finally, the Extender Enzyme B Mix 14 (3 µl of DEPC-H_2O, 1 µl of 5x RT Buffer and 1 µl of Extender Enzyme B) was added to each sample, mixed well, incubated at 37°C for 30 minutes, 65°C for 15 minutes and placed on ice.

cDNA purification and ethanol precipitation of the purified cDNA

cDNA purification was achieved by adding 275 µl of Binding buffer and 3 µl of Carrier DNA to the Second Strand cDNA Reaction and continued by RNA-Purification using RNeasy Mini Kit and ethanol precipitation of purified antisense RNA as described on page 100-101. cDNA for each sample was dissolved in 8 µl of Solubilisation Buffer.

Third Round Amplification

If required, the third round amplification was performed by repeating the same steps as described for the second round of amplification.

2.5.7.2. Synthesis of cRNA-in vitro transcription (IVT)

The purified double-stranded cDNA was in vitro-transcribed in the presence of biotin-labelled nucleotides using an IVT labelling kit (Affymetrix, Santa Clara, CA, USA). The cDNA for each sample was mixed with IVT master mix (12 µl of DEPC-H_2O, 4 µl of (10x) IVT labelling Buffer, 12 µl of IVT labelling NTP mix, 4 µl of IVT labelling enzyme mix) and incubated overnight at 37°C. The biotinylated cRNA was purified using a Sample Cleanup Module (Affymetrix, Santa Clara, CA, USA). The IVT reaction for each sample was mixed with 60 µl of RNase-free water, 350 µl of cRNA binding buffer, 250 µl of absolute ethanol and each time mixed thoroughly. The mixture was transferred to IVT cRNA cleanup columns and centrifuged at full speed for 15 seconds. The columns were then washed with 500 µl of cRNA wash buffer, 500 µl of 80% ethanol and eluted twice with 10 µl of RNase-free water. The quality and quantity of eluted cRNA was determined using NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE, USA) and a Bioanalyzer 2100 (Agilent, Waldbronn, Germany).

2.5.7.3. cRNA fragmentation

25 µg of biotin-labelled cRNA for each sample was mixed with 10 µl of (5x) Fragmentation buffer (Santa Clara, CA, USA) and topped up to 50 µl with DEPC-treated water. Samples were then subjected to random fragmentation in a PCR block at 94°C for 35 minutes. Finally, 1-2 µg of fragmented cRNA was run on 1.2% agarose gel and fragments were found to be approximately 35-200 bp.

2.5.7.4. Hybridisation and analysis of hybridisation signal

Biotinylated cRNA was hybridized to Affymetrix (Santa Clara, CA, USA) HG-U133 Plus2 microarrays. Scanned images of microarray chips were analyzed using Affymetrix GeneChip Operating Software (GCOS). Gene expression signal was calculated using the MicroArray Suite 5 (MAS5) algorithm of GCOS with the default settings except the target signal was set to 100. For two separate patients, the gene expression profiles of LMP1-transfected or BLIMP1α-transfected cells were compared with that of the control pcDNA3.1-transfected GC B cells. Differentially expressed genes were identified using the GCOS pairwise analysis with the default settings.

2.5.8. Identification of differential gene expression in B cell subsets using a published data set

Differentially expressed genes among centrocytes, plasma cells and memory cells were identified using statistical analysis of microarrays (SAM) (Tusher *et al.*, 2001) analysis with a fold-change threshold of 1.5 and a q-value threshold of 5%, following robust multi-array average (RMA, Irizarry *et al.*, 2003) reprocessing of the raw data of Brune *et al.* (2008) under GEO series no GSE12453.

2.6. Cell line studies

2.6.1. Transfection of cell lines by electroporation

Electroporation

Cell lines were transfected using a Bio-Rad Gene Pulser® II electroporator (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Cells were seeded at a concentration of 5 x 10^5/ml 24 hours prior to transfection and then pelleted by centrifugation at 900 rpm for 10 minutes. 10^7 cells were used for each transfection, although the conditions of each experiment differed for individual cell lines and are summarized in Appendix 7. Cells were twice washed with washing buffer or media (specified in Appendix 7), pelleted by centrifugation and resuspended in transfection media containing individual plasmids. In some experiments, pMACS CD4.1 vector (Miltenyi Biotec Ltd., Surrey, UK) was co-transfected with a plasmid of interest at ratio 9:1. Electroporation of cells was then carried out in cuvettes with a 4-mm gap between electrodes (GENEFLOW INC., Alexandria, USA) at a voltage and high capacity dependent upon the cell line. Following the pulse, the content of the cuvette was immediately transferred into 8 ml of pre-warmed cultivation media and placed at 37°C in 5% CO_2 for the required length of time. Details specific to individual transfection experiments are specified in result chapters.

Purification of viable lymphocytes

In the case that transfection of individual cell lines led to the death of substantial percentage of electroporated cells, viable cells were isolated using Lymphoprep® centrifugation. Lymphoprep® solution (Axis-Shield Diagnostics Ltd. UK, Dundee, UK) was

pipetted into universal tubes and carefully overlaid with media containing transfected cells. Tubes were then centrifuged at 2200 rpm at room temperature for 30 minutes. The layer of viable cells which formed between the lower layer (media) and upper layer (lymphoprep) was transferred to a fresh tube and washed with PBS.

Magnetic enrichment of transfected cells

In the case that transfection with a plasmid of interest was carried out in the presence of pMACS CD4.1 vector, expression of pMACS CD4.1 was used to positively enrich transfected cells. Media with transfected cells were diluted in three volumes of ice-cold autoMACS™ Rinsing Solution (autoMACS; Miltenyi Biotec Ltd, Surrey, UK) supplemented with 1% penicillin-streptomycin solution (Sigma-Aldrich Company Ltd., Gillingham, UK), 0.5% ciprofloxacin (Bayer, Newbury, UK) and 5% MACS® BSA Stock Solution (Miltenyi Biotec Ltd, Surrey, UK) and centrifuged at 900 rpm and 4°C for 10 minutes. Following two more washes, 10^7 cells were resuspended in 80 µl of cold autoMACS and 20 µl of CD4 multisort beads (Miltenyi Biotec Ltd, Surrey, UK) and incubated at 4°C for 15 minutes. Following the incubation, cells were washed with ten volumes of cold autoMACS and pelleted by centrifugation at 900 rpm at 4°C for 10 minutes. The cell pellets were resuspended in 500 µl of cold autoMACS and transferred onto filters (Partec UK Limited, Canterbury, UK) placed on Mini-MACS MS columns fitted into a magnet (both Miltenyi Biotec Ltd, Surrey, UK). When the cells passed through, columns and filters were washed three times with 500 µl of cold autoMACS. Columns were removed and retained cells eluted with 500 µl of cold autoMACS. Transfection efficiency of electroporated cells was determined by Immunofluorescence using anti-CD4 antibody; Immunotech, Marseille,

France (as described in section 2.5.6.), real-time PCR, Western blotting or immunohistochemical analysis (see sections 2.1., 2.3. and 2.4.) and is specified in the individual result chapters.

2.6.2. Luciferase assays

To measure activity of BLIMP1α and BLIMP1β promoters, the Dual-Luciferase® Reporter (DLR™) Assay System (Promega UK Ltd, Hampshire, UK) was used. In this assay, the activity of firefly (Photinus pyralis) and Renilla (Renilla reniformis) luciferases were measured sequentially from the same samples. The firefly luciferase signal was quantified first by adding Luciferase Assay Reagent II (LAR II). After the reaction was quenched, the Renilla luciferase signal reaction was simultaneously initiated by adding Stop & Glo® Reagent to the same tube. The luminescence produced by Renilla luciferase was then used as an internal control to which expression of the experimental firefly luciferase reporter gene was normalized.

Briefly, all cells were electroporated as described on page106-107. In each reaction, 1 x 10^7 cells were co-transfected with 100 ng of pcDNA3.1 or pcDNA3.1-LMP1 vector together with 10 µg of either BLIMP1α or BLIMP1β promoter construct. All transfections were carried out in triplicate and in the presence of 0.5 µg pRL-null Vector. Cells were then incubated at 37°C in 5% CO_2 for 24 or 48 hours. Following incubation, cells were pelleted by centrifugation at 4°C and 1200 rpm for 10 minutes. The cell pellets were resuspended in 100 µl of 1x passive lysis buffer (part of the DLR™ Assay System), vortexed

and placed at -80°C for at least 24 hours. When required, cell lysates were vortexed and centrifuged again to remove cell debris. 10 µl of supernatant for each sample was transferred into 96 well plates and luciferase assay performed using Orion L Microplate Luminometer (Geneflow Ltd, Staffordshire, UK) according to manufacturer's instructions. All data were presented as relative luciferase units (RLU), which were calculated by dividing the firefly luciferase activity by the renilla luciferase activity.

2.6.3. Chromatin Immunoprecipitation

Preparation of cross-linked chromatin

To determine if BLIMP1 protein directly binds into BZLF1 promoter of EBV, Chromatin Immunoprecipitation (ChIP) was performed. B95.8 and SL3-LCL cells were electroporated with 20 µg of pcDNA3.1 or pcDNA3.1-BLIMP1α plasmids (see Appendix 7) and cultivated at 37°C in 5% CO_2 for 48 hours prior to ChIP (see Appendix 7). Untransfected B95.8 cells were split 24 hours prior to ChIP.

4 x 10^6 cells from each sample were harvested and washed twice in ice cold PBS/5 mM sodium butyrate. To reversibly cross-link, 54 µl of formaldehyde was added and cells were incubated for 15 minutes at room temperature on a rotator. The fixation reaction was then quenched with 228 µl of 1.25 M glycine for 8 minutes at room temperature. Cells were centrifuged at 470 g at 4°C for 10 minutes and washed twice with PBS/5 mM sodium butyrate. Cell pellets for each sample were lysed in 520 µl of lysis buffer (50 mM Tris-HCl,

pH 8.1, 10 mM EDTA, 1% SDS, 5 mM sodium butyrate, and protease inhibitors) and incubated on ice for 1 hour.

Fragmentation of the chromatin

Cell lysates were sonicated in a water bath Bioruptor^{TM} sonicator (Diagenode, Liege, Belgium). The sheared chromatin was centrifuged at 10000 g at 4°C for 10 minutes to remove cell debris, measured using a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE, USA) and stored at -80°C.

Checking for correct fragmentation

2.5 µg of chromatin was topped up to 150 µl with lysis buffer and 2 µl of proteinase K (final concentration 50 µg/ml; Sigma-Aldrich Ltd., Gillingham, UK) was added. The mixture was incubated in a thermomixer at 68°C and 1300 rpm for 16 hours (overnight). DNA was extracted with **ChIP DNA Clean & Concentrator™** kit (Cambridge Bio Science, Cambridge, UK) and 1 µg of extracted DNA run on 1.5% agarose gel to check shearing of the chromatin (as described on pages 84-85).

Immunoprecipitation of resulting chromatin fragments

Protein G Dynabeads® (Invitrogen Ltd., Paisley, UK) were resuspended and washed three times with RIPA buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM EGTA, 1% Triton, 0.1% SDS, 0.1% sodium deoxycholate and 150 mM NaCl). All beads were resuspended in RIPA buffer and divided into 100 µl aliquots. 2.4 µg of antibody was added to the beads and incubated on a rotator at 4°C for 16 hours (overnight). The antibodies used were:

BLIMP1 R21 rabbit polyclonal antibody and anti-HA (Y-11) rabbit polyclonal antibody; normal rabbit IgG antibody and no antibody (water) were used alongside as negative controls (Appendix 6). Following incubation, beads with bound antibody were captured on a magnet, the supernatant removed and 10 µg of chromatin in 100 µl of RIPA buffer was added to the antibody bound beads. Immune complexes were incubated on a rotator at 4°C for 8 hours (during day). Following the incubation, beads were captured on a magnet, washed three times with RIPA buffer for 4 minutes and resuspended in TE buffer (pH 8.0).

DNA elution, cross-link reversal, proteinase K digestion

Immune complexes were eluted with 150 µl of elution buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM sodium butyrate, 50mM NaCl and 1% SDS). The cross-links were reversed by incubation on a thermo-mixer at 68°C and 1300 rpm for 16 hours (overnight) in the presence of and 2 µl of proteinase K (final concentration 50 µg/ml; Sigma-Aldrich Ltd., Gillingham, UK). Following the incubation, DNA was extracted with **ChIP DNA Clean & Concentrator™** kit (Cambridge Bio Science, UK) and measured using a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE, USA).

Sybr green real-time PCR

1 µl aliquots of purified DNA were analysed by sybr green real-time PCR using primers designed to amplify 3 different regions of the BZLF1 promoter. The real-time PCR assays were performed using an ABI Prism 7700 sequence detection system (Applied Biosystems, Warrington, UK). All reactions were set up in MicroAmp Fast optical 96-well

reaction plates covered with MicroAmp Optical Adhesive Film. A final reaction volume of 25 µl contained 12.5 µl of QuantiTect SYBR Green PCR master mix (Qiagen, Crawley, UK), 5 µl of BZLF1 primers at concentration 10 pmol/µl (see Appendix 8 for details on primer sequences used in ChIP), 6.5 µl of PCR grade water and 1 µl of DNA. Thermal-cycling conditions were: initial 95°C incubation for 15 minutes and 50 rounds of amplification (denaturation for 15 seconds at 95°C, annealing for 30 seconds at 58°C and extension for 1 minute at 72°C). All test samples were run in triplicate.

2.6.4. DNA methylation analysis by pyrosequencing

The methylation status of CpG islands in the BLIMP1β promoter region was analysed by pyrosequencing. DNA was extracted from a panel of HL cell lines and B95.8 cells and bisulfite converted. This treatment led to deamination of cytosines and their conversion to uracils, while 5-methylcytosines remained unchanged. After bisulfite treatment and PCR amplification, the proportion of methylated/unmethylated CpGs in the BLIMP1β promoter sequence was assessed using PyroMark ID sequencer (Biotage UK Ltd., Hertford, UK).

Phenol extraction and ethanol precipitation of DNA

2 x 10^6 cells per each cell line were harvested, washed in 1 ml of PBS and centrifuged at 3000 rpm for 5 minutes. Cell pellets were resuspended in 400 µl of lysis buffer (40 µl of 10x PCR buffer, 360 µl of PCR grade water and 2 µl of 1% Tween) and 13 µl of proteinase K (Sigma-Aldrich Ltd., Gillingham, UK) was added. Samples were incubated at 55°C on a

heat block for 16 hours (overnight). 400 µl of phenol was added and samples vortexed until they had a milky appearance. Following centrifugation at 13000 rpm for 5 minutes, the upper phase was aspirated and transferred into a new eppendorf containing 400 µl of phenol and 400 µl of chloroform. Samples were vortexed and centrifuged again, upper phase aspirated, transferred into a new eppendorf containing 400 µl of chloroform only, vortexed and centrifuged. The upper phase was mixed with precipitation solution (1 ml of absolute ethanol, 40 µl of sodium acetate, pH 5.2 and 1 µl of glycogen) and stored at -80°C. When required, samples were centrifuged at 13000 rpm at 0°C for 20 minutes. DNA pellets were washed with 800 µl of cold 70% ethanol, centrifuged at 13000 rpm at 4°C for 20 minutes, washed with 400 µl of 70% ethanol at room temperature and centrifuged at 13000 rpm and 4°C for 10 minutes. Eppendorfs with precipitated DNA were placed on a heat block at 37°C with lids open until the ethanol evaporated. DNA pellets were then reconstituted with PCR grade water and stored at -20°C.

Bisulfite modification and PCR reaction

Bisulphite conversion of DNA was performed using EZ DNA Methylation-Gold™ Kit (Cambridge Bio Science, UK). Briefly, 20 µl of DNA (corresponding to 1 µg of DNA) was mixed with 130 µl of CT Conversion Reagent and incubated in a thermal cycler at 98°C for 10 minutes and then at 64°C for 2.5 hours. A water sample was also included as a negative control. All samples were loaded onto Zymo-Spin™ IC Columns containing 600 µl of M-binding buffer and centrifuged at full speed for 30 seconds. 100 µl of M-Wash buffer was added and columns were centrifuged again. 200 µl of M-Desulphonation Buffer was added to columns and allowed to stand for 15-20 minutes at room temperature. After the

incubation, samples were washed twice with 200 µl of M-Wash buffer and eluted into fresh eppendorfs with 10 µl of M-Elution buffer. The eluted volume was topped up to 50 µl with PCR grade water and subjected to PCR. PCR mix consisted of 25 µl of Thermostart Mastermix (Fisher Scientific UK Ltd., Loughborough, UK), 2 µl of non biotinylated and 1 µl of biotinylated primer both at concentration 10 pmol/µl (see Appendix 9 for details on primer sequences used in pyrosequencing analysis), 10 µl of bisulfite modified DNA and 12 µl of PCR grade water. Cycling conditions were: 95°C for 15 minutes, followed by 70 rounds of amplification (denaturation for 15 seconds at 95°C, annealing for 30 seconds at 51°C and extension for 30 seconds at 72°C) and terminated at 72°C for 10 minutes.

Following PCR, 5 µl of product per each cell line was run on a 2% agarose gel (see pages 84-85 details) at 120 V for 1 hours resulting in one single band appearing.

Pyrosequencing

40 µl of PCR products were loaded onto a 96 well plate (Applied Biosystems, Warrington, UK). 40 µl of mastermix consisting of 3 µl of streptavidin beads and 37 µl of binding buffer was added into each well. The 96 well plate was incubated at 4°C for at least 1 hour followed by agitating at 1300 rpm for 5 minutes. A sequencing plate (Biotage UK Ltd., Hertford, UK) was loaded with sequencing mastermix (containing 1.5 µl of sequencing primer and 38.5 µl of annealing buffer) in the exactly same order as the 96 well plate. The following controls were also run on each plate: 1 µl of biotinylated primer, 1.5 µl of sequencing primer and a control with both 1 µl of biotinylated primer + 1.5 µl of sequencing primer. Samples were run on the PyroMark pyrosequencer (Biotage UK Ltd., Hertford, UK) according to the manufacturer's instructions.

CHAPTER 3

AN INVESTIGATION OF THE INFLUENCE OF EBV

AND THE EBV-ENCODED LMP1 ON THE EXPRESSION OF BLIMP1α

AND ITS DOWN-STREAM TARGETS

AN INVESTIGATION OF THE INFLUENCE OF EBV AND THE EBV-ENCODED LMP1 ON THE EXPRESSION OF BLIMP1α AND ITS DOWN-STREAM TARGETS

3.1. Introduction

In normal carriers, EBV persists in memory B cells (Babcock *et al.,* 1998; Thorley-Lawson and Gross, 2004; Souza *et al.,* 2007). However, the exact mechanism by which EBV gains access to the memory compartment is controversial, the most widely held model is that the virus drives newly infected B cells into a GC reaction, and then subsequently induces their differentiation into memory cells (Thorley-Lawson and Gross, 2004). EBV-infected B cells may also differentiate into plasma cells, an event associated with induction of the EBV lytic cycle (Anagnostopoulos *et al.,* 1995; Crawford and Ando, 1986; Niedobitek *et al.,* 1997; Niedobitek *et al.,* 2000; Laichalk and Thorley-Lawson, 2005).

The importance of the GC reaction in the life cycle of EBV is supported by studies which show that tonsils from persistently infected individuals contain EBV-infected cells expressing the GC marker, CD10 (Babcock *et al.,* 2000); in these cells virus gene expression is limited to a subset of latent genes including the latent membrane proteins, LMP1 and LMP2 (Thorley-Lawson and Gross, 2004; Roughan and Thorley-Lawson, 2009). LMP1 and LMP2 have been shown to possess, respectively, the CD40 and BCR signalling functions necessary for the survival of GC B cells. Indeed, studies *in vitro* and from transgenic mice suggest that LMP1 and LMP2 alone might be capable of driving the GC process in the absence of antigen (Gires *et al.,* 1997; He *et al.,* 2003; Panagopoulos *et al.,* 2004; Caldwell *et al.,* 1998; Casola *et al.,* 2004; Swanson-Mungerson *et al.,* 2005).

Work from our laboratory has shown that LMP1 expression in primary human GC B cells induces a global down-regulation of B cell-associated genes (Vockerodt *et al.,* 2008). This observation is consistent with the ability of LMP1 to drive B cell differentiation towards the post-GC stages, which are characterized by the loss of B cell identity. However, it is not clear if LMP1 drives B cell differentiation in the direction of memory cells, plasma cells, or both.

Plasma cell differentiation is regulated by a small number of essential transcription factors which include BLIMP1. The full-length BLIMP1α isoform orchestrates plasma cell differentiation by repressing genetic programs associated with the GC stages, while at the same time activating those programs associated with plasma cell functions (Shapiro-Shelef *et al.,* 2003; Calame *et al.,* 2003). Forced expression of BLIMP1 alone is capable of driving mature B cells to differentiate into plasma cells (Turner *et al.,* 1994). BLIMP1 is essential to extinguish many aspects of the mature B cell gene-expression program, including the silencing of C-MYC, PAX5 and BCL6, and for the exit from the cell cycle characteristic of terminal differentiation (Shaffer *et al.,* 2002; Lin *et al.,* 1997; Lin *et al.,* 2000; Lin *et al.,* 2002; Sciammas *et al.,* 2004). BLIMP1 also activates IRF4 which is required for the completion of plasma cell differentiation (Shaffer *et al.,* 2002; Klein *et al.,* 2006). Furthermore, BLIMP1 can apparently prime plasma cells for apoptosis by down-regulating the expression of anti-apoptotic genes (e.g. BCL2A1) (Shaffer *et al.,* 2002).

In this chapter, I have focused on the possibility that the EBV-encoded LMP1 might regulate BLIMP1α in GC B cells. There are several reasons to believe that LMP1 might influence BLIMP1α expression. First, LMP1 is a functional homologue of CD40 which is

known to down-regulate BLIMP1 expression and to suppress plasma cell differentiation (Bishop and Hostager, 2001; Lam and Sugden, 2003; Panagopoulos *et al.*, 2004; Callard, *et al.*, 1995; Randall *et al.,* 1998; Satpathy *et al.,* 2010). Second, LMP1 is known to suppress induction of EBV lytic cycle, an event which is associated with plasma cell differentiation (Adler *et al.,* 2002; Prince *et al.,* 2003). Third, LMP1 is expressed in several GC-derived EBV positive lymphomas including HL which has previously been shown to express only low levels of BLIMP1 (Pallesen *et al.,* 1991; Murray *et al.,* 1992; Buettner *et al.,* 2005).

3.2. Results

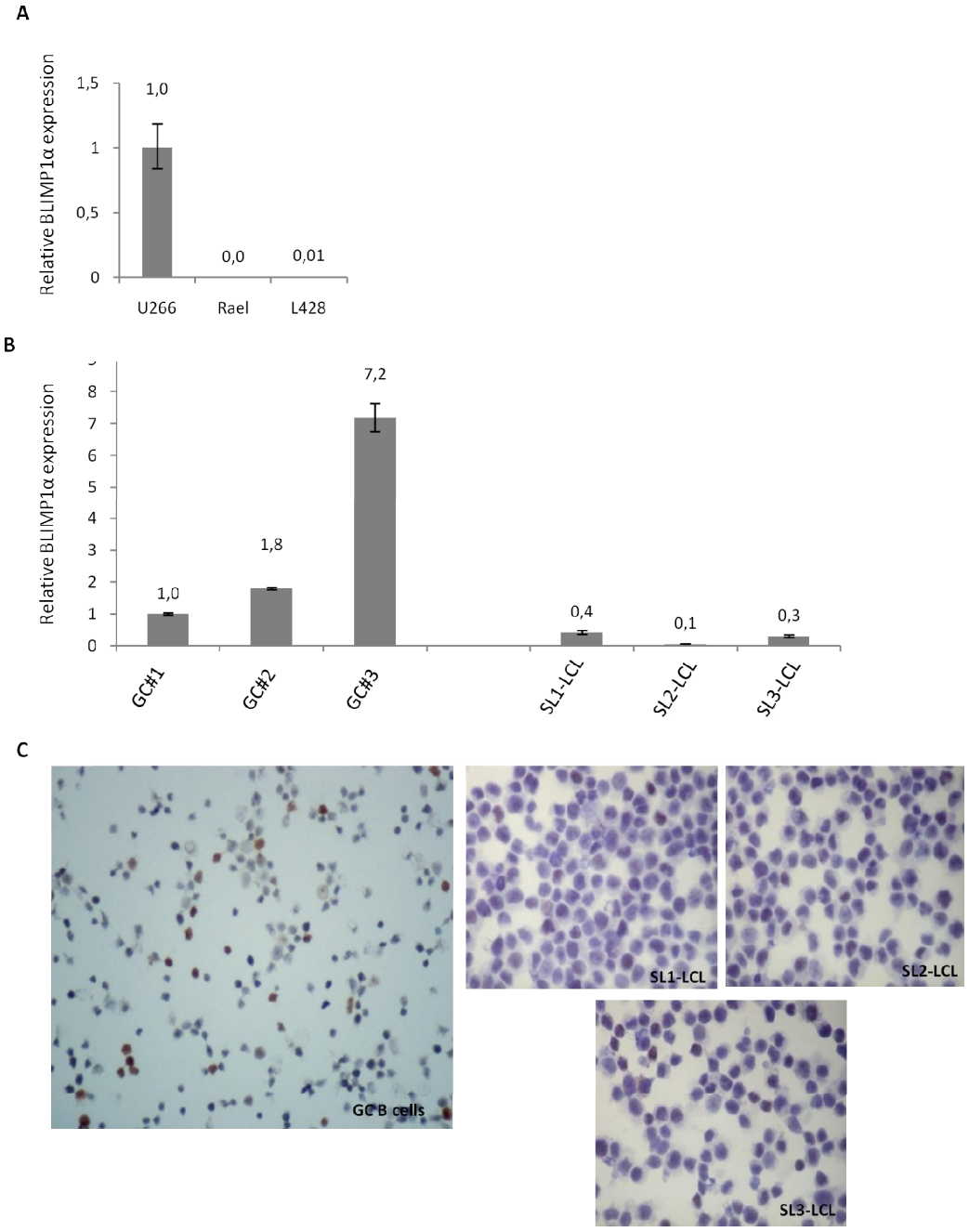
3.2.1. Loss of BLIMP1α expression in EBV-transformed germinal centre B cells

I first compared the expression of BLIMP1α in a panel of EBV-transformed LCLs derived from GC B cells, with that in purified, but un-infected, GC B cells. These LCLs were previously generated by Dr. Sarah Leonard from the GC B cells of three separate donors by infection with a recombinant wild-type EBV. Analysis of the *Ig* gene rearrangements in these LCLs six weeks post-infection confirmed their polyclonal nature. For one of these LCL (SL1-LCL) RNA from matched un-infected GC B cells was available. The other two GC B samples were isolated previously by other group members and are not matched to the LCLs.

I used a commercially available qRT-PCR assay for the detection of BLIMP1α mRNA having first shown BLIMP1α expression in the positive control cell line, U266 and its low level expression in BL and HL cell lines (Figure 3.1A). I then showed that compared to GC B cells, BLIMP1α was decreased in all three GC-derived LCLs (Figure 3.1B).

Immunohistochemistry was then performed to study protein expression in these cells. To do this, I used a monoclonal antibody which recognizes both BLIMP1α and BLIMP1β isoforms. Figure 3.1C shows that as expected this antibody stained a sub-population of the un-infected GC B cells (15-20%). In contrast, and in agreement with the qRT-PCR data, the GC-derived LCLs showed homogenously weaker BLIMP1α expression (Figure 3.1C).

Figure 3.1: Differential expression of BLIMP1α in normal and transformed GC B cells. A) BLIMP1α is expressed in positive control U226 cells and is very low in BL and HL cell lines. B) qRT-PCR analysis shows that EBV infection of GC B cells was associated with the down-regulation of BLIMP1α transcription. Shown here are three LCLs (SL1-LCL, SL2-LCL, and SL3-LCL) established following the infection of GC B cells isolated from three separate donors. C) Immunohistochemical analysis revealed strong BLIMP1α expression in a sub-population of the un-infected GC B cells. In contrast, the LCLs showed homogenously weaker BLIMP1α expression.



3.2.2. The EBV-encoded LMP1 suppresses BLIMP1α expression in germinal centre B cells.

Having shown that EBV infection of GC B cells was followed by the down-regulation of BLIMP1α; I next investigated if LMP1 was responsible for this effect. I chose to study the influence of LMP1 on the expression of BLIMP1α in primary GC B cells.

Tonsils from children were collected from Birmingham Children's Hospital, United Kingdom with informed consent and under appropriate ethical approval (ref No 06/Q2702/50). Tonsils were transported on ice, minced and tonsillar mononuclear cells isolated by Ficoll-Isopaque centrifugation. I used MACS technology and anti-CD10-microbeads to separate CD10+ GC B cells. It has previously been shown by Dr. Martina Vockerodt in our laboratory that this isolation procedure gives a purity >95% (Vockerodt *et al.,* 2008). I confirmed that the isolated cells contained less than 2% of CD3+ cells (Figure 3.2A). I also showed that the isolated CD10+ cells contained both centroblasts (CD77+) and centrocytes (CD77-) and had a viability greater than 90% (Figure 3.2A).

CD10+ cells were nucleofected with either pcDNA3.1 (control) or pcDNA3.1-LMP1 together with LNGFR vector. Western blotting was later used to demonstrate the expression of LMP1 in CD10+ cells prior to the enrichment (Figure 3.2B). Following a 16-hour incubation LNGFR-positive cells were sorted on the MoFlo sorter. The transfection efficiency of the viable cells (PI negative) was generally between 5%-20% and the purity of cells co-expressing CD10 and LNGFR was >95% (Figure 3.2C). The viability of LMP1-expressing GC B cells was similar to that of empty vector transfected cells (Figure 3.3).

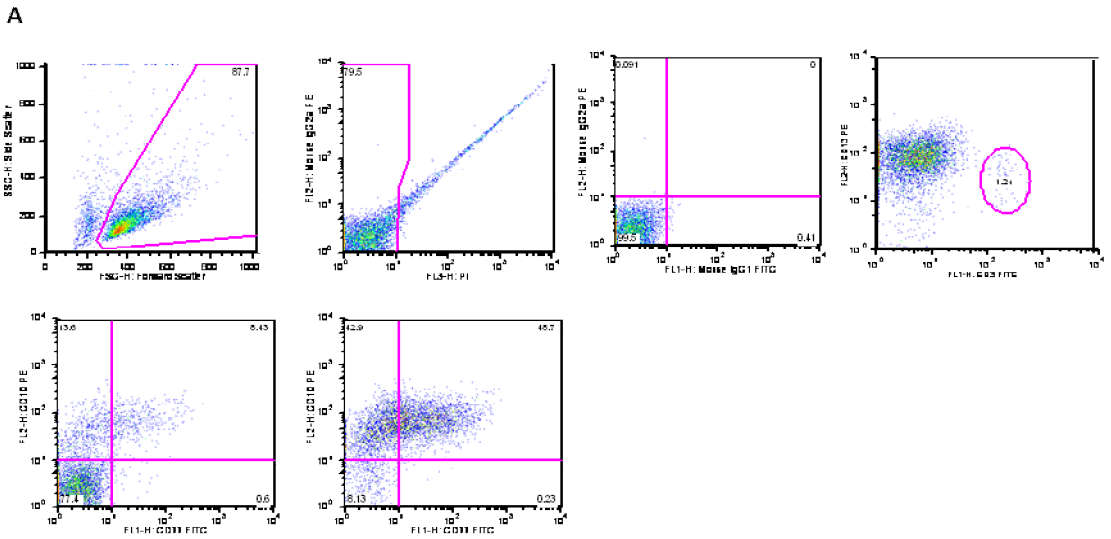
RNA was extracted from at least $1x10^4$ transfected and purified cells. In some cases, the isolated RNA was amplified using two rounds of amplification (as indicated in individual figures). Prior to amplification, the RNA quality was determined on an Agilent 2100 Bioanalyzer (performed by Sim Sihota). Only RNA of sufficient quantity and with a RNA Integrity Number (RIN) value of 7.0 or above was amplified.

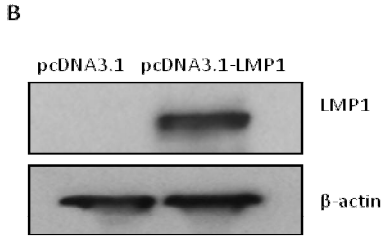
qRT-PCR analysis showed that in CD10+ cells from six separate donors the transfection of LMP1 was followed by the down-regulation of BLIMP1α mRNA expression (Figure 3.4).

Given that in the GC BLIMP1α protein is predominantly expressed by centrocytes, I repeated this experiment, but this time the transfected CD10+ cells were separated on a MoFlo sorter into CD77+ (centroblasts) and CD77- (centrocytes) subpopulations. I observed that LMP1 down-regulated BLIMP1α in both sub-populations (Figure 3.5A).

It is generally not possible in a single experiment to detect protein changes using immunoblotting, due to the low numbers of GC B cells obtained after transfection and enrichment. For these reasons, I investigated the expression of BLIMP1α protein in the B95.8 cell line which I had shown in preliminary experiments to be unusual in so far as it expresses both BLIMP1α and LMP1. I performed dual immunohistochemistry for BLIMP1α and LMP1 on cytospin preparations of these cells. This analysis revealed that the expression of BLIMP1α and LMP1 is mutually exclusive in B95.8 cells (Figure 3.5B).

Figure 3.2: Enrichment and transfection of CD10+ GC B cells. A) Flow cytometric dot blot analysis of tonsillar mononuclear cells before enrichment and after enrichment of CD10+ cells. Upper dot blots: tonsillar mononuclear cells were gated by size (Side Scatter/Forward Scatter) and PI staining to select viable cells. Isotype control was used to determine the levels of unspecific background staining. The lower dot blots: show the gates for CD10+ and CD77+ and before and after the enrichement of CD10+ cells. CD10+ contained less than 2% of CD3+ cells. B) Ectopic expression of LMP1 in unsorted GC B cells was confirmed by western blotting (here shown for one representative example). C) MoFlo sorting dot blot graph of CD10+ cells transfected with plasmid of interest together with LNGFR. The vertical axis indicates LNGFR expression visualized by anti-LNGFR-APC staining, the horizontal axis shows CD10 expression (anti-CD10-PE).





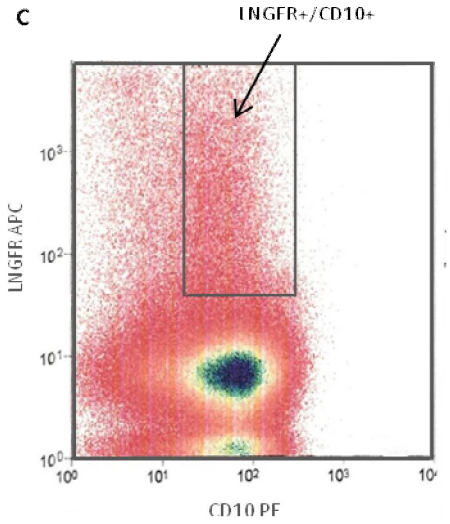


Figure 3.3: Viability of LMP1-transfected GC B cells compared with empty vector control-transfected GC B cells. Flow cytometric analysis of CD10+ cells transfected with LNGFR along with vector control only (pcDNA3.1) or LMP1 (pcDNA3.1-LMP1). Three different experiments are shown. Cells were stained with anti-LNGFR-APC, anti-CD10-PE and PI. The upper dot blots show the gates for LNGFR+ and CD10+ cells. The lower dot blot shows the percentage of PI positive (dead) cells in the fraction of LNGFR+ and CD10+ cells.

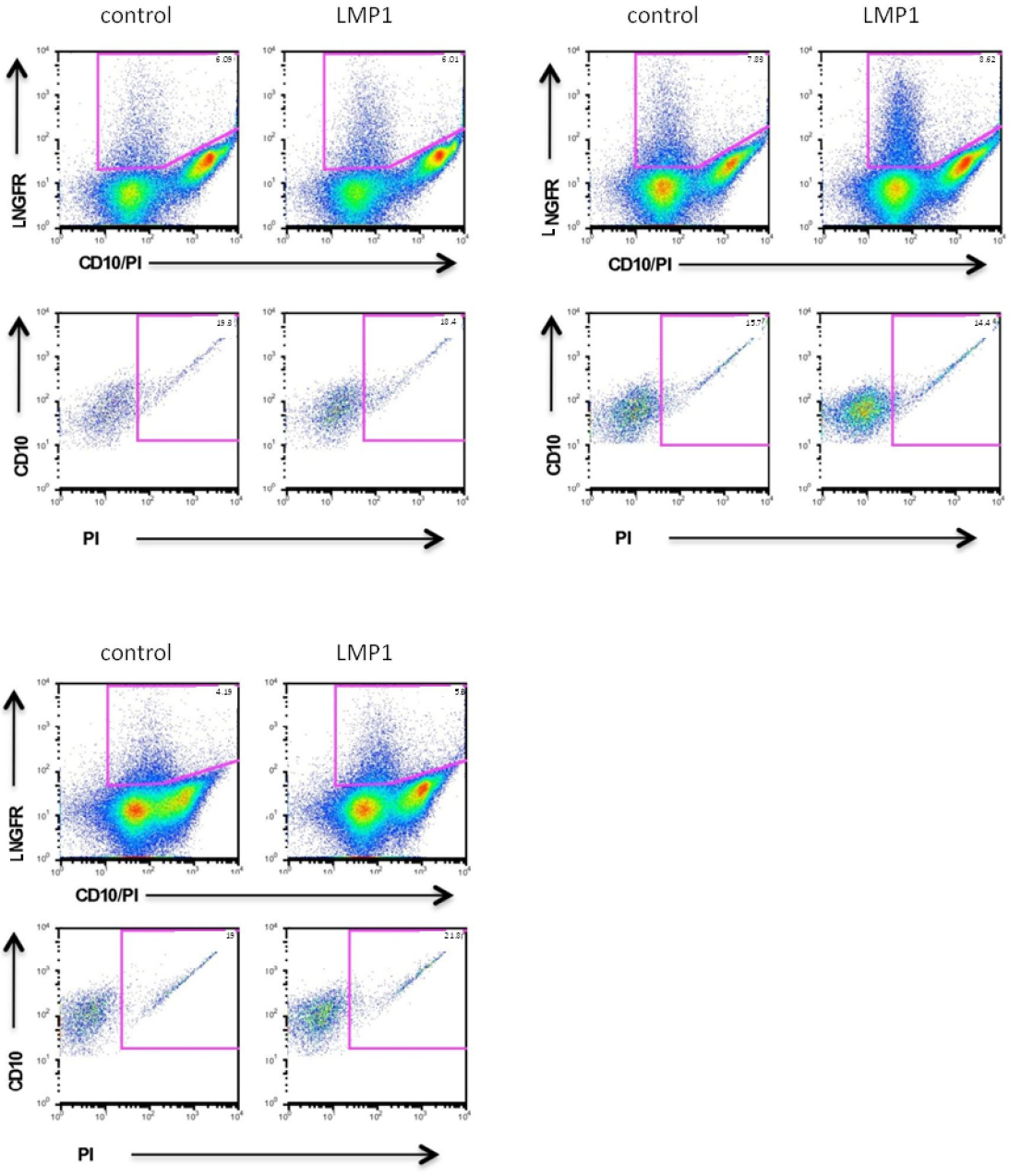


Figure 3.4: LMP1 down-regulates BLIMP1α expression in primary GC B cells. qRT-PCR analysis of BLIMP1α mRNA in CD10+ GC B cells from six separate donors following transfection with LMP1 (pSG5-LMP1 or pcDNA3.1-LMP1) or vector control only (pSG5 or pcDNA3.1). The data are presented as $2^{-\Delta\Delta}$ CT values compared to vector only-transfected GC B cells of Tonsil 1. LMP1 down-regulated BLIMP1α mRNA in all samples. RNA isolated from Tonsils 1, 2 and 29 was amplified using two runs of amplification, RNA isolated from tonsils 24, 27 and 28 was not amplified.

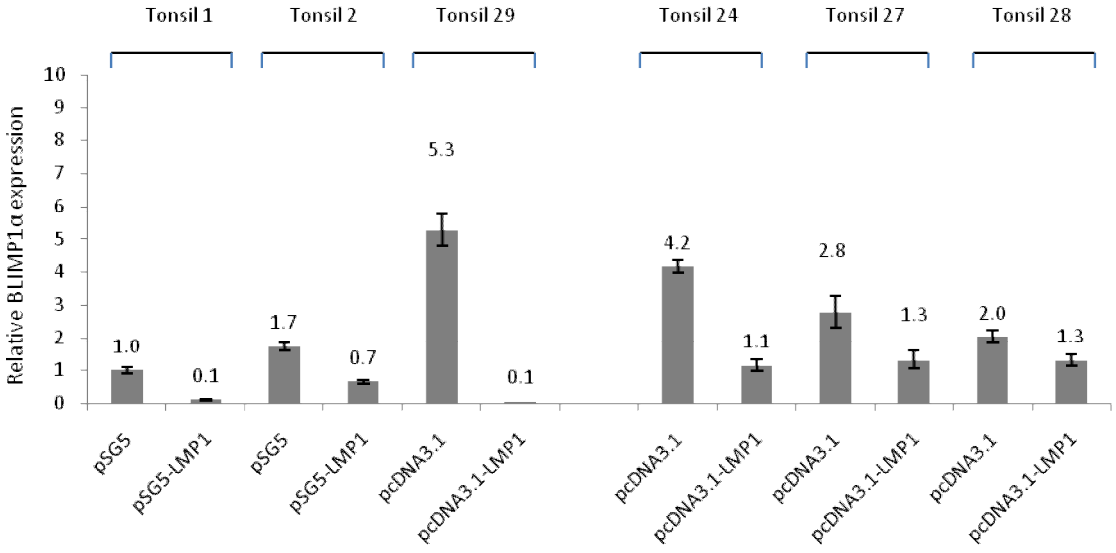
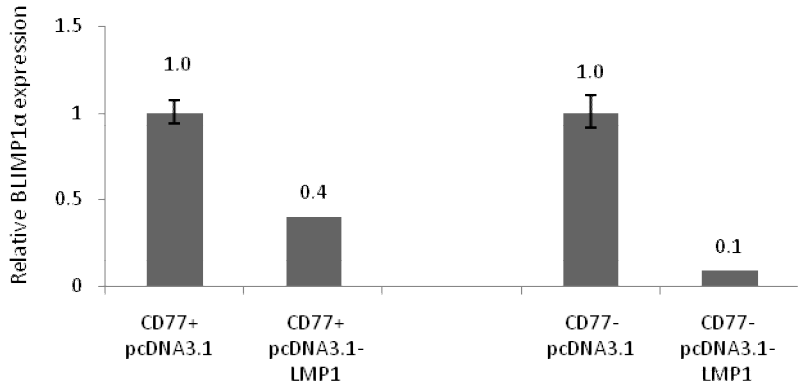
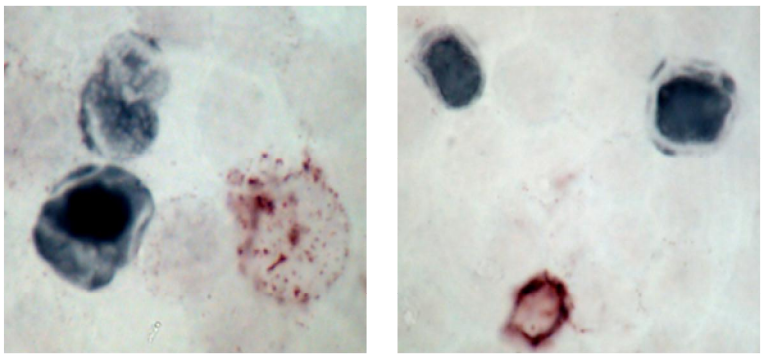


Figure 3.5: LMP1 down-regulates BLIMP1α expression in primary GC B cell subsets and in transformed B cells. A) LMP1 also down-regulated BLIMP1α mRNA in CD77-and CD77+ subsets of CD10+ cells. B) Dual immunohistochemistry revealed that the expression of BLIMP1α (grey) and LMP1 (red) were mutually exclusive in B95.8 cells.

A



B



3.3.3. Differential regulation of BLIMP1α target genes by LMP1 in germinal centre B cells

BLIMP1α is known to regulate plasma cell differentiation by modulating the transcription of key B cell transcription factors. Therefore, I investigated if the LMP1-mediated down-regulation of BLIMP1α also influenced the expression of important BLIMP1α target genes in GC B cells. Four B cell-associated transcription factors, C-MYC, BCL6, PAX5 and IRF4 were selected. BLIMP1α has been shown to down-regulate C-MYC, BCL6, PAX5 and to up-regulate IRF4 in transformed B cells; the silencing of C-MYC, BCL6 and PAX5, and the up-regulation of IRF4 are necessary for plasma cell differentiation (Lin *et al.,* 1997; Shaffer *et al.,* 2002; Lin *et al.,* 2002; Sciammas *et al.,* 2004).

I used qRT-PCR to study the expression of these transcription factors following the transfection of GC B cells with either pcDNA3.1-BLIMP1α, pcDNA3.1-LMP1 or pcDNA3.1 (control) vector.

To express BLIMP1α in GC B cells, I used the same approach as described above for LMP1. I used qRT-PCR to confirm the expression of BLIMP1α and RT-PCR to show LMP1 expression in transfected cells (Figure 3.6). I also showed that in some cases, the viability of BLIMP1α-transfected GC B cells was reduced as compared to control-transfected cells (Figure 3.6B). However, the analysis of the effects of BLIMP1α in GC B cells was restricted to viable transfected cells.

I observed that both BLIMP1α and LMP1 down-regulated BCL6 and PAX5, and up-regulated IRF4. However, whereas C-MYC was down-regulated by BLIMP1α, it was up-regulated by LMP1 in GC B cells (Figure 3.7).

3.3.4. Identification of genes differentially regulated by LMP1 and BLIMP1α in germinal centre B cells

I next explored the influence of LMP1 and BLIMP1α on the global transcriptional programme of GC B cells using genome-wide expression profiling. CD10+ GC B cells isolated from two donors were transfected with either pcDNA3.1-LMP1, pcDNA3.1-BLIMP1α or pcDNA3.1 (control) vector as described above. Isolated RNA was amplified using three rounds of amplification and hybridised to Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays. Differentially expressed genes were identified using the GCOS pairwise analysis with the default settings using the criteria set out in Materials and Methods (page 105). The gene expression profiles of LMP1-transfected or BLIMP1α-transfected cells were compared with that of the control vector-transfected GC B cells from both patients.

Transfection of GC B cells with LMP1 was followed by the up-regulation of 365 genes and the down-regulation of 1094. Transfection of GC B cells with BLIMP1α was followed by the up-regulation of 321 genes and the down-regulation of 654.

Figure 3.6: Enrichment and transfection of CD10+ GC B cells. A) Representative example of detection of BLIMP1α or LMP1 in transfected and MoFlo-sorted CD10+ cells. B) Flow cytometric analysis of CD10+ cells transfected with LNGFR along with vector control only (pcDNA3.1) or BLIMP1α (pcDNA3.1-BLIMP1α). One representative experiment is shown. Cells were stained with anti-LNGFR-APC, anti-CD10-PE and PI. The upper dot blots show the gates for LNGFR+ and CD10+ cells. The lower dot blots show the percentage of PI positive (dead) cells in the fraction of LNGFR+ and CD10+ cells.

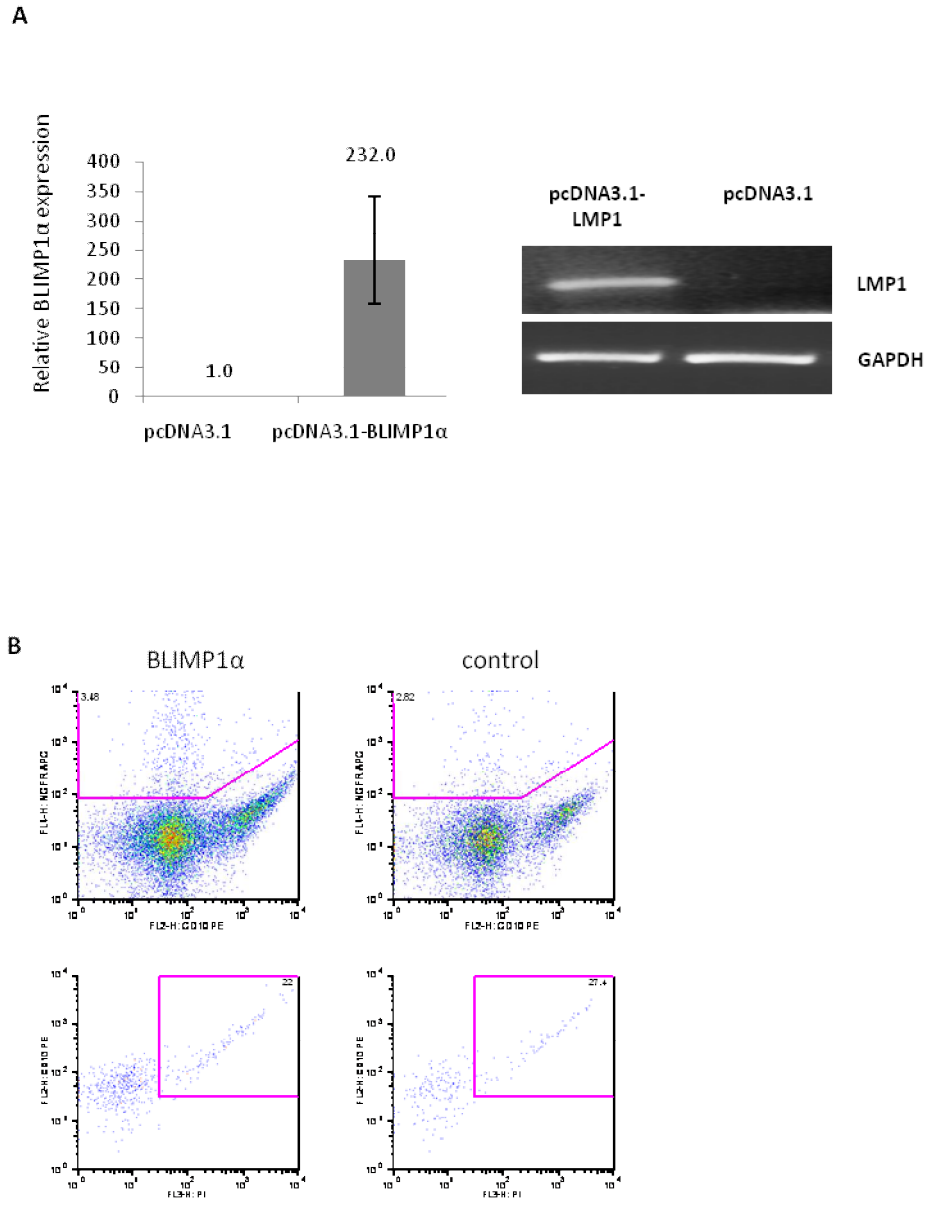
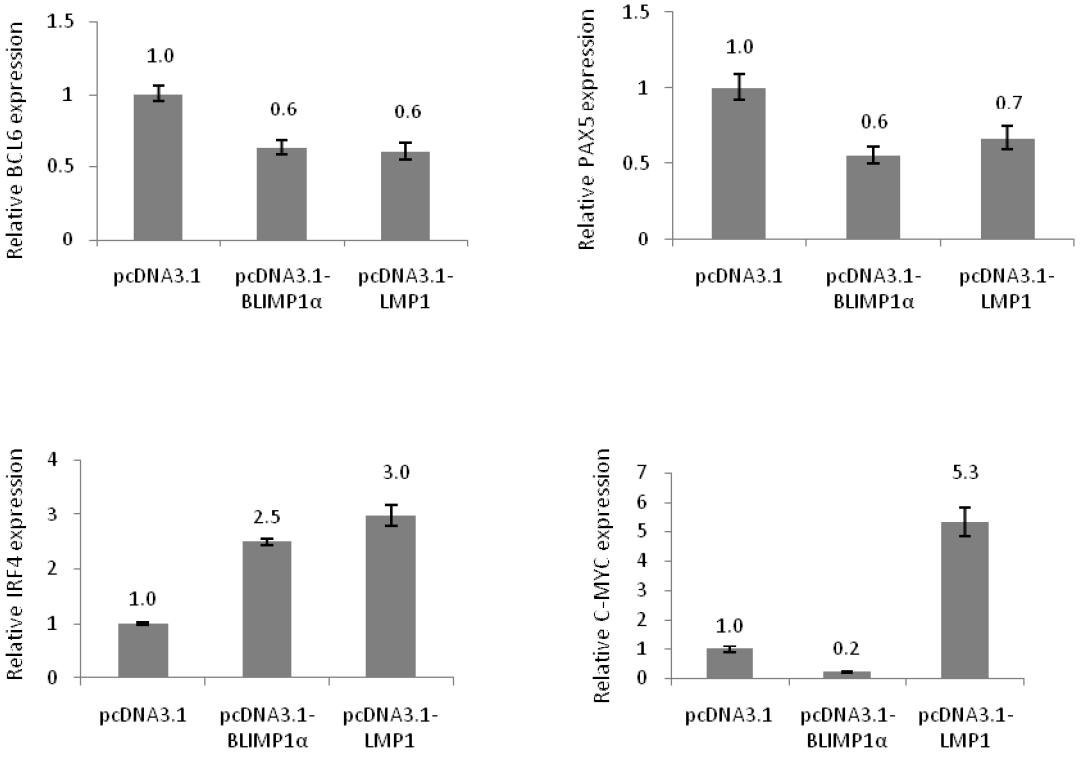


Figure 3.7: Differential regulation of BLIMP1α target genes by LMP1 in GC B cells. qRT-PCR was used to measure the relative quantity of BCL6, PAX5, IRF4 and C-MYC mRNA in LMP1-expressing GC B cells or BLIMP1α-expressing GC B cells presented as $2^{-\Delta\Delta CT}$ values compared with vector only-transfected GC B cells. The expression of BCL6 and PAX5 was down-regulated, and that of IRF4 up-regulated by both LMP1 and BLIMP1α. In contrast, while C-MYC was down-regulated by BLIMP1α, it was up-regulated by LMP1. These results are representative of three independent experiments.



3.3.5. LMP1 partially disrupts the BLIMP1α transcriptional programme in germinal centre B cells.

When the LMP1 and BLIMP1α transcriptional targets identified in GC B cells were compared, 230 genes were found to be concordantly regulated by LMP1 and BLIMP1α (Figure 3.8). However, 57 genes were found to be down-regulated by BLIMP1α and up-regulated by LMP1, or *vice versa* (Figure 3.8 and Table 3.1).

LMP1 GC B cell array	BLIMP1α GC B cell array	Number of genes significantly changed by LMP1 and BLIMP1
up-regulated	up-regulated	36
up-regulated	down-regulated	29
down-regulated	up-regulated	28
down-regulated	down-regulated	194

Table 3.1: LMP1 and BLIMP1α overlapping targets in GC B cells. 230 genes were found to be concordantly regulated by LMP1 and BLIMP1α. However, 57 genes were found to be down-regulated by BLIMP1α and up-regulated by LMP1, or *vice versa.* For a complete list of differentially expressed genes see Appendix 10, 11 and 12.

qRT-PCR was used to confirm the transcriptional changes in selected genes (Figure 3.9). I observed that BCL2A1, CIITA, CCL22 and RFX5 were down-regulated by BLIMP1α, but up-regulated by LMP1 in the same GC B cells. However, both LMP1 and BLIMP1α up-regulated HSPA1A and down-regulated CD20.

Figure 3.8: LMP1 partially disrupts the BLIMP1α transcriptional programme in GC B cells. Genome-wide expression profiling revealed that LMP1 expression in GC B cells was followed by the up-regulation of 365 genes and the down-regulation of 1094. BLIMP1α expression was followed by the up-regulation of 321 genes and the down-regulation of 654.

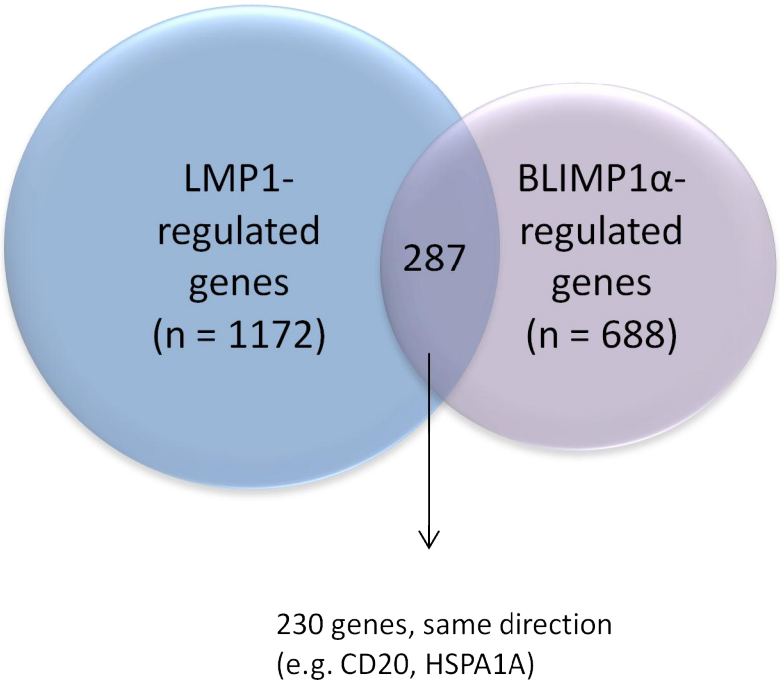
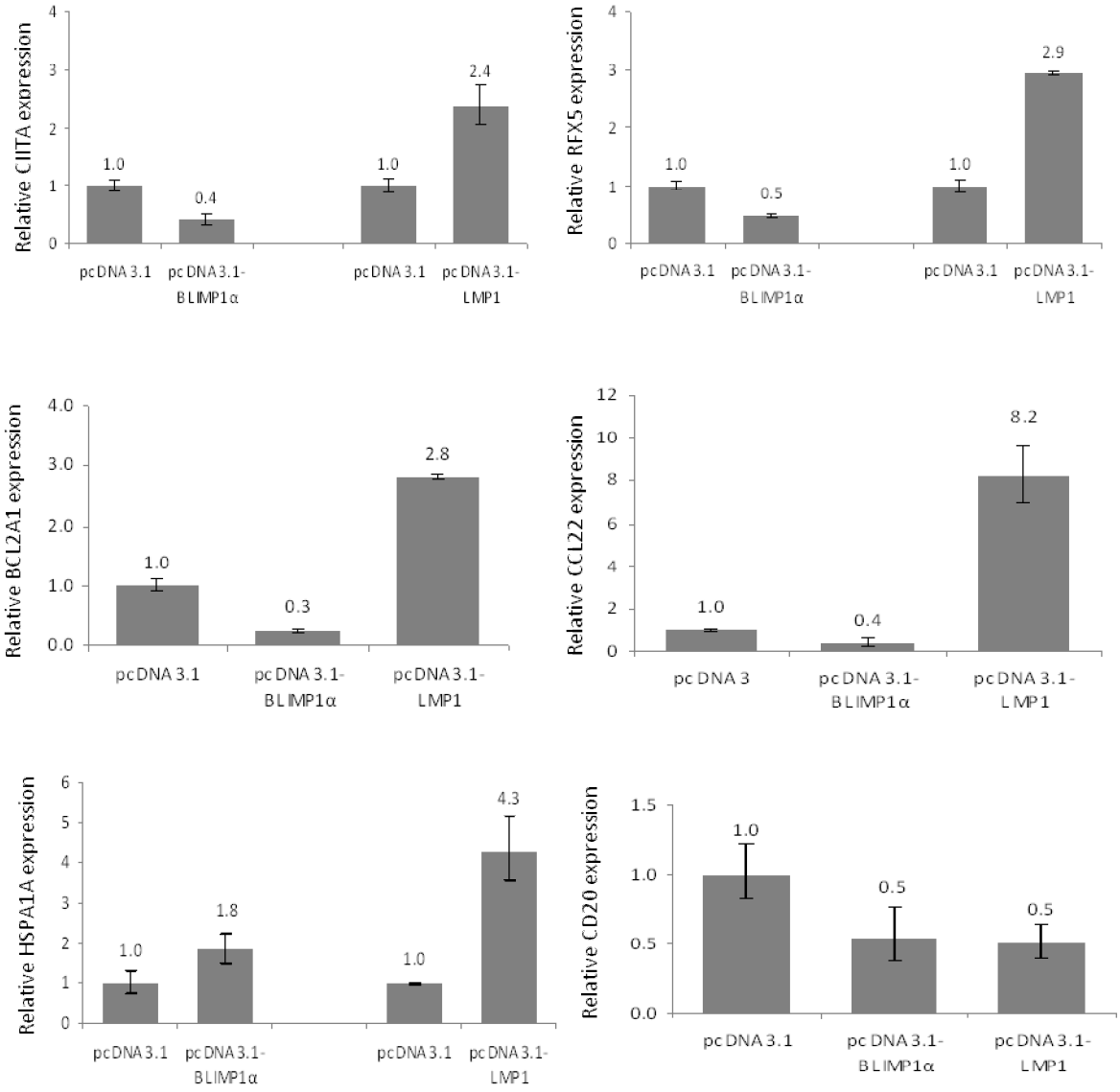


Figure 3.9: Validation of genes from the microarray analysis. LMP1 expression in GC B cells resulted in the up-regulation of a number of genes repressed by BLIMP1α, including CIITA, RFX5, BCL2A1 and CCL22. Both LMP1 and BLIMP1α down-regulated CD20 and up-regulated HSPA1A. With exception of CCL22 performed only twice, these results are representative of three independent experiments.



3.3.6. Validation of LMP1 and BLIMP1α target genes in germinal centre B cells

I next compared the transcriptional targets of LMP1 and BLIMP1α identified in GC B cells with the transcriptional targets of LMP1 and BLIMP1α which have been previously reported in the literature.

Comparison of LMP1 transcriptional targets

Transcriptional targets of LMP1 in primary human GC B cells have been previously reported by Vockerodt et al. (2008). In this study, LMP1 was expressed in GC B cells isolated from three different donors, extracted RNA was amplified using two runs of amplification and hybridised on Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays. Comparison of LMP1 transcriptional targets identified in the present study with those identified by Vockerodt et al. (2008) revealed a remarkable overlap (Table 3.2).

LMP1 GC B cell array Vockerodt et al. (2008) (622 gene increased; 1304 genes decreased)	LMP1 GC B cell array This study (365 genes increased; 1094 genes decreased)	Number of genes significantly changed by LMP1 in both LMP1 GC B cell arrays
up-regulated	up-regulated	207
up-regulated	down-regulated	7
down-regulated	up-regulated	7
down-regulated	down-regulated	471

Table 3.2: Concordantly and discordantly regulated genes in LMP1 GC B cell arrays.

Comparison of BLIMP1 transcriptional targets

Two studies have indentified BLIMP1 target genes in B cells. In the first of these, 260 genes were significantly changed following the transfection of transformed human B cell lines with BLIMP1. The transcriptional targets of BLIMP1 were identified using a lymphochip (Shaffer *et al.,* 2002). In the second, 378 named genes were significantly changed following transfection of the mouse M12 cell line with BLIMP1 and following cytokine-induced differentiation of mouse BCL1 cells (Sciammas *et al.,* 2004). The gene lists were abstracted from the supplemental data accompanying these published reports and re-annotated using the gene symbol provided on the NCBI database (performed by Prof. Ciaran Woodman). The BLIMP1 target genes identified in transformed B cell lines were compared with the BLIMP1α target genes identified in human GC B cells (Table 3.3). The overlap between these three arrays is extremely modest and may well reflect the fact that BLIMP1 has been transfected into very different cell backgrounds.

Genes differentially expressed following transfection of GC B cells with BLIMP1α (this study) (975 genes changed)	Genes differentially expressed following transfection of transformed mouse B cells with BLIMP1 (Shaffer et al., 2002) (260 genes changed)	Genes differentially expressed following transfection of transformed human B cells with BLIMP1 (Sciammas et al., 2004) (378 genes changed)	Number of genes concordantly changed in two or more BLIMP1 studies
down-regulated	down-regulated	down-regulated	5
down-regulated	down-regulated	N/A	23
down-regulated	N/A	down-regulated	4
N/A	down-regulated	down-regulated	4
up-regulated	up-regulated	up-regulated	0
up-regulated	up-regulated	N/A	4
up-regulated	N/A	up-regulated	6
N/A	up-regulated	up-regulated	3

Table 3.3: BLIMP1 down-stream targets in primary and transformed B cells. Comparison of the transcriptional changes induced by BLIMP1α in GC B cells with those induced by BLIMP1 in transformed human B cell lines (Shaffer *et al.,* 2002) and in transformed mouse cell lines (Sciammas *et al.,* 2004).

3.3.7. Identification of genes differentially expressed among centrocytes, plasma and memory cells

I next wished to re-interpret the BLIMP1α- and the LMP1-induced transcriptional changes in GC B cells in the context of the global transcriptional changes which occur during post-GC B cell differentiation into plasma and memory cells.

To do this, I took advantage of a previous study which compared gene expression in centrocytes, plasma cells and memory cells using the same array platform as I used above (Brune et al., 2008). This dataset is held on GEO database under GEO series no GSE12453. Re-analysis of the raw data of Brune et al. (2008) (performed by Dr. Wenbin Wei and Prof.

Ciaran Woodman), identified 4,403 genes to be significantly changed in memory cells or plasma cells, or both, compared with centrocytes. 11 genes were excluded from further analyses because they were listed as both up-regulated and down-regulated either in the arrays comparing plasma cells with centrocytes, or in those comparing memory cells with centrocytes.

The genes were disaggregated into groups based on the following criteria:

Genes considered to be differentially expressed during plasma cell differentiation but not memory cell differentiation were:

- significantly changed in plasma cells compared with centrocytes
- not significantly changed in memory cells compared with centrocytes
- significantly changed in plasma cells compared with memory cells

Genes considered to be differentially expressed during memory cell differentiation but not plasma cell differentiation were:

- significantly changed in memory cells compared with centrocytes
- not significantly changed in plasma cells compared with centrocytes
- significantly changed in memory cells compared with plasma cells

Genes considered up-regulated or down-regulated significantly in both plasma cells and memory cells compared with centrocytes were considered components of both programmes irrespective of whether their expression varied significantly between plasma

cells and memory cells. Table 3.4 reveals an unexpectedly large overlap between the plasma and memory cell differentiation programmes.

Genes differentially expressed during plasma cell differentiation	Genes differentially expressed during memory cell differentiation	Genes differentially expressed in plasma cells compared with memory cells	Number of genes differentially expressed
NSC	up-regulated	MC > PC	517
up-regulated	up-regulated	NAD	659
down-regulated	down-regulated	NAD	245
NSC	down-regulated	MC < PC	208
up-regulated	NSC	PC > MC	978
down-regulated	NSC	PC < MC	103
NSC*: no significant change; NAD* does not apply			

Table 3.4: Number of genes differentially expressed during plasma and memory cell differentiation. Genes differentially expressed between centrocytes and plasma cells (PC) and between centrocytes and memory cells (MC) were identified using a GEO dataset describing gene expression profiles of B cell subsets (GEO series no GSE12453; Brune *et al.,* 2008).

Comparison of BLIMP1 transcriptional targets indentified in different cellular backgrounds

Having defined the genes differentially expressed during plasma cell differentiation, I next compared BLIMP1α target genes identified in primary human GC B cells or transformed B cell lines (Table 3.3) with the genes found to be differentially expressed in plasma cells when compared with centrocytes (Table 3.4).

Table 3.5 suggests that transfection with BLIMP1α alone is insufficient to reveal the full plasma cell differentiation programme in all three cell backgrounds. However, BLIMP1α

transcriptional targets identified in GC B cells clearly recapitulate more of the plasma cell differentiation programme than do those experiments performed in transformed cell lines.

Genes differentially expressed following transfection with BLIMP1α	Genes differentially expressed during plasma cell differentiation	Genes differentially expressed following transfection of GC B cells with BLIMP1α (this study) (975 genes changed)	Genes differentially expressed following transfection of transformed mouse B cells with BLIMP1 (Shaffer et al., 2002) (260 genes changed)	Genes differentially expressed following transfection of transformed human B cells with BLIMP1 (Sciammas et al., 2004) (378 genes changed)
up-regulated	up-regulated	102	11	82
up-regulated	down-regulated	0	5	26
down-regulated	up-regulated	45	27	9
down-regulated	down-regulated	100	36	32

Table 3.5: BLIMP1 down-stream targets in primary and transformed B cells. Comparison of the transcriptional changes induced by BLIMP1α in GC B cells, or in transformed B cell lines (Shaffer *et al.,* 2002; Sciammas *et al.,* 2004) with those genes differentially expressed when plasma cells were compared with centrocytes.

3.3.8. BLIMP1α-induced transcriptional changes in germinal centre B cells recapitulated many of those observed during plasma cell, but not memory cell differentiation.

To measure the extent to which the plasma cell and memory cell differentiation programme is recapitulated following the expression BLIMP1α in GC B cells, transcriptional targets of BLIMP1α were compared to the genes identified in the study of Brune et al. (2008) and disaggregated into groups as shown in Table 3.4.

Figure 3.10 demonstrates that 30% of the genes down-regulated in plasma cells compared with centrocytes are also down-regulated following transfection of GC B cells with BLIMP1α. In keeping with the role of BLIMP1α as a transcriptional repressor and

with previous reports, this overlap was mainly restricted to genes down-regulated during plasma cell differentiation. No gene up-regulated by BLIMP1α in GC B cells was down-regulated in plasma cells compared with centrocytes. A small proportion of those genes up-regulated in plasma cells compared with centrocytes are also up-regulated by BLIMP1α in GC B cells.

In contrast, only a small number of genes which are differentially expressed in memory cells but not in plasma cells compared with centrocytes were also found to be de-regulated following transfection of GC B cells with BLIMP1α.

These observations demonstrate the ability of BLIMP1α to induce transcriptional changes associated with plasma cell differentiation in GC B cells and confirm the validity of this comparative approach.

3.3.9. LMP1-induced transcriptional changes in GC B cells recapitulated many of those observed during plasma cell and memory cell differentiation.

The overlap between LMP1-induced transcriptional changes in GC B cells and the plasma and memory cell transcriptional programmes was also considered. Given the substantial overlap between LMP1 down-stream targets identified in the recent study and in the study by Vockerodt et al. (2008) (Table 3.2), it appeared reasonable to include LMP1 down-stream targets identified in both studies in next analyses, to allow use of all of the available evidence.

Figure 3.11 shows how often LMP1-induced changes in GC B cells overlap distinct and shared components of the plasma and memory cell transcriptional programmes. LMP1 concordantly and discordantly de-regulates in GC B cells, a substantial proportion of those genes which are down-regulated in plasma cell compared with centrocytes. There is also an overlap albeit less substantial between those genes up-regulated by LMP1 in GC B cells and those up-regulated in plasma cells compared with centrocytes. However, LMP1 also down-regulates genes which are up-regulated during plasma cell differentiation.

However, unlike BLIMP1α, LMP1-induced transcriptional changes also substantially overlapped with those gene expression changes observed when memory B cells were compared with centrocytes. In conclusion, LMP1 and BLIMP1α regulate subset of genes associated with plasma cell differentiation, but that LMP1 also induces transcriptional changes in GC B cells that are characteristic of memory B cells.

Figure 3.10: BLIMP1α-induced transcriptional changes in GC B cells recapitulated many of those changes observed during plasma cell, but not memory cell differentiation. BLIMP1α expression in GC B cells recapitulated many of the transcriptional changes associated with plasma cell differentiation, including those specific for plasma cell differentiation and those common to both plasma and memory cell differentiation. In contrast, only a substantially fewer number of those transcriptional changes induced by BLIMP1α showed overlap with the group of genes specific for memory cell differentiation.

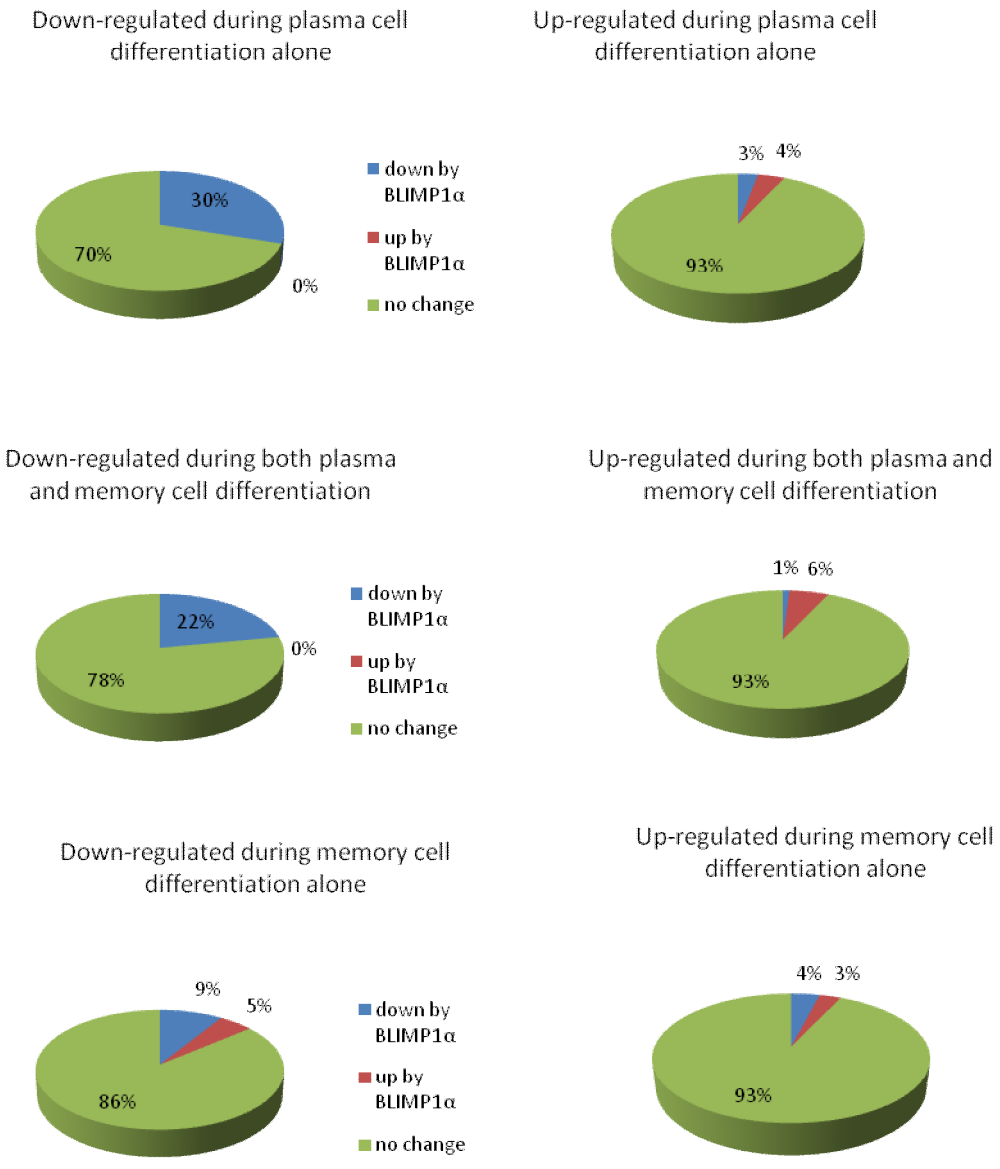
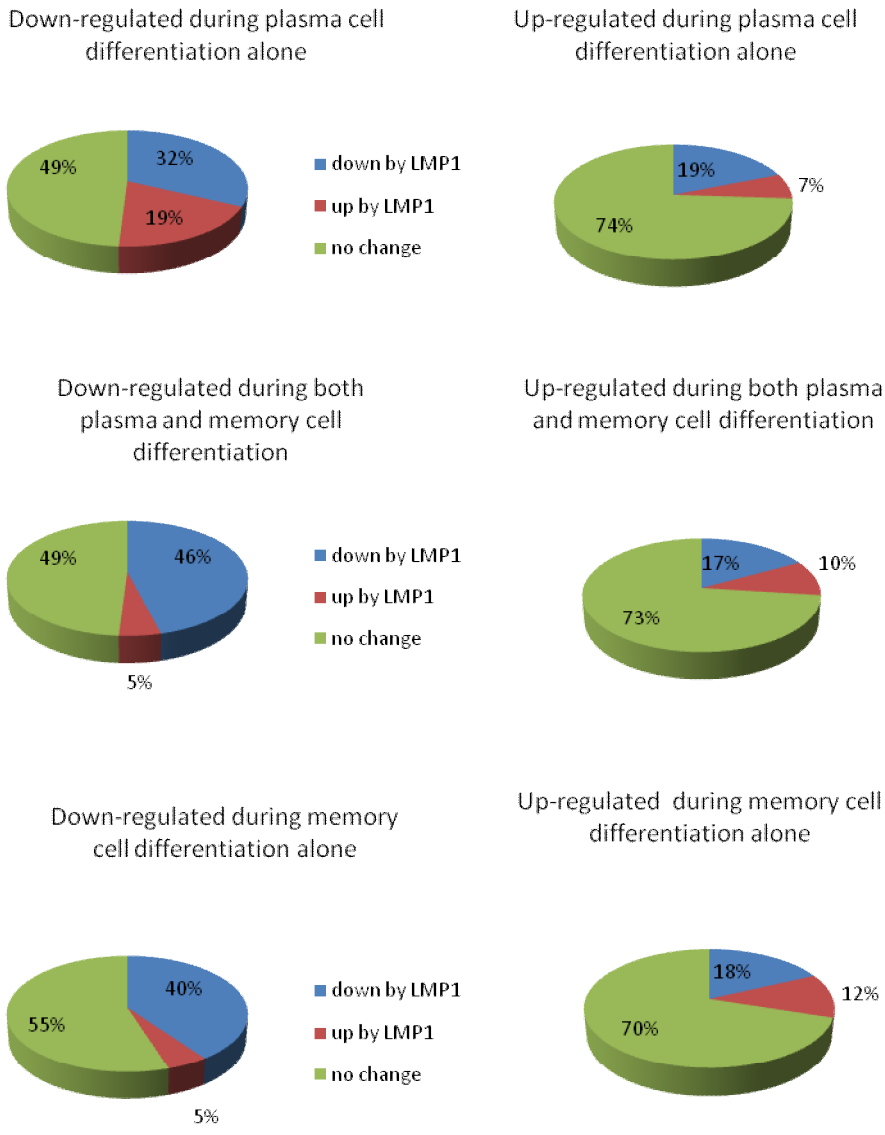


Figure 3.11: LMP1-induced transcriptional changes in GC B cells recapitulated many of those observed during plasma cell and memory cell differentiation. LMP1 expression in GC B cells recapitulated many of the transcriptional changes associated with plasma cell differentiation, including those specific for plasma cell differentiation and those common to both plasma cell and memory cell differentiation. However, unlike BLIMP1α, LMP1-induced transcriptional changes that also substantially overlapped with genes specific for memory cell differentiation.



3.3. Discussion

In this study I have shown that BLIMP1α, a key regulator of plasma cell differentiation, is down-regulated in primary GC B cells by the EBV oncogene, LMP1. In this respect, LMP1 would appear to mimic closely the effects of CD40, which can direct the differentiation of GC B cells towards memory B cells while at the same time suppressing plasma cell differentiation, an effect associated with the down-regulation of BLIMP1 (Arpin *et al.,* 1995; Randall *et al.,* 1998; Knödel *et al.,* 2001). CD40 also contributes to the NF-κB/IRF4-mediated down-regulation of BCL6, which is necessary to terminate the GC transcriptional programme (Saito *et al.,* 2007). However, it should be noted that the LMP1 signal is constitutive, whereas that induced by CD40 is regulated by the availability of ligand. Furthermore, although CD40 is expressed in GC B cells, its activation occurs only in a subset of centrocytes at the final stages of the GC reaction (Basso *et al.,* 2004). It remains to be established how the nature and timing of these signals influence the eventual outcome of post-GC B cell differentiation.

When interpreting the results of the LMP1 transfection experiments it is important to remember that in unsorted GC B cells the proportion of BLIMP1α-positive cells is only around 15-20% (own immunohistochemistry data and Angelin-Duclos *et al.,* 2000; Högerkorp and Borrebaeck, 2006). Therefore, one possible explanation of these data is that LMP1 preferentially induces the death of BLIMP1α-expressing GC B cells. Were this is the case then I might have expected LMP1 to reduce the viability of GC B cells accordingly. However, I consistently observed no change in the viability of LMP1-expressing GC B cells compared to empty vector transfected cells.

I also observed that BLIMP1α was down-regulated following the infection of GC B cells with EBV suggesting that LMP1 might at least initiate the silencing of BLIMP1α in EBV-infected B cells. However, an alternative interpretation is that during the establishment of the LCL there is selective immortalisation of BLIMP1α-negative GC B cells. This could result from 1) viral replication in BLIMP1α-positive cells followed by cell death. 2) differentiation of BLIMP1α-positive cells and their gradual loss from the culture. 3) the EBV induced de-differentiation of BLIMP1α-positive cells, a possibility supported by a study which shows that infection of BLIMP1α-positive multiple myeloma cells with EBV leads to the down-regulation of BLIMP1 and to a partial reprogramming of these cells to a mature B cell phenotype (Anastasiadou *et al.,* 2009).

Consistent with a role for LMP1 in hijacking the B cell transcriptional programme I found a striking overlap between the LMP1 and BLIMP1α transcriptional programmes in GC B cells. Although I have not investigated this further, the commonality between the transcriptional programmes of BLIMP1α and LMP1 is probably best explained by their concordant regulation of transcription factors including BCL6, IRF4 and PAX5 all of which are important in regulating post-GC B cell differentiation (Shaffer *et al.,* 2002; Lin *et al.,* 1997; Lin *et al.,* 2000; Lin *et al.,* 2002; Reljic *et al.,* 2000; Klein *et al.,* 2006; Panagopoulos *et al.,* 2004; Cahir-McFarland *et al.,* 2004). However, I observed that the down-regulation of PAX5 and BCL6 that followed the ectopic expression of both LMP1 and BLIMP1α in GC B cells, although reproducible, was relatively modest when compared to the effects on C-MYC. These observations suggest that BLIMP1α and LMP1 are alone insufficient to mediate the complete repression of PAX5 and BCL6 in GC B cells. This is consistent with previous studies which show that the repression of PAX5 that occurs during plasma cell

differentiation is dependent not only on BLIMP1α but also on the presence of other signals which suppress PAX5 independently of BLIMP1α (Angelin-Duclos *et al.,* 2000). In particular, it has been shown that the repression of PAX5 and BCL6 that occurs early after the initiation of plasma cell differentiation in pre-plasmablasts does not require BLIMP1α (Angelin-Duclos *et al.,* 2000; Kallies *et al.*, 2007). Furthermore, in other experiments I observed that LMP1 expression in GC B cells up-regulated ID2 (data not shown), which acts to suppress PAX5 function (Renné *et al.,* 2006; Vockerodt *et al.,* 2008). Therefore, LMP1 apparently acts to suppress PAX5 by two distinct mechanisms.

The microarray analysis also revealed other genes that were repressed by BLIMP1α, but induced by LMP1, including several genes that are known to be down-regulated during plasma cell differentiation (e.g. BCL2A1, CIITA; Piskurich *et al.,* 2000; Martins and Calame, 2008) as well as C-MYC, the suppression of which has been shown to be essential for plasma cell differentiation (Lin *et al.,* 2000). These findings suggest that LMP1 can partially disrupt the BLIMP1α transcriptional programme in GC B cells and in doing so prevent plasma cell differentiation.

Of the four transcription factors I originally showed to be regulated by both LMP1 and BLIMP1α by qRT-PCR analysis (figure 3.7), only BCL6 was shown to be a target of both LMP1 and BLIMP1α on array analysis, suggesting that the array is less sensitive than qRT-PCR. Furthermore, it is also possible that heterogeneity between GC B cells isolated from different donors could have contributed to the failure to detect transcriptional targets in some experiments (Angelin-Duclos *et al.,* 2000).

Although I found a substantial overlap between the plasma and memory cell differentiation programmes, transcriptional changes specific to each of these programmes could be identified. I found that a third of those genes down-regulated in plasma cells compared with centrocytes were also down-regulated following the transfection of GC B cells with BLIMP1α. However, transfection with BLIMP1α alone was not sufficient to induce the full plasma cell differentiation programme. The limitation of this approach is that I was comparing changes which occur following the expression of BLIMP1α in GC cells on average 16 hours after transfection. Whereas, the differentiation of GC B cells into terminally differentiated memory or plasma cells occurs after several days. Therefore, some gene expression changes between centrocytes and plasma or memory cells might have not been observed in my experiments.

I also found that the LMP1 transcriptional programme in GC B cells substantially overlapped with that of the plasma and memory cell differentiation programmes. LMP1 concordantly and discordantly regulated many genes which are modulated during plasma and memory cell differentiation.

These results suggest that LMP1 expression in GC B cells might initiate post-GC differentiation. However, it is not clear if LMP1 can complete this process. Failure to do so would be compatible with the notion that LMP1-mediated arrest of B cell differentiation might allow time for the accumulation of pathogenic mutations (Thorley-Lawson, 2001).

The observation that LMP1 can promote post-GC differentiation might help explain the contribution of this viral oncogene to the pathogenesis of HL, a GC B cell-derived

malignancy which is characterised by an abortive plasma cell differentiation programme and a loss of B cell identity (Schwering *et al.,* 2003; Buettner *et al.,* 2005).

Inactivation of the PRDM1 gene encoding BLIMP1 has been detected in DLBCL of the activated B cell type (Tam *et al.,* 2006; Pasqualucci *et al.,* 2006). Translocations de-regulating the BCL6 gene have not been found in DLBCL which carry BLIMP1 mutations, but are restricted to un-mutated cases, suggesting that BCL6 de-regulation and BLIMP1 inactivation might represent alternative pathogenic mechanisms, both leading to a block in post-GC differentiation and, ultimately, to lymphomagenesis (Tam *et al.,* 2006). Our observations suggest that LMP1 expression in progenitor GC B cells might provide an alternative mechanism to block terminal B differentiation in EBV-positive lymphomas.

Finally, preliminary data generated recently in our laboratory suggest that BLIMP1α can also be down-regulated by another EBV-encoded latent membrane protein, LMP2A, which is also expressed in EBV-associated HL (Vockerodt *et al.,* unpublished). I showed in another experiment which is not presented in this thesis that in contrast to the LMPs the EBV maintenance protein EBNA1 up-regulates BLIMP1α in GC B cells. It remains to be established if switching between different forms of EBV latency can determine the fate of an EBV-infected GC B cells. In the absence of the LMPs (latency I), EBV infected GC B cells may be preferentially driven to differentiate into plasma cells. However, when the LMPs are expressed (e.g. in latency II), the infected cells may be prevented from undergoing plasma cell differentiation, but instead differentiate into memory B cells.

CHAPTER 4

AN INVESTIGATION OF THE INFLUENCE OF BLIMP1α

ON THE EBV LYTIC CYCLE IN B CELLS

AN INVESTIGATION OF THE INFLUENCE OF BLIMP1α ON THE EBV LYTIC CYCLE IN B CELLS

4.1. Introduction

As well as maintaining latency in B lymphocytes, EBV can also induce its replicative cycle in these cells. Thus, at any one time a small proportion of cells in an LCL may spontaneously enter the lytic cycle, or be induced to do so by treatment with chemical agents such as phorbol esters, or by ligation of surface Ig (Kieff and Rickinson, 2001). The replicative cycle of EBV is induced by BZLF1, the immediate-early protein critical for triggering the switch from latency to lytic cycle in EBV infected B cells. BZLF1 is alone sufficient to activate downstream lytic genes and complete viral replication in a permissive cell type (Countryman *et al.,* 1985; Takada *et al.,* 1986).

A number of studies suggest that EBV replicates in terminally differentiated plasma cells (Anagnostopoulos *et al.,* 1995; Crawford and Ando, 1986; Niedobitek *et al.,* 1997; Niedobitek *et al.,* 2000; Laichalk and Thorley-Lawson, 2005). For example, it has been shown that the BZLF1 promoter is active in memory cells only after they have been differentiated into plasma cells (Laichalk and Thorley-Lawson, 2005).

The intimate association between terminal differentiation and EBV replication in B cells suggests that the switch from latency to the lytic cycle is controlled by factors which normally regulate plasma cell differentiation. Given that BLIMP1α expression in GC B cell induces plasma cell differentiation and that plasma cell differentiation is associated with induction of the EBV lytic cycle, I have investigated if BLIMP1α can induce the viral lytic cycle.

4.2. Results

4.2.1. Ectopic expression of BLIMP1α induces BZLF1 in LCLs.

I first studied if BLIMP1α could induce the lytic cycle in EBV-transformed B cells. I transfected two established LCLs, OKU-LCL and SAL-LCL, with either BLIMP1α (pcDNA3.1-PRDM1α) or control (pcDNA3.1) vector together with CD4.1 vector (9:1). Cells were cultivated for 24 hours before the enrichment of CD4.1-expressing cells. The expression of BLIMP1α in transfected cells was confirmed by qRT-PCR (Figure 4.1A) and by immunohistochemistry for HA-tag of BLIMP1α (Figure 4.1D, shown for OKU-LCL). I then demonstrated that in both cell lines the ectopic expression of BLIMP1α was accompanied by increased levels of BZLF1 mRNA (Figure 4.1B). I next used immunohistochemistry to study if the ectopic expression of BLIMP1α in these LCLs was associated with a change in BZLF1 protein expression. Figure 4.1C and Table 4.1 show an increase in the number of BZLF1-expressing cells in BLIMP1α transfected cells compared to control transfected cells. I also used immunohistochemistry to demonstrate HA expression in transfected cells. Surprisingly, I observed that HA was expressed only in a fraction of the transfected and enriched cells (Table 4.1). This suggests that transfection with the larger BLIMP1α expression vector is less efficient than with the CD4.1 expression vector. However, I observed that in most cases the increase in the number of BZLF1-expressing cells that followed BLIMP1α transfection correlated with the number of HA-positive cells. The obvious exception to this was B95.8 cells where the increase in the number of BZLF1-expressing cells was substantially higher than the number of HA-expressing cells. One

explanation for this discrepancy might be that the HA staining fails to detect transfected cells expressing low amounts of BLIMP1α.

I repeated this experiment in three more LCLs and in the B95.8 cell line. In all these cells I observed an increase in the number of BZLF1 expressing cells in BLIMP1α transfected cells compared to controls (Table 4.1 and Figure 4.2).

Cell line	No. of BZLF1 + cells in control-transfected cells (%)	No. of BZLF1 + cells in BLIMP1α-transfected cells (%)	No. HA-tag + cells in BLIMP1α-transfected cells (%)
HK-LCL	3.56	8.12	6.96
OKU-LCL	1.02	7.14	11.36
SL1-LCL	0.0	6.32	9.9
PER213	3.02	5.68	2.25
SAL-LCL	1.22	2.55	4.13
B95.8	14.69	28.2	3.72

Table 4.1: Increased frequency of BZLF1-expressing cells following expression of BLIMP1α in LCLs. Immunohistochemistry was used to measure the frequency of BZLF1-expressing cells in BLIMP1α-transfected cell lines. At least 1000 cells were counted in each sample. Staining for the HA-tag provided an estimate of the transfection efficiency.

Figure 4.1: BLIMP1α up-regulates BZLF1 expression in LCLs. qRT-PCR analysis of BLIMP1α (A) and BZLF1 (B) mRNA levels in OKU-LCL and SAL-LCL following transfection with BLIMP1α or empty vector. C) Immunostaining revealed that ectopic BLIMP1α expression increased the frequency of BZLF1-expressing cells in OKU-LCL. D) Staining for HA-tag of BLIMP1α was used to demonstrate the expression of BLIMP1α vector in the cell line. These results are representative of two (SAL-LCL) or three (OKU-LCL) independent experiments.

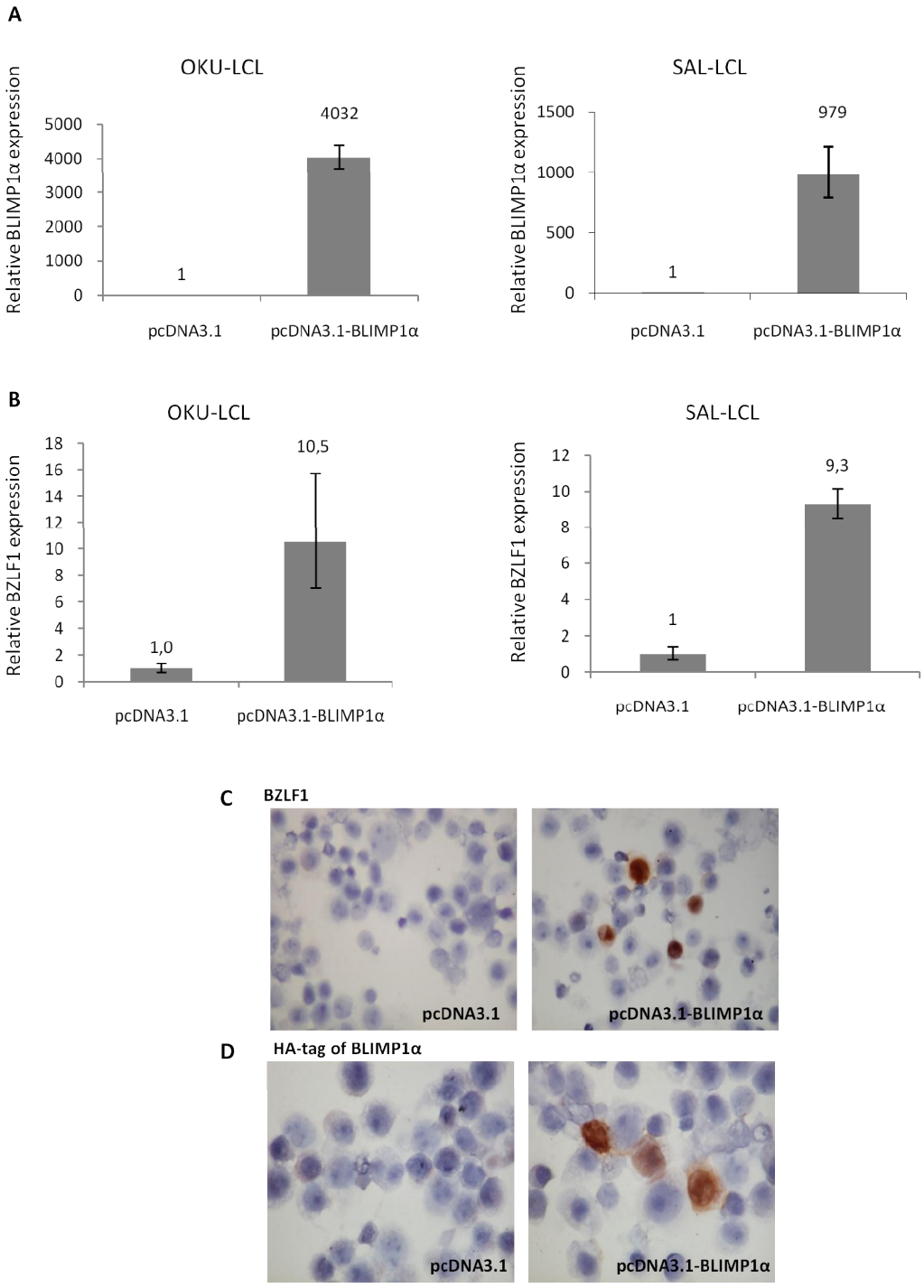
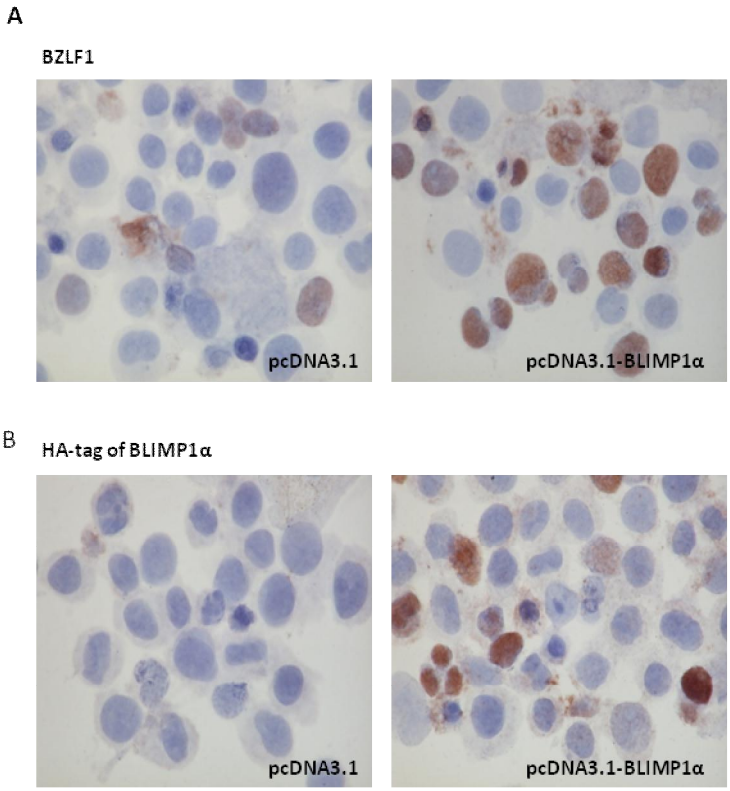


Figure 4.2: BLIMP1α increases BZLF1 expression in B95.8 cells. A) Immunohistochemical staining of B95.8 cells following tranfection with either BLIMP1α or control vector increased the numbers of BZLF1 protein-expressing cells. B) Staining for HA-tag of BLIMP1α was used to demonstrate the expression of BLIMP1α vector in B95.8 cells.



4.2.2. Ectopic expression of BLIMP1α induces the EBV lytic cycle in LCLs.

Having shown that BLIMP1α was able to induce BZLF1 expression in LCLs, I next investigated if BLIMP1α could also induce the expression of other EBV lytic cycle genes. For this analysis I chose to study four genes representative of the different stages of the EBV lytic cycle; BRLF1 (immediate-early), BMLF1 and BNLF2a (early) and BALF4 (late). OKU-LCL was transfected as described before and cultivated for 24 and 48 hours prior to enrichment. I then performed qRT-PCR for each of the viral genes. Figure 4.3 shows that the expression of BLIMP1α in OKU-LCL was followed by the increased expression of all these lytic cycle genes. The expression of BRLF1 was highest 24 hours after transfection and appeared to decline at 48 hours. BMLF1 and BNLF2a were up-regulated at both 24 and 48 hours. However, BALF4, showed only a modest induction following BLIMP1α transfection. I observed similar results following the transfection of both SAL-LCL and B95.8 cells (Figure 4.4).

I also used dual immunohistochemistry to study the expression of the late lytic gene products, gp350/220 and viral capsid antigen (VCA). Consistent with the modest induction of BALF4, I observed expression of gp350 and VCA in only a minor fraction of the BLIMP1α transfected cells. For example, VCA expression could be detected in only 0.16% of BLIMP1α-expressing cells (data not shown).

I next used immunohistochemistry to study the co-expression of either gp350 or VCA with endogenous BLIMP1α in B95.8 cells. Figure 4.5 shows that whereas late lytic gene products, VCA and gp350 were rarely detectable in BLIMP1α-negative B95.8 cells (0.4%

and 0.6%, respectively) they were commonly present in BLIMP1α-positive B95.8 cells (36.9% and 55.5%, respectively).

4.2.3. BLIMP1α induces EBV lytic cycle in Akata Burkitt's lymphoma cells.

I next investigated if BLIMP1α could activate the lytic cycle in the Akata BL cell line (Takada and Ono, 1989). These cells were co-transfected with either BLIMP1α (pcDNA3.1-PRDM1α) or control (pcDNA3.1) vector together with CD4.1 vector and cultivated for 72 hours prior to enrichment of transfected cells. Figure 4.6 demonstrates that the ectopic expression of BLIMP1α in Akata cells increased the transcription of BZLF1, BRLF1, BNLF2a, BMLF1 and BALF4. As before, I observed that only a minority of transfected and enriched Akata cells expressed the HA tagged protein. However, I was able to show using dual immunohistochemistry that VCA was expressed in 70.6% of BLIMP1α-expressing Akata cells and gp350 in 52.1%.

Figure 4.3: BLIMP1α induces the EBV lytic cycle in OKU-LCL. Ectopic expression of BLIMP1α led to the increased expression of BRLF1, BMLF1, BNLF2a and BALF4.

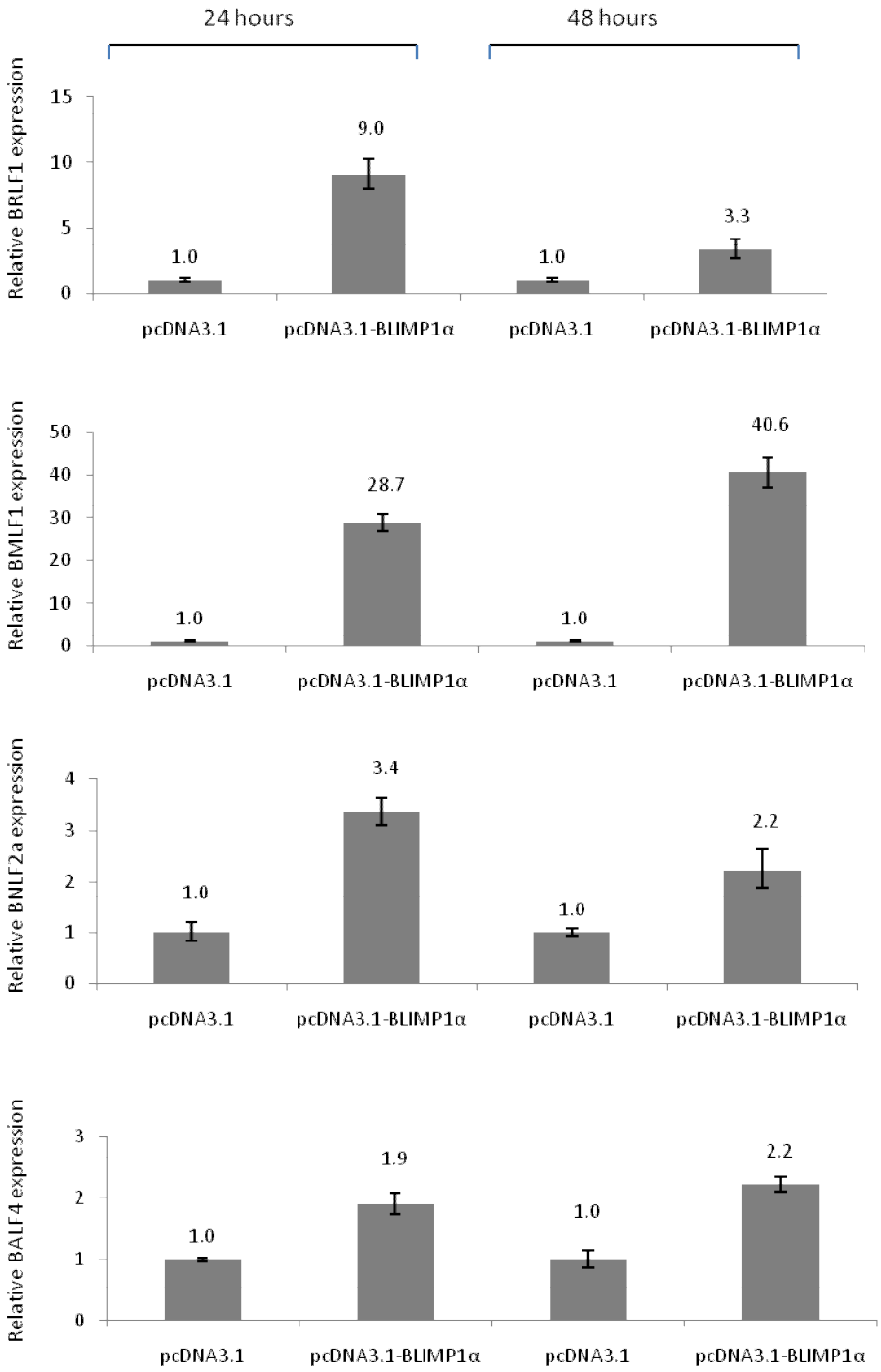


Figure 4.4: BLIMP1α induces EBV lytic cycle in SAL-LCL and B95.8 cells. A) Ectopic expression of BLIMP1α in SAL-LCL resulted in the up-regulation of BRLF1, BMLF1 and BLNF2a as demonstrated by qRT-PCR. B) A similar effect was also observed in B95.8 cells in which the expression of BRLF1, BMLF1 and BALF4 viral lytic genes was increased in BLIMP1α-transfected cells.

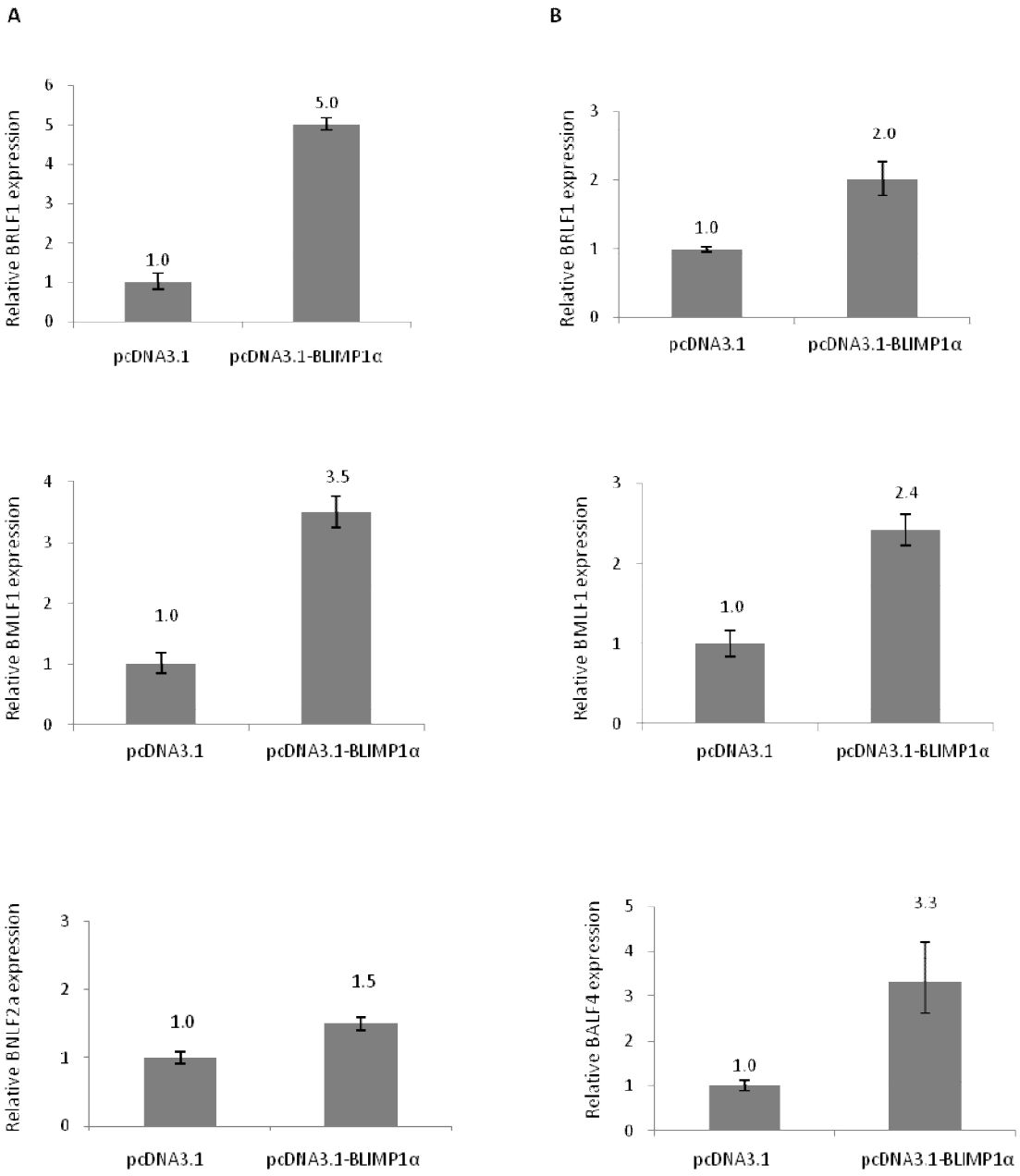


Figure 4.5: BLIMP1α and EBV lytic cycle genes are co-expressed in B95.8 cells. Double immunohistochemical staining for BLIMP1α (grey) and VCA (red) (A) or BLIMP1α (grey) and gp350 (red) (B) demonstrates co-expression of BLIMP1α and VCA or gp350 proteins at the single cell level. These results are representative of three independent experiments.

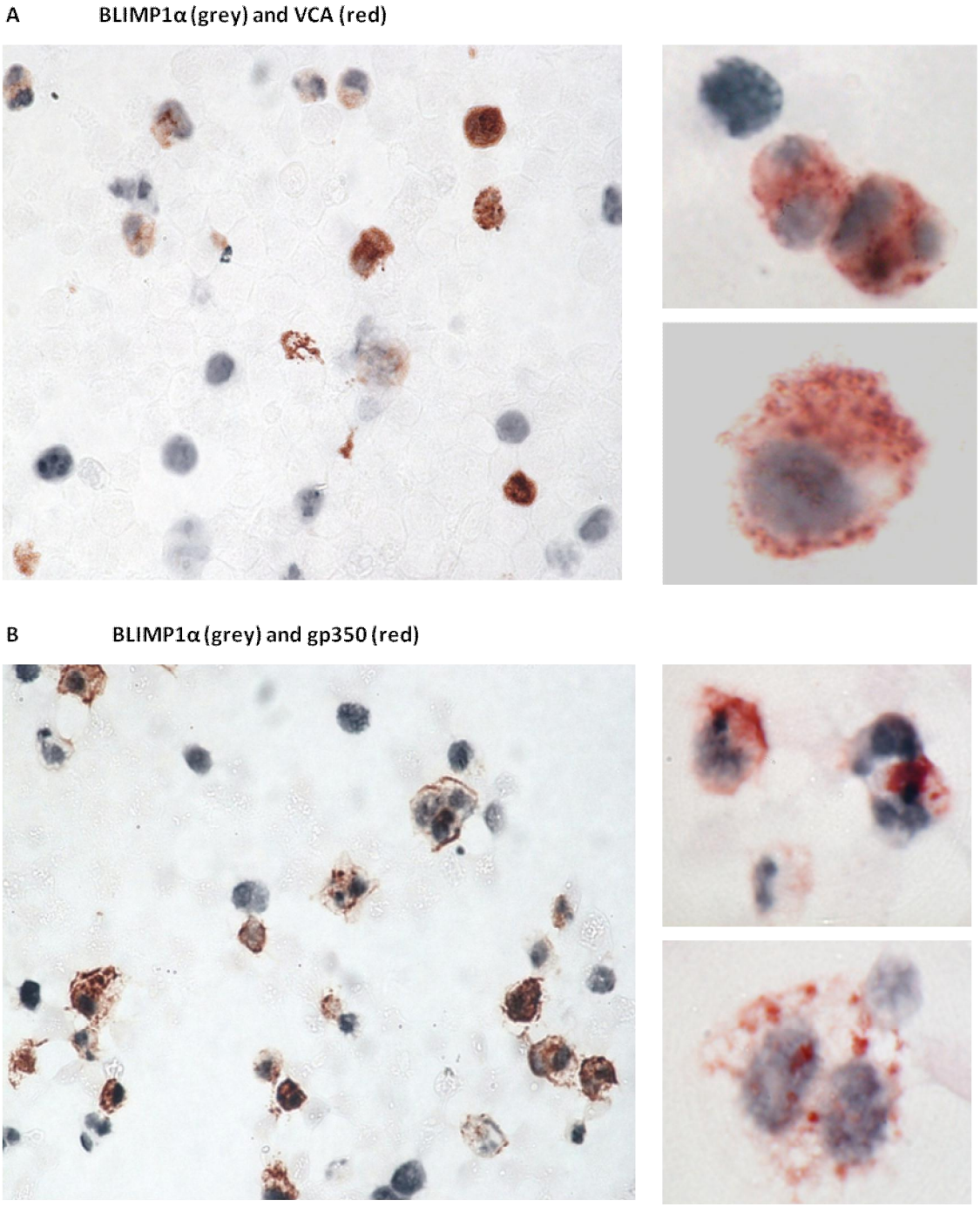
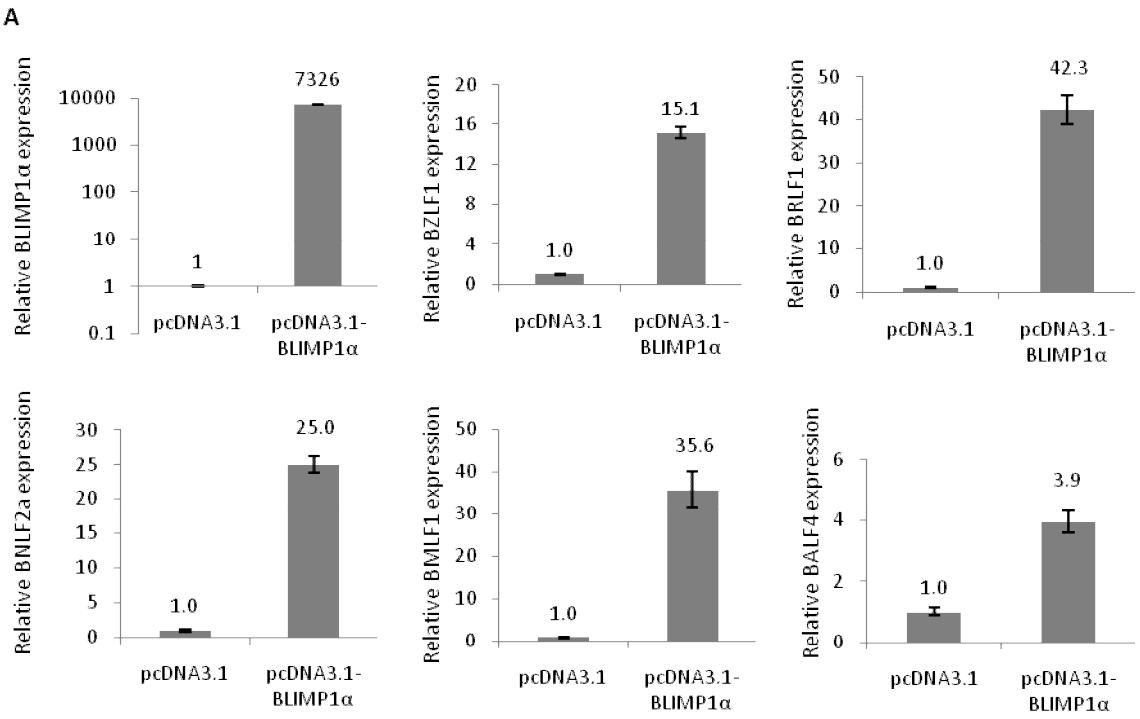
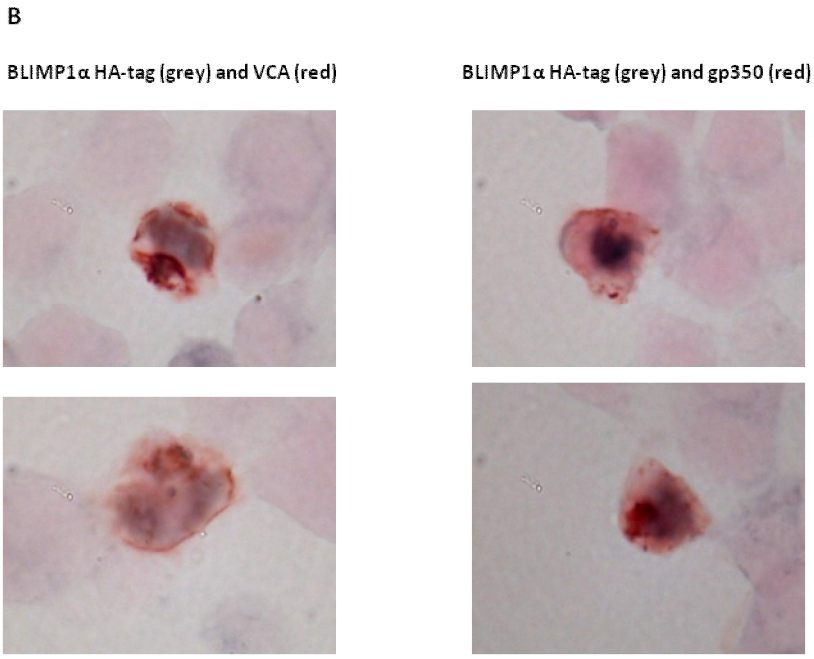


Figure 4.6: BLIMP1α induces EBV lytic cycle in Akata BL cells. (A) qRT-PCR demonstrates induction of BZLF1, BRLF1, BNLF2a, BMLF1 and BALF4 mRNA following BLIMP1α expression in Akata BL cells. (B) Immunohistochemistry shows co-staining for BLIMP1α HA-tag (grey) and VCA or gp350 (red) in transfected Akata cells. Representative examples of double-positive cells taken from one of two experiments are shown.

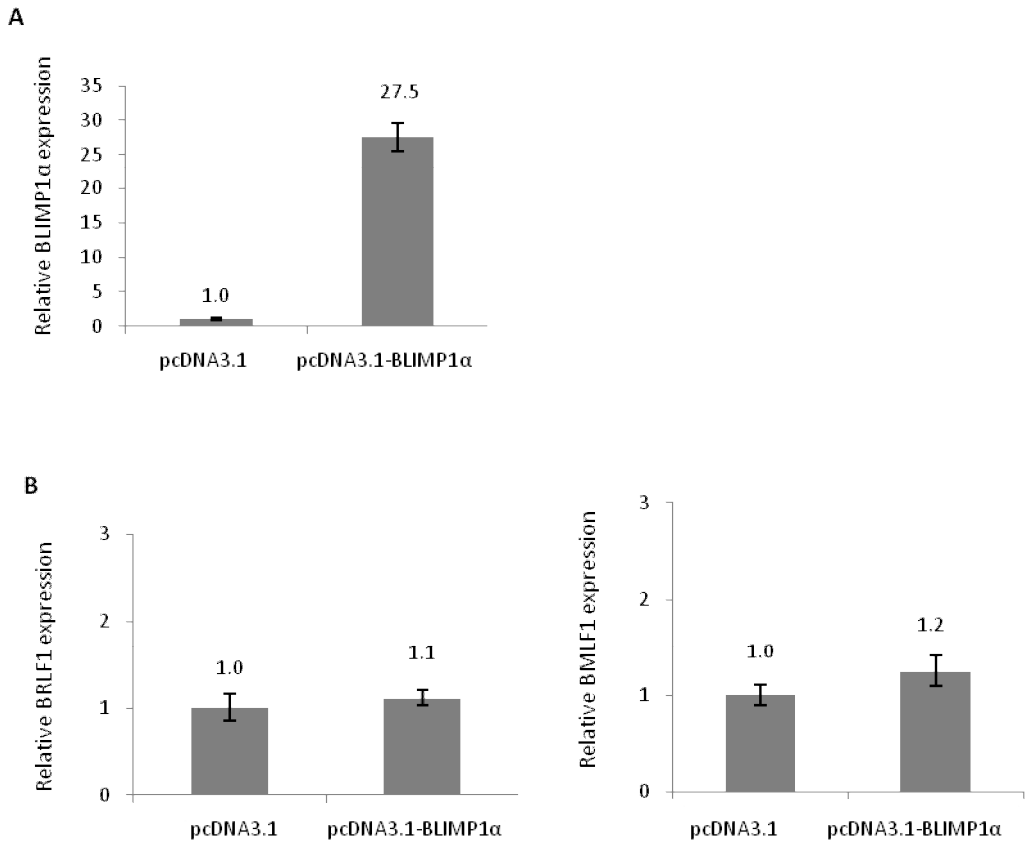




4.2.4. BLIMP1α does not induce EBV lytic cycle in L591 Hodgkin's lymphoma cells.

I next studied if BLIMP1α could also induce the lytic cycle in L591 cells, the only naturally occurring EBV positive HL cell line. These cells were co-transfected with either BLIMP1α (pcDNA3.1-PRDM1α) or control (pcDNA3.1) vector together with CD4.1 vector and cultivated for 48 hours prior to enrichment. The ectopic expression of BLIMP1α in L591 cells was confirmed by qRT-PCR (Figure 4.7A) and by immunohistochemistry for the HA-tag of BLIMP1α (data not shown). Figure 4.7B demonstrates that the ectopic expression of BLIMP1α in L591 cells did not induce the expression of BRLF1 or BMLF1. The levels of BZLF1 transcript in transfected cells were below the limit of detection (assessed by qRT-PCR) and therefore these data are not shown. Immunohistochemical staining showed no BZLF1, VCA or gp350 protein expression. These data suggest that BLIMP1α is not sufficient to trigger the viral lytic cycle in EBV-positive HL cells.

Figure 4.7: BLIMP1α expression in L591 HL cells does not induce EBV lytic cycle. A) qRT-PCR analysis of BLIMP1α expression in BLIMP1α- or control-transfected L591 cells. B) Expression of BLIMP1α had no effect on BRLF1 and BMLF1 mRNA levels. These results are representative of two independent experiments.



4.2.5. BLIMP1α does not bind to the BZLF1 promoter in SL3-LCL and B95.8 cells.

I next explored the possibility that BLIMP1α regulated BZLF1 expression directly by binding to BZLF1 promoter. To do this, I performed chromatin immunoprecipitation (ChIP) on extracts of BLIMP1α-transfected and control-transfected SL3-LCL and B95.8 cells using either a rabbit polyclonal antibody directed to BLIMP1α and which has previously been used in ChIP experiments (gift of Dr. Reuben Tooze, St James's University Hospital, Leeds, UK), or an antibody which recognizes the HA-tag of BLIMP1α (Tooze *et al.,* 2006). An isotype control (IgG) antibody or no antibody were used as negative controls. ChIP was followed by qPCR to amplify three overlapping regions (referred to as regions 1-3) encompassing the BZLF1 promoter. The ectopic expression of BLIMP1α and induction of BZLF1 was confirmed in both cell lines by immunohistochemistry (Figure 4.8 and data not shown).

Figure 4.9 shows that when compared to isotype control or no antibody control, none of the three regions of the BZLF1 promoter examined were found to be enriched for BLIMP1α binding in either SL3-LCL or B95.8 cells. I repeated this experiment in untransfected B95.8 cells using the BLIMP1α antibody. Although I observed an apparent enrichment of BLIMP1α across all three regions of the BZLF1 promoter, I also observed an enrichment of BLIMP1α at the Cp promoter which I used as a negative control in this experiment (Figure 4.10).

Figure 4.8: Confirmation of ectopic BLIMP1α expression in B95.8 cells. Prior to ChIP, ectopic BLIMP1α expression was confirmed in both SL3-LCL and in B95.8 cells. Shown here is the data for B95.8 cells (co-stained for the HA-tag of BLIMP1α and VCA).

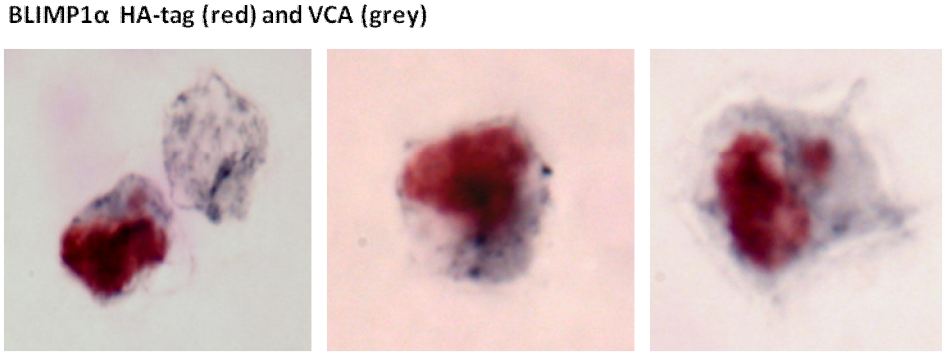


Figure 4.9: Ectopically expressed BLIMP1α does not bind to the BZLF1 promoter in SL3-LCL and B95.8 cells. Chromatin immunoprecipitation analysis of SL3-LCL (A) and B95.8 (B) cells following the ectopic expression of pcDNA3.1-BLIMP1α or vector control (pcDNA3.1). HA-tag and BLIMP1α antibodies were used to immunoprecipitate BLIMP1α protein. No antibody and isotype control (IgG ATB) were used as negative controls. Input was determined by qRT-PCR; the values obtained from BLIMP1α-transfected samples were normalized to that from control-transfected samples and represent fold enrichment over negative control (no antibody of vector only-transfected cells). The signal produced by HA-tag and BLIMP1α antibodies was compared to the background signal of isotype control IgG and no antibodies. These results are representative of three independent experiments.

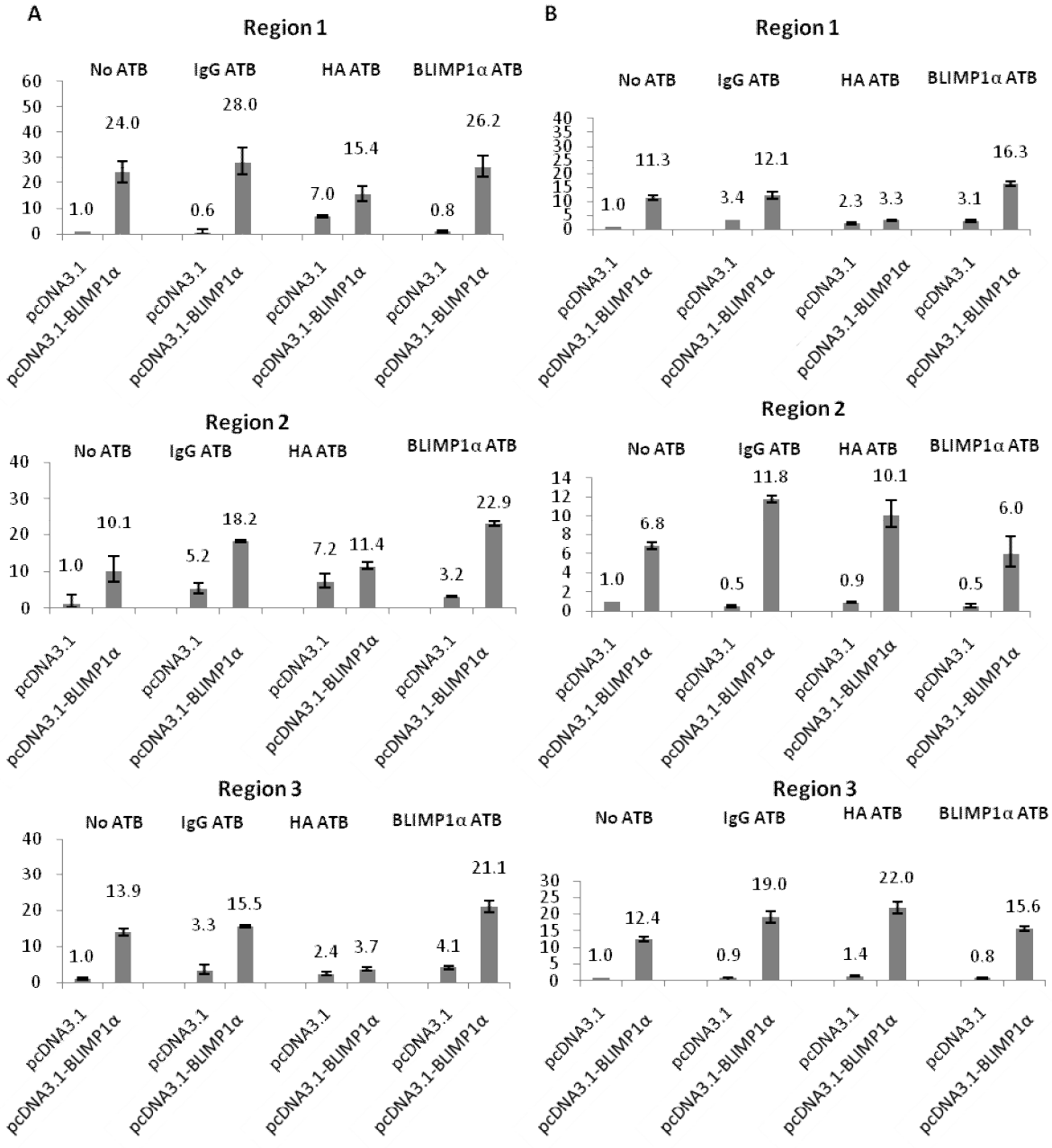
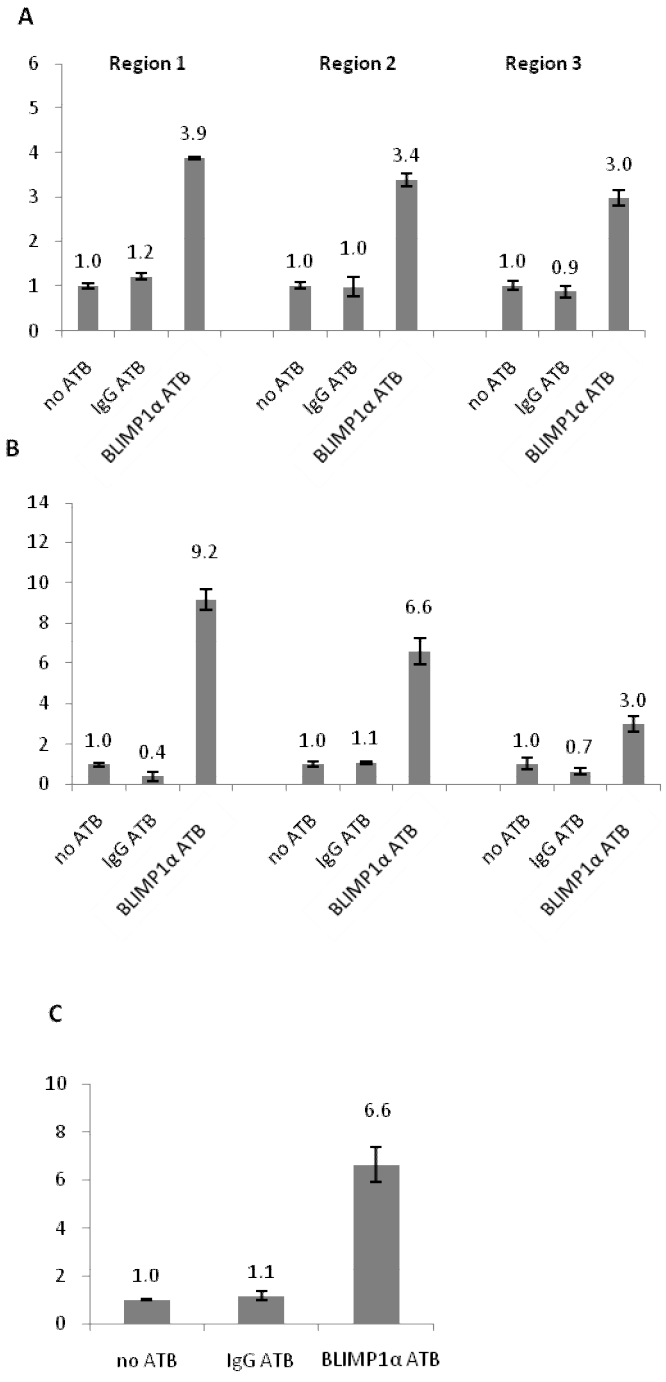


Figure 4.10: Endogenous BLIMP1α does not bind to the BZLF1 promoter in B95.8 cells. Chromatin immunoprecipitation analysis of untransfected B95.8 cells using an antibody for BLIMP1α. Two separate experiments (A and B) are shown. Although I observed an apparent enrichment of BLIMP1α across all three regions of the BZLF1 promoter, I also observed an enrichment of BLIMP1α at the Cp promoter which I used as a negative control in this experiment (C).



4.3. Discussion

I have shown that the ectopic expression of BLIMP1α in EBV-transformed B cells and in EBV-positive BL cells induces the viral lytic cycle. For herpesviruses, viral replication leading to the production of infectious virions ultimately results in cell death. Therefore, both terminal B cell differentiation and lytic replication are likely to be incompatible with the transformed state.

Expression of the immediate-early genes BZLF1 and BRLF1 was used to demonstrate induction of the viral lytic cycle in both LCLs and BL cell lines following BLIMP1α expression. Analysis of BLIMP1α and BZLF1 protein expression using immunohistochemistry suggested that the majority of BLIMP1α-expressing cells probably induced the virus lytic cycle. However, I observed only a modest increase in the expression of the late lytic cycle gene, BALF4 in OKU-LCL. Furthermore, the late lytic proteins, gp350/220 and viral capsid antigen (VCA) were expressed in only a fraction of these cells. These results suggest that, at least in this cell line, progression to the late lytic cycle occurs only in a minority of BLIMP1α-expressing cells. These data are consistent with a previous report suggesting that viral replication is abortive in most plasma cells (Laichalk and Thorley-Lawson, 2005). However, an alternative explanation of my results is that these cells were analysed at a point in time when the majority of cells had not yet entered into the late lytic cycle.

However, two observations suggested that the efficiency with which BLIMP1α-expressing cells can enter the late lytic cycle might be dependent upon cell type. First, I observed in untransfected B95.8 cells that whereas the late lytic cycle genes products, VCA and gp350

were rarely detectable in BLIMP1α-negative B95.8 cells they were commonly present in BLIMP1α-positive B95.8 cells. Second, I showed that the majority of BLIMP1α-expressing Akata BL cells also expressed VCA and gp350. However, the induction of the viral lytic cycle by BLIMP1α does not seem to be a universal feature of B cells since I was not able to induce lytic cycle gene expression in L591 HL cells following ectopic BLIMP1α expression. This observation is in contrast to a more recent study which showed a constitutive expression of BZLF1 in L591 cells which was only moderately increased following TPA treatment (Uphoff *et al.,* 2010). The observation that EBV positive HL tumours consistently lack BZLF1 expression suggests that these tumours might be inherently more resistant to lytic cycle induction (Herbst *et al.,* 1996). However, this possibility requires further investigation.

An important question is how BLIMP1α induces the EBV lytic cycle in B cells. One mechanism might involve an acute reactivation in which B cells respond to cellular stress by initiating virus replication thereby allowing the virus to escape quickly before the cell dies (Takada and Ono, 1989). However, it is more likely that BLIMP1α induces B cells into lytic cycle because it drives their differentiation towards plasma cells (Laichalk and Thorley-Lawson, 2005). This is supported by my observation that in GC B cells BLIMP1α expression induced changes characteristic of plasma cell differentiation. I did not observe direct binding of BLIMP1α to the BZLF1 promoter suggesting that the activation of BZLF1 by BLIMP1α is indirect. This latter mechanism might be mediated by XBP1, a transcription factor known to be induced by BLIMP1α and which has been shown to bind to the BZLF1 promoter and to activate the EBV lytic cycle (Takada and Ono, 1989; Sun and Thorley-Lawson, 2007; Bhende *et al.,* 2007; Vallabhapurapu *et al.,* 2006). Consistent with this it

has been shown that the expression of XBP1 in EBV latently infected cell lines leads to the induction of the virus lytic cycle (Reimold, *et al.,* 2001; Bhende *et al.,* 2007).

My data are also consistent with previous reports showing that in B cells, LMP1 can block entry into the lytic cycle, and that it does so primarily by suppressing BZLF1 expression at the transcriptional level (Adler *et al.,* 2002; Prince *et al.,* 2003). Interestingly, it has previously been shown that CD40 ligation can also suppress induction of the EBV lytic cycle in B cells (Adler *et al.,* 2002).

Identification of the cellular factors that regulate the switch from latency to lytic cycle in B cells is important for a better understanding of the pathogenesis of EBV-associated lymphomas, such as BL and HL, in which the lytic cycle is apparently suppressed. My results identify BLIMP1α as one such factor. The ability of BLIMP1α to activate the EBV lytic cycle would seem to represent a hitherto undescribed tumour suppressor function for BLIMP1α in the context of EBV-associated lymphomas.

CHAPTER 5

AN INVESTIGATION OF THE CONTRIBUTION OF C-MYC TO THE REGULATION OF BLIMP1α BY LMP1

AN INVESTIGATION OF THE CONTRIBUTION OF C-MYC TO THE REGULATION OF BLIMP1α BY LMP1

5.1. Introduction

Work presented in chapter 3 showed that LMP1 can down-regulate BLIMP1α expression in primary human germinal centre B cells. In the next part of the study, I investigated the mechanism responsible for this effect. I considered the possibility that the up-regulation of C-MYC by LMP1 might be responsible for the down-regulation of BLIMP1α. This seemed a reasonable proposition given that I had already shown that LMP1 could up-regulate C-MYC expression in GC B cells and that BLIMP1α is known to form reciprocal regulatory loops with other transcription factors involved in B cell differentiation, including BCL6, PAX5 and IRF4. It should be noted the experiments presented in this chapter were not performed in the order described. Therefore, while the first section considers the repression of BLIMP1α by C-MYC, the second half presents experiments in which no discrimination was made between the expression of the different isoforms following LMP1 transfection.

5.2. Results

5.2.1. C-MYC represses BLIMP1α in untransformed and transformed GC B cells.

I first attempted to confirm the up-regulation of C-MYC by LMP1 in primary GC B cells. To do this I performed qRT-PCR for C-MYC expression using RNA from two of the GC B cell samples I had previously used to demonstrate the down-regulation of BLIMP1α by LMP1

(Tonsil 1 and Tonsil 2, Figure 3.4, page 124). Figure 5.1A shows that in both samples, LMP1 up-regulated C-MYC expression. I also performed qRT-PCR analysis for C-MYC expression using RNA isolated from the LMP1 transfected CD77-positive and CD77-negative GC B cells described in Figure 3.5A (page 125). I observed that LMP1 up-regulated C-MYC in both GC B cell subsets (Figure 5.1B).

I next compared the expression of C-MYC in GC-derived LCLs with that in primary uninfected GC B cells. I used RNA taken from a previous experiment in which I had shown that EBV infection of GC B cells was followed by the down-regulation of BLIMP1α (Figure 3.1B, page 119). Figure 5.1C shows that C-MYC mRNA levels were elevated following the infection of GC B cells with EBV.

Having shown that LMP1 could up-regulate C-MYC expression in GC B cells, I next investigated if C-MYC regulated BLIMP1α expression in these cells. To do this, primary GC B cells isolated from three separate donors were transfected with either pcDNA3.1-C-MYC or pcDNA3.1 (control) together with LNGFR vector (these experiments were performed by Alexandra Schrader in our laboratory). After confirming the expression of C-MYC in transfected GC B cells (Figure 5.2A), qRT-PCR was used to show that the ectopic expression of C-MYC in GC B cells was followed by the down-regulation of BLIMP1α expression (Figure 5.2B).

Figure 5.1: LMP1 induces C-MYC expression in GC B cells. A) qRT-PCR analysis of GC B cells transfected with LMP1 or vector control. LMP1 expression led to the induction of C-MYC expression. These same samples were previously used to describe the down-regulation of BLIMP1α by LMP1 (Figure 3.4, page 124). B) LMP1 also up-regulated C-MYC mRNA in CD77-and CD77+ subsets of CD10+ cells. C) Infection of GC B cells with EBV resulted in up-regulation of C-MYC.

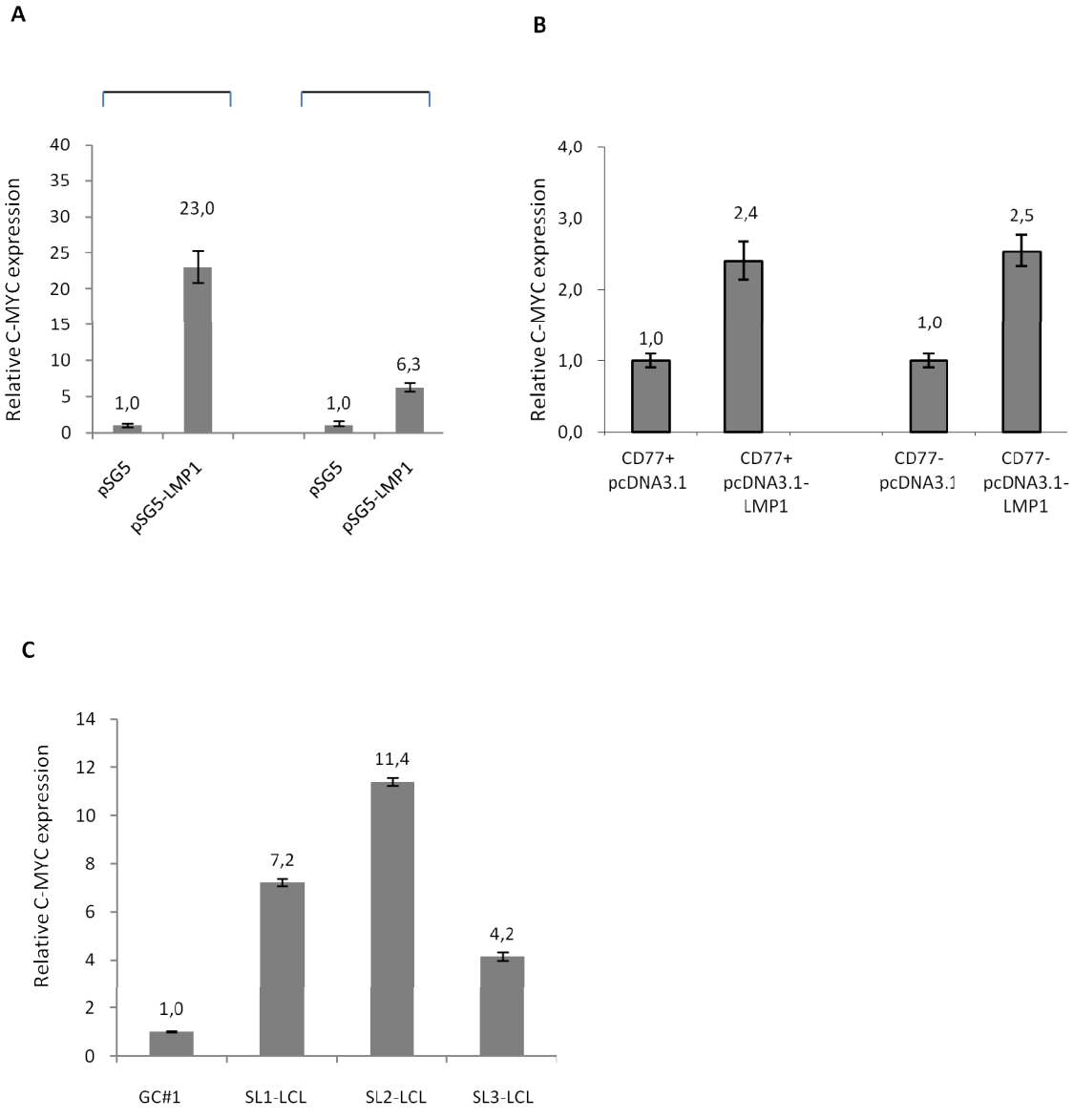
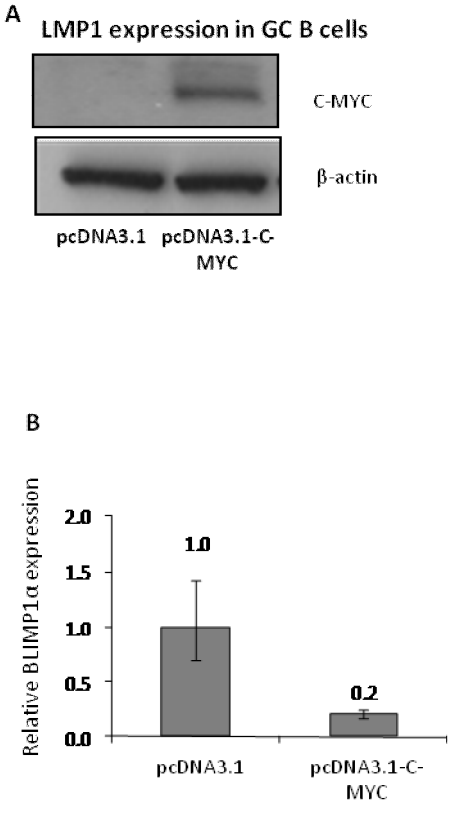


Figure 5.2: C-MYC represses BLIMP1α in GC B cells. A) A representative western blot for C-MYC and β-actin following the transfection of primary human GC B cells with C-MYC expression vector or control vector. B) qRT-PCR for BLIMP1α expression in GC B cells after ectopic C-MYC expression (one representative experiment of three is shown).



5.2.2. Regulation of C-MYC and BLIMP1 by LMP1 in Burkitt's lymphoma cells

I next investigated if LMP1 could also regulate C-MYC and BLIMP1 in BL cells. I first compared the expression of BLIMP1 in the EBV-positive BL cell lines, MUTU I and MUTU III. The MUTU I line expresses a latency I viral gene expression programme in which LMP1 is not detectable. In contrast, MUTU III, which is derived from MUTU I, expresses a latency III viral gene expression programme in which LMP1 is present and C-MYC is down-regulated. I confirmed the expression of LMP1 in MUTU III and its absence in MUTU I cells (data not shown). Figure 5.3A shows the down-regulation of C-MYC in MUTU III cells. Figure 5.3B shows that BLIMP1 mRNA levels were higher in MUTU III compared with MUTU I cells.

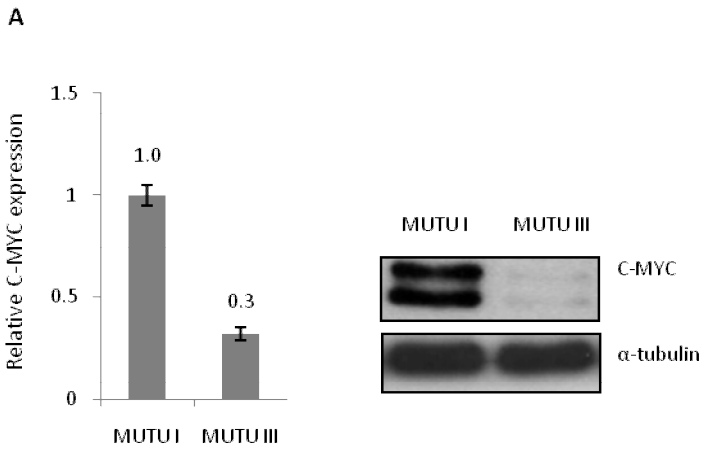
I next studied if LMP1 could regulate BLIMP1 and C-MYC in two EBV negative BL cell lines, BL2 and DG75. Figure 5.4 shows that the transient transfection of BL2 cells with LMP1 was followed by the up-regulation of BLIMP1 and the down-regulation of C-MYC. To study the effects of LMP1 in DG75 cells, I took advantage of a system already established in our laboratory in which DG75 cells are stably transduced with a tetracycline-regulatable LMP1 construct (Floettmann *et al.,* 1996). These cells were maintained in the continuous presence of tetracycline to prevent LMP1 expression. Removal of tetracycline from the culture induced LMP1 expression (Figure 5.5A) and was followed by the up-regulation of BLIMP1 (Figure 5.5B,C) and by the down-regulation of C-MYC (Figure 5.5C). The small induction of BLIMP1 observed in DG75 cells carrying the LMP1 expression vector in the presence of tetracycline is almost certainly due to leaky expression of LMP1 from this

vector and is consistent with the low levels of LMP1 mRNA present in these cells (Figure 5.5A).

5.3.3. LMP1 does not regulate BLIMP1 or C-MYC expression in L428 Hodgkin's lymphoma cells.

Finally, I studied if LMP1 could also regulate C-MYC and BLIMP1 expression in L428 HL cells. However, I did not observe any consistent change in the expression of either BLIMP1 or C-MYC following the transient transfection of L428 cells with LMP1 (Figure 5.6).

Figure 5.3: Comparison of C-MYC and BLIMP1 expression in MUTU BL cells. A) Relative quantity of C-MYC and BLIMP1 in MUTU I and MUTU III cells. Down-regulation of C-MYC in MUTU III cells (A), which express LMP1 (data not shown), was associated with the increased expression of BLIMP1 (B).



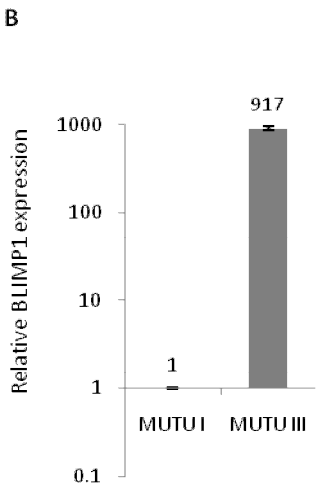


Figure 5.4: LMP1 up-regulates BLIMP1 and down-regulates C-MYC expression in BL2 cells. Ectopic expression of LMP1 is shown in A). B) qRT-PCR analysis of BL2 cells following transient transfection with LMP1 or control vector showed that LMP1 down-regulated C-MYC, but up-regulated BLIMP1 mRNA levels.

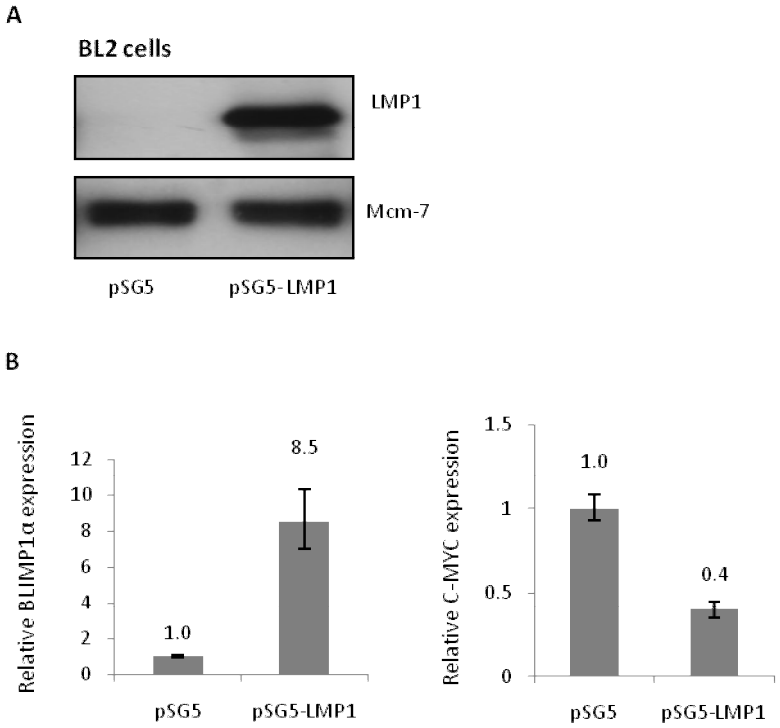


Figure 5.5: LMP1 up-regulates BLIMP1 and down-regulates C-MYC expression in DG75 cells. A) Expression of LMP1 in DG75 stably transduced with tetracycline regulated LMP1 expression vector in the presence/absence of tetracycline (TET). Removal of tetracycline was followed by induction of LMP1. B) shows that BLIMP1 mRNA was increased in the presence of LMP1. This experiment also shows that BLIMP1 expression was not regulated by tetracycline itself. C) shows that the up-regulation of BLIMP1 in LMP1-expressing cells was accompanied by the up-regulation of C-MYC

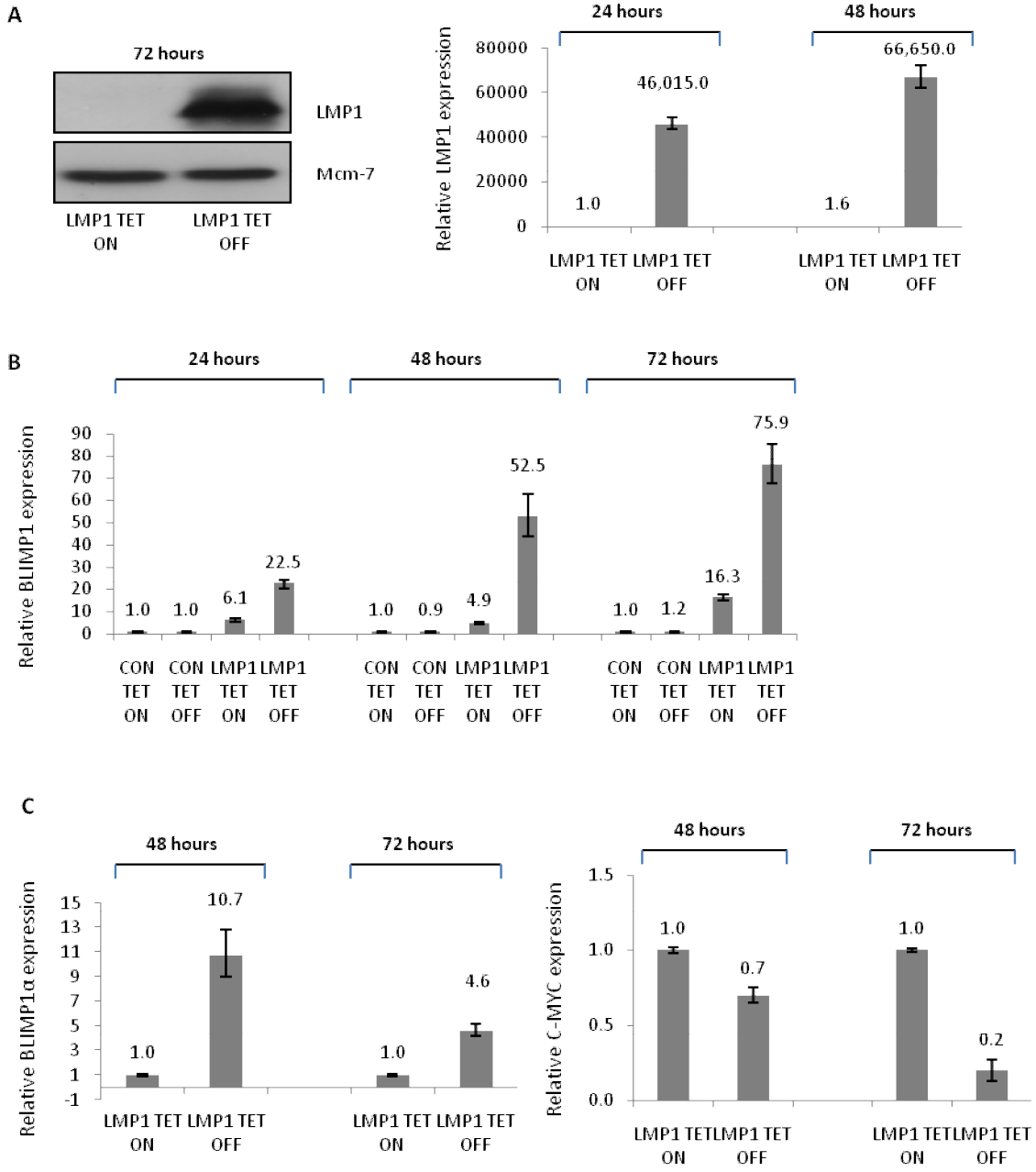
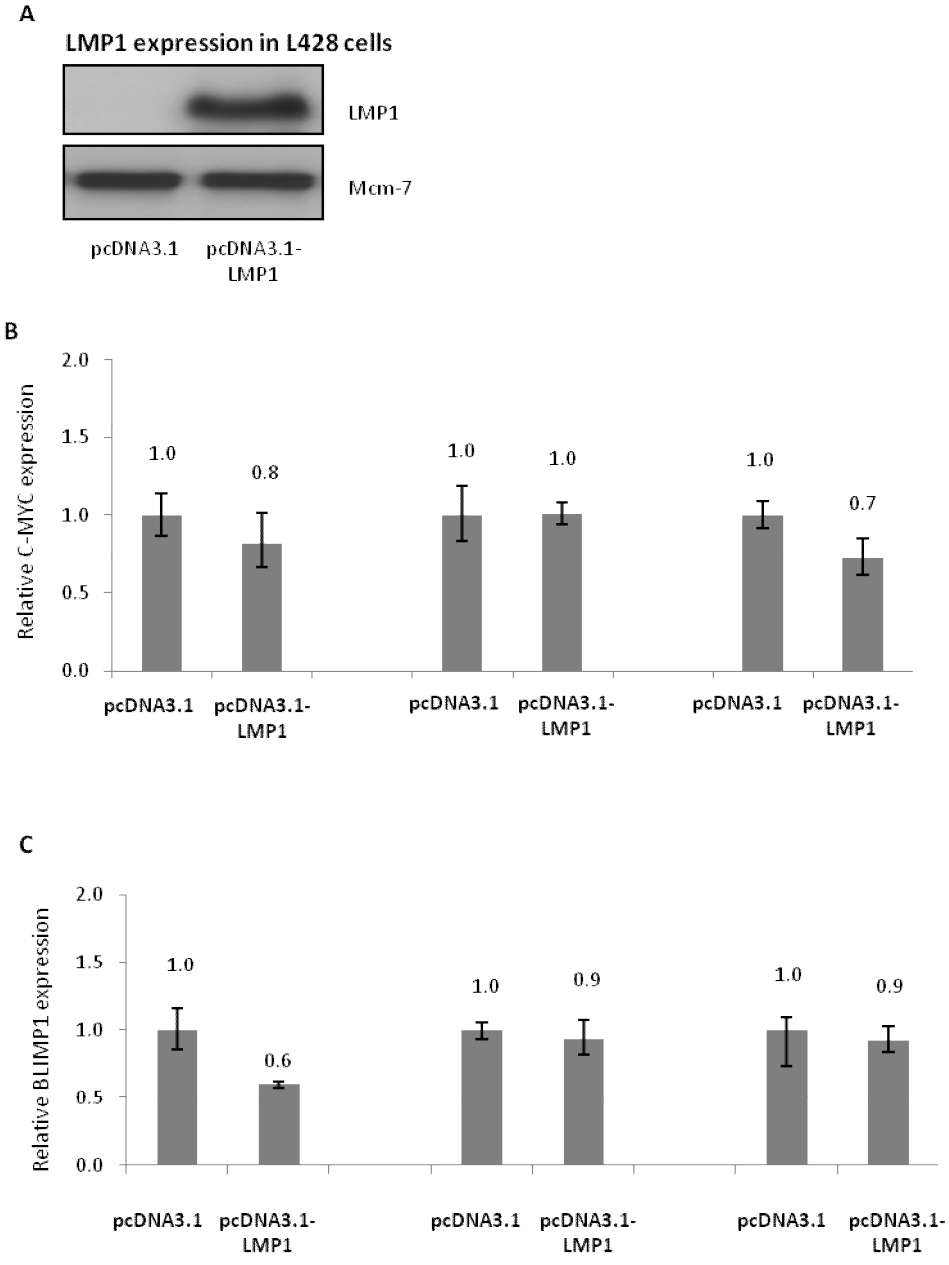


Figure 5.6: LMP1 does not regulate BLIMP1 and C-MYC expression in L428 HL cells. Ectopic expression of LMP1 is shown in A). qRT-PCR analysis of L428 cells following transient transfection with LMP1 or control vector showed that LMP1 did not regulate either C-MYC (B) or BLIMP1 (C) mRNA levels. This experiment was repeated three times.



5.3. Discussion

In this chapter, I have shown that the ectopic expression of LMP1 in primary GC B cells leads not only to the down-regulation of BLIMP1, but also to the up-regulation of C-MYC. I observed this effect in both CD77-positive and CD77-negative GC B cells. Furthermore, I observed that the down-regulation of BLIMP1 that occurs following the infection of GC B cells with EBV is also accompanied by the up-regulation of C-MYC. These data are consistent with observations made in chapter 3, in which I showed that in two separate GC B cell samples LMP1 up-regulated C-MYC expression.

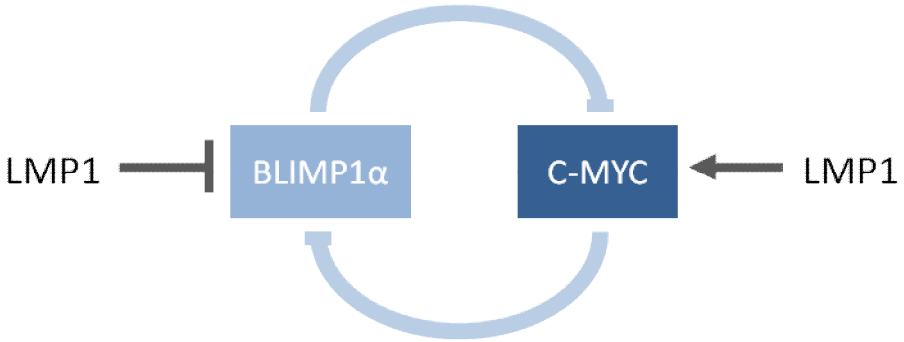
I explored if the up-regulation of C-MYC might account for the down-regulation of BLIMP1 that follows the ectopic expression of LMP1 in GC B cells. I observed that the transfection of C-MYC into GC B cells down-regulated BLIMP1 expression. It has already been shown that BLIMP1 can repress C-MYC in both transformed human B cells and in primary mouse B cells (Lin *et al.,* 1997; Shaffer *et al.,* 2002). Furthermore, I showed in chapter 3 that BLIMP1 can also repress C-MYC in primary untransformed GC B cells. Therefore, BLIMP1 and C-MYC are capable of negatively regulating each other's expression. Further evidence in support of this has been provided elsewhere by the demonstration that the knock-down of C-MYC in BL cells leads to the up-regulation of BLIMP1α (Vrzalikova *et al.,* 2011).

Because the repression of C-MYC is required for terminal B cell differentiation (Lin et al., 2000), my data suggest that LMP1 may antagonize plasma cell differentiation by up-regulating C-MYC which then represses plasma cell differentiation by suppressing BLIMP1α. This not only provides a plausible explanation for the down-regulation of BLIMP1α by LMP1 but also a potentially novel mechanism which might explain the

impaired differentiation characteristic of B cell lymphomas harbouring C-MYC abnormalities. Alternatively, it is possible that LMP1 initially drives the down-regulation of BLIMP1α which then leads to the up-regulation of C-MYC. In either case LMP1 would drive a reciprocal regulatory loop involving BLIMP1α and C-MYC which would ultimately lead to the activation of MYC and the repression of BLIMP1α. Figure 5.7 shows a schematic illustration of the proposed regulation of this loop by LMP1.

During the course of this study, I was also able to confirm previous reports that LMP1 suppresses C-MYC expression in BL cells (Floettmann *et al.,* 1996). However, I also observed that the suppression of C-MYC by LMP1 was accompanied by the induction of BLIMP1 expression, a finding independently verified by Dr. Gemma Kelly in our Institute (personal communication). These observations may provide an explanation for the consistent lack of LMP1 expression in EBV-positive BL, since expression of this viral oncogene would lead not only to the loss of the proliferative effects of C-MYC, but also to the induction of plasma cell differentiation and potentially to the activation of the viral lytic cycle. It is not clear why BLIMP1 is up-regulated by LMP1 in BL cells, but down-regulated by this viral protein in GC B cells. However, one explanation might be that a translocated C-MYC gene present in BL cell is regulated differently from the normal C-MYC gene present in GC B cells.

Figure 5.7: Schematic illustration of the potential regulation by LMP1 of a reciprocal regulatory loop involving BLIMP1α and C-MYC. LMP1 might act initially to down-regulate BLIMP1α which would release the BLIMP1α-mediated suppression of C-MYC. The up-regulation of C-MYC would lead to further suppression of BLIMP1α. Alternatively, LMP1 might initially activate C-MYC expression.



CHAPTER 6

AN INVESTIGATION OF BLIMP1β EXPRESSION IN EBV-TRANSFORMED GERMINAL CENTRE B CELLS AND HODGKIN'S LYMPHOMA CELLS

AN INVESTIGATION OF BLIMP1β EXPRESSION IN EBV-TRANSFORMED GERMINAL CENTRE B CELLS AND HODGKIN'S LYMPHOMA CELLS

6.1. Introduction

The PRDM1 gene encodes at least two isoforms, designated BLIMP1α and BLIMP1β, which are expressed from alternate promoters (György *et al.,* 2003). The full-length BLIMP1α protein orchestrates plasma cell differentiation by repressing genetic programs associated with the germinal centre (GC) stages, while at the same time activating those programs associated with plasma cell functions (Shapiro-Shelef *et al.,* 2003; Calame *et al.,* 2003). In contrast, BLIMP1β is transcribed from a different promoter and exon located upstream of exon 4 of the gene (György *et al.,* 2003). The BLIMP1β protein lacks the first 101 amino acids of BLIMP1α and instead contains 3 novel amino acids fused to amino acids 102–789 of BLIMP1α. BLIMP1β, which lacks most of the proline rich (PR) domain, has a diminished capacity to repress target genes (György *et al.,* 2003). Since BLIMP1β contains the DNA-binding domain but bears a disrupted regulatory domain it has been suggested to behave as an inhibitor of BLIMP1α (György *et al.,* 2003).

Other members of the PRDM family, including PRDM2 (RIZ), PRDM3 (MDS1-EVI1) and PRDM16 (MEL1) can also express a full length protein containing the PR domain as well as a truncated protein missing the PR domain (Morishita *et al.,* 2007). The balance of expression of these different PRDM isoforms is disrupted in many cancers and results from both the over-expression of the truncated proteins as well as the loss of expression of the full length proteins (Huang *et al.,* 1999; Chadwick *et al.,* 2000; Steele-Perkins *et al.,*

2001; Sasaki *et al.,* 2002; He *et al.,* 1998; Cuenco *et al.,* 2000; Soderholm *et al.,* 1997; Kurokawa *et al.,* 1998a). For example, the transcript of the long form of the *MDS1-EVI1/PRDM3* gene is expressed at very low levels in leukaemia cells, whereas the short form of the *EVI1* gene is over-expressed in human leukaemias with chromosome 3q abnormalities (Soderholm *et al.,* 1997; Kurokawa *et al.,* 1998a; Kurokawa *et al.,* 1998b; Nucifora*,* 1997; Sood *et al.,* 1999). Likewise, the human *MEL1/PRDM16* also has two alternative protein forms, a long form, MEL1, and a short form, MEL1S. The latter is over-expressed in leukaemia cells carrying the t(1;3) translocation (Barjesteh van Waalwijk van Doorn-Khosrovani *et al.,* 2003; Mochizuki *et al.,* 2000). In some cases, the full length PRDM proteins have been shown to have tumour supressive functions, whereas the truncated variants can have tumour promoting activity. For example, the product of PRDM2, RIZ1, is a tumour suppressor protein because it demonstrates a loss of function in many types of human cancers with genomic deletions or point mutations and because RIZ1-deficient mice have been shown to develop DLBCL (Huang*,* 1999; Chadwick *et al.,* 2000; Steele-Perkins *et al.,* 2001). In contrast, RIZ2 which lacks the PR domain is over-expressed in breast cancer and in acute lymphoblastic leukaemias (Sasaki *et al.,* 2002; He *et al.,* 1998).

The over-expression of BLIMP1β has been reported in multiple myeloma, DLBCL and in some T cell lymphomas (György *et al.,* 2003; Ocana *et al.,* 2006; Zhao *et al.,* 2008; Liu *et al.,* 2007). BLIMP1β over-expression is associated with advanced Ann Arbor stage and a high-risk International Prognostic Index in T cell lymphomas and with a shorter patient survival in both DLBCL and T cell lymphoma patients (Zhao *et al.,* 2008; Liu *et al.,* 2007). In both B- and T-cell lymphomas, BLIMP1β expression is also associated with *in vitro* resistance to

chemotherapeutic agents (Zhao *et al.,* 2008; Liu *et al.,* 2007). Here I have investigated the expression of the BLIMP1β isoform in EBV-transformed GC B cells and in HL cells.

6.2. Results

6.2.1. BLIMP1β levels are low in normal tonsillar B cells.

I first compared the expression of BLIMP1α and BLIMP1β mRNA in normal tonsillar B cells using qRT-PCR. I found that BLIMP1α mRNA levels were low in naive B cells and in CD77-positive GC B cells, but higher in CD77-negative GC B cells and in memory B cells (Figure 6.1A). Although BLIMP1β levels appeared to follow a similar trend (Figure 6.1B), comparison of the relative amounts of transcript within each subset revealed that the levels of BLIMP1β mRNA in all cells were substantially lower than those of BLIMP1α (Figure 6.1C). I was not able to differentiate between BLIMP1α and BLIMP1β protein expression in these cells since all available BLIMP1 specific antibodies recongnize either only the BLIMP1α isoform or both.

6.2.2. BLIMP1β expression is up-regulated following EBV infection of primary human germinal centre B cells.

Having shown that the levels of BLIMP1β mRNA are very low in normal B cell subsets, including GC B cells, I next explored the expression of this isoform following the *in vitro* infection of B cells with EBV. To do this I measured expression of the BLIMP1β isoform in the three GC-derived LCLs and in the uninfected GC B cells previously described in chapter

3 in which I had demonstrated the down-regulation of BLIMP1α by EBV (Figure 3.1, page 119). Figure 6.2 shows that compared to un-infected GC B cells, the GC-derived LCL showed increased expression of the BLIMP1β isoform. Figure 6.3 shows that the up-regulation of BLIMP1β and the down-regulation of BLIMP1α reduced the BLIMP1α:BLIMP1β ratio in the EBV infected cells. For example, in the matched pair, GC#1 and SL1-LCL, the BLIMP1α:BLIMP1β ratio fell from 25 (1/0.04) in normal GC B cells to 1.47 (1/0.68) in EBV-transformed GC B cells. However, it should be noted that Figure 6.3 describes the expression of BLIMP1β relative to BLIMP1α in each cell type and does not necessarily reflect the modest increase in BLIMP1β levels compared to the pronounced down-regulation of BLIMP1α.

6.2.3. Over-expression of BLIMP1β in Hodgkin's lymphoma cells

I next studied the expression of the two BLIMP1 isoforms in GC B cells and HL cell lines. I found that in 3/4 HL cell lines, BLIMP1β levels were increased and those of BLIMP1α unchanged compared to GC B cells (Figure 6.4A,B). The exception was the KMH2 cell line in which BLIMP1β levels were unchanged, but those of BLIMP1α increased. However, I also observed that EBV infection increased the levels of BLIMP1β mRNA in KMH2 cells. These changes in the expression of the different BLIMP1 isoforms in HL cells resulted in a decreased BLIMP1α:BLIMP1β ratio (Figure 6.5). U266 was used as a ppositive control in these experiments.

Figure 6.1: BLIMP1β levels are low in normal B cells. qRT-PCR analysis of the relative quantity of (A) BLIMP1α mRNA and B) BLIMP1β mRNA in individual subsets of B cells (IgD+ naïve cells, CD77+GC B cells, CD77-GC B cells and CD27+memory cells) isolated from the same tonsil. C) Comparison of mRNA levels of BLIMP1α and BLIMP1β isoforms relative to total BLIMP1 (tBLIMP1) in individual B cell subsets.

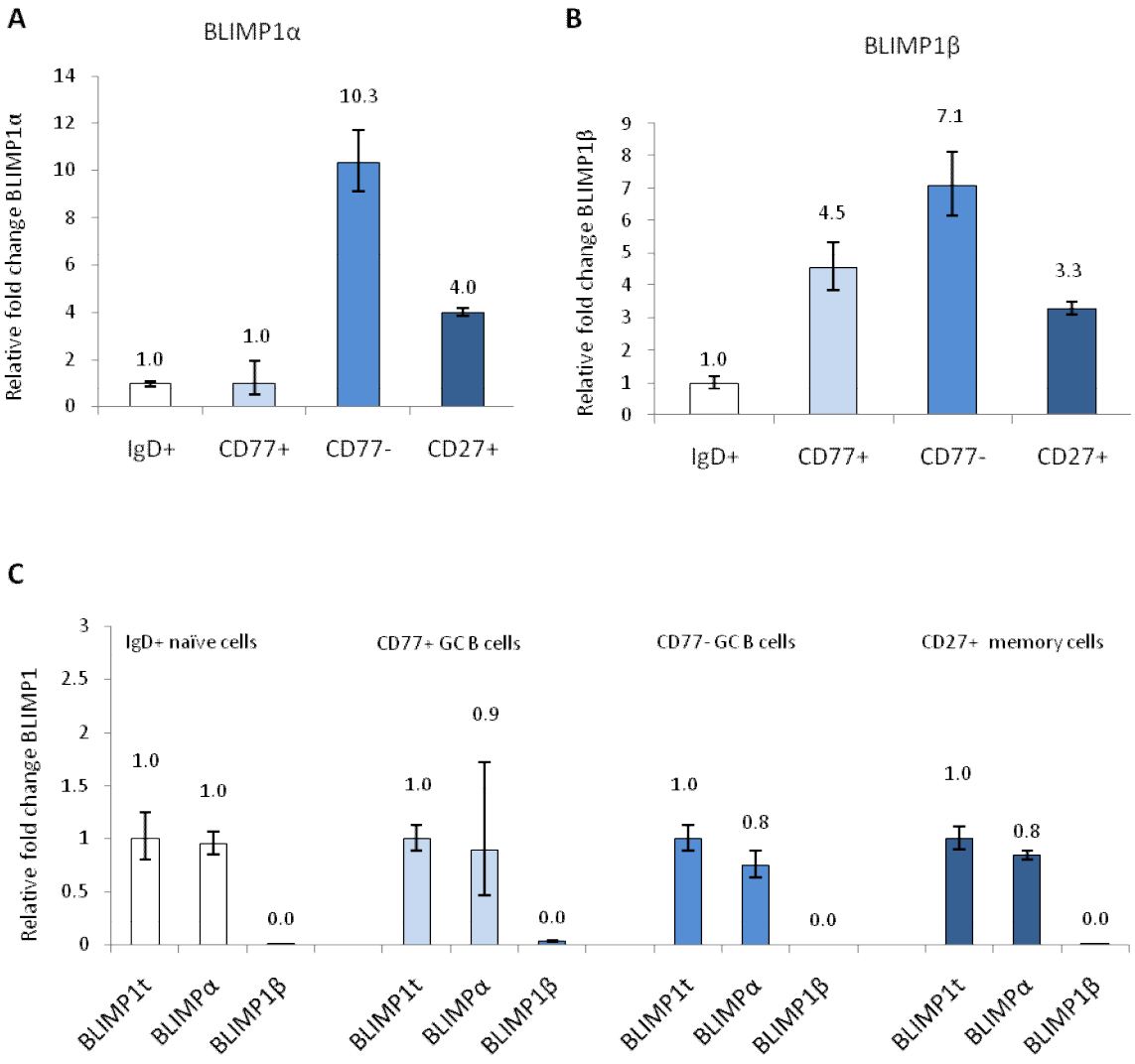


Figure 6.2: BLIMP1β mRNA expression is up-regulated following EBV infection of primary human GC B cells. qRT-PCR analysis shows increased expression of the BLIMP1β isoform in three LCLs derived from GC B cells compared to three samples of normal CD10+ GC B cells.

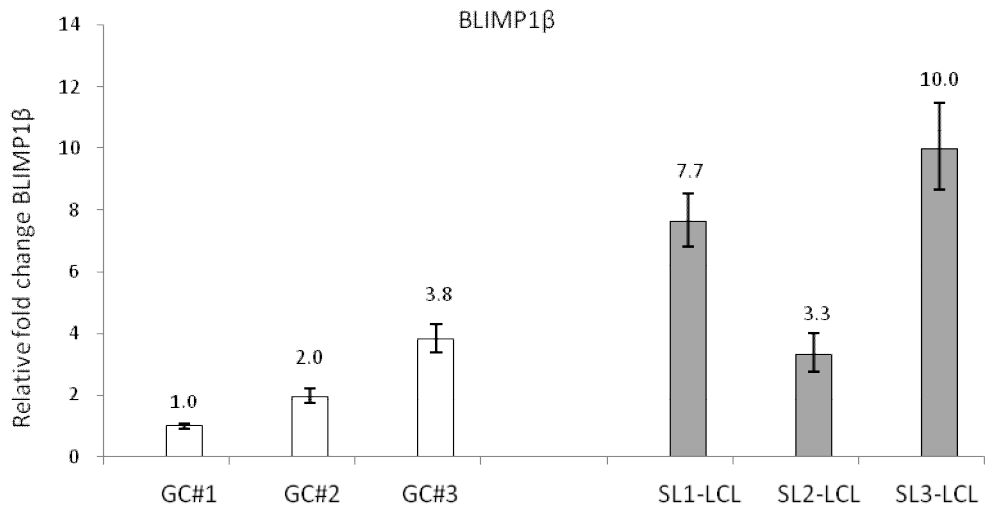


Figure 6.3: Comparison of the relative levels of BLIMP1α and BLIMP1β isoforms in primary GC B cells and in EBV-infected GC B cells. Relative levels of BLIMP1α and BLIMP1β isoforms in GC B cells (A) and GC B cell-derived LCLs (B); EBV infection reduced the BLIMP1α:BLIMP1β ratios.

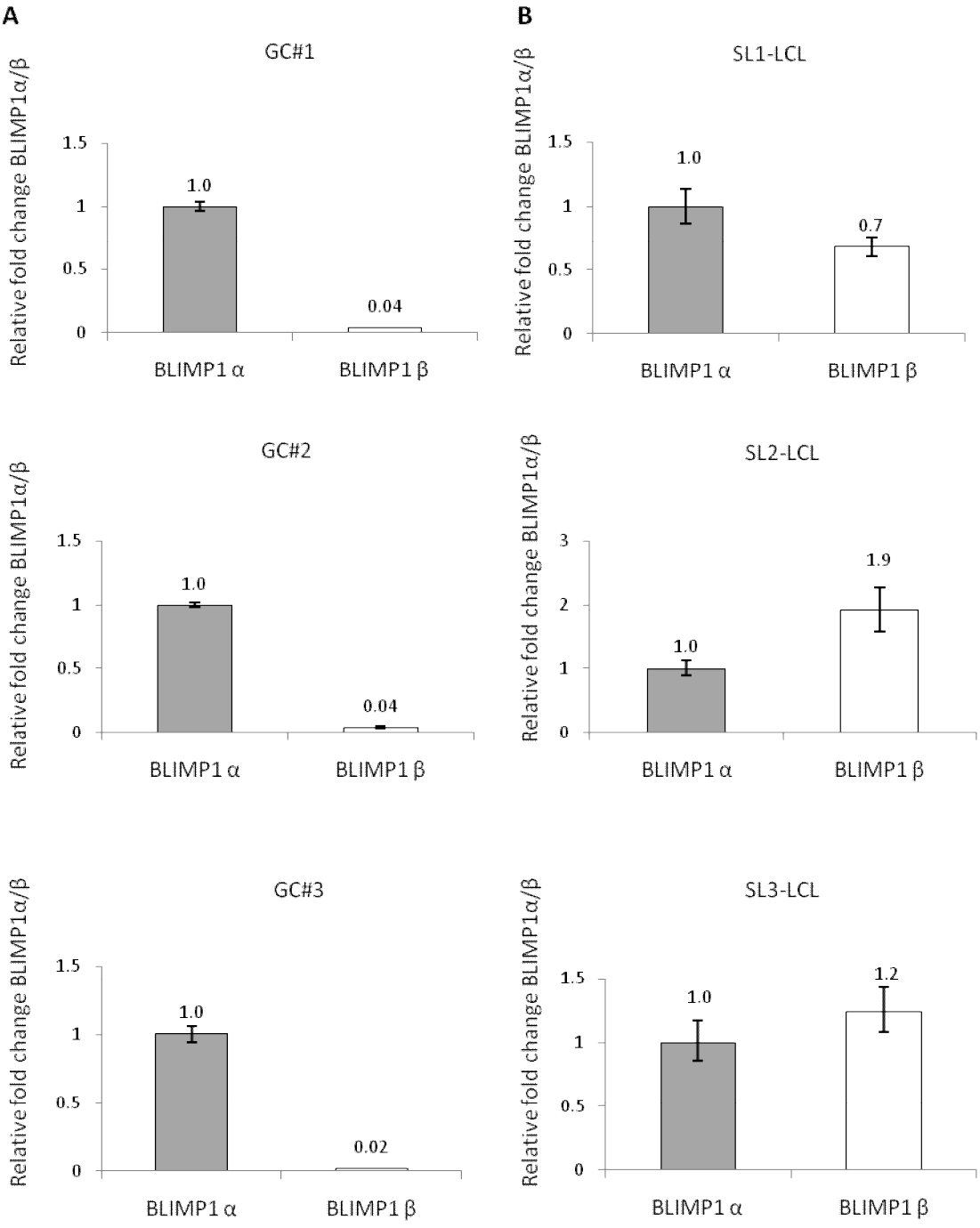


Figure 6.4: Over-expression of BLIMP1β in HL cells. qRT-PCR analysis of the relative quantity of (A) BLIMP1β, and B) BLIMP1α isoforms in GC B cell derived lymphoma lines compared to a representative sample of normal CD10+ GC B cells. When compared to normal GC B cells, BLIMP1β mRNA was increased in three HL cell lines (L428, L1236, and L591) and decreased in one HL cell line (KMH2) and in both BL cell lines. EBV infection of KMH2 cells increased BLIMP1β expression. In contrast, the levels of BLIMP1α mRNA were not significantly changed in L428, L1236 and L591 cells but were decreased in KMH2 cells and in both BL cell lines (BL2 and Rael).

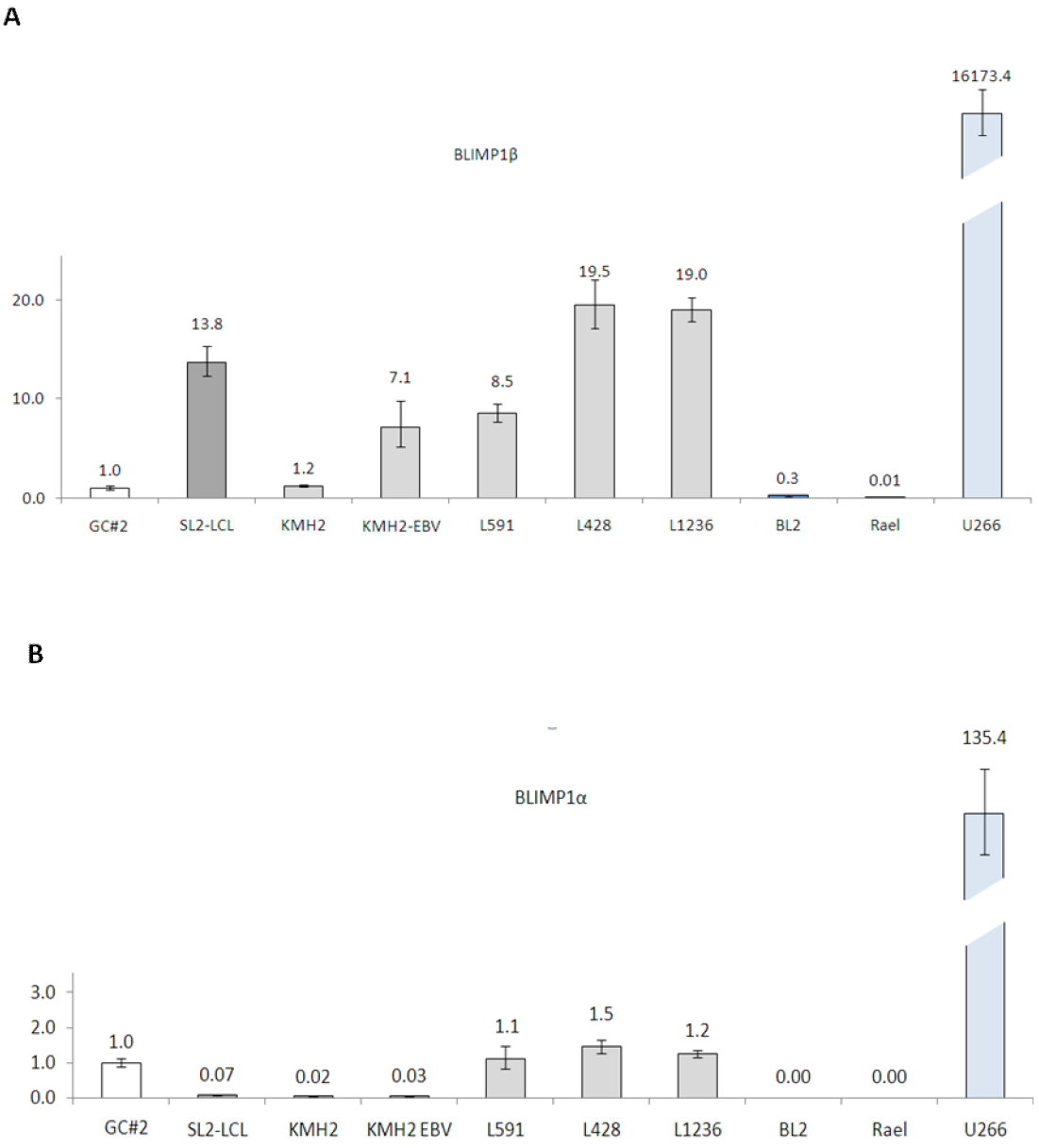
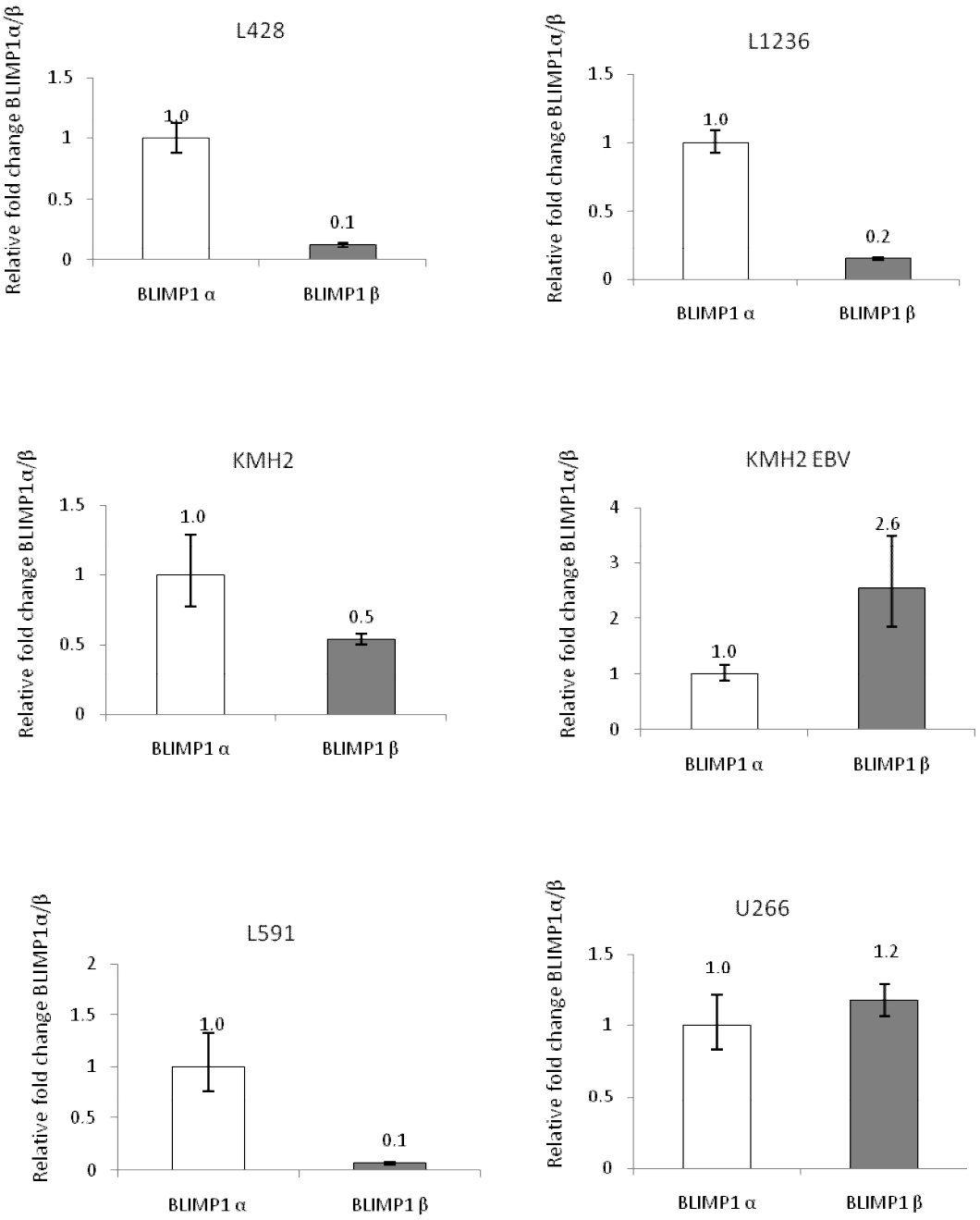


Figure 6.5: Over-expression of BLIMP1β in HL cells. The BLIMP1α:BLIMP1β ratio was decreased in HL cell lines compared to normal GC B cells (compare to ratios for GC B cells shown in upper panel of Figure 6.3).

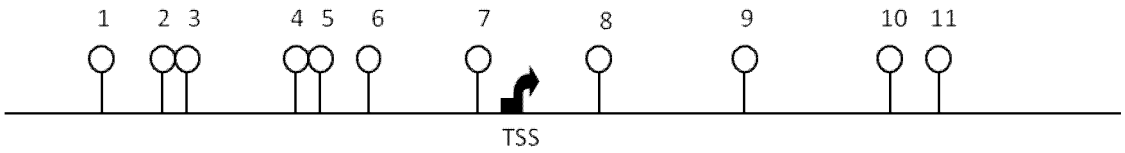


6.2.4. Increased expression of BLIMP1β in Hodgkin's lymphoma cells is associated with hypomethylation of the BLIMP1β-specific promoter.

Previous studies in our laboratory have shown that when compared to uninfected GC B cells the BLIMP1β-specific promoter is hypomethylated in EBV-transformed GC B cells (Leonard *et al.*, unpublished). I explored if the increased expression of BLIMP1β in HL cell lines was associated with a change in the methylation status of the BLIMP1β-specific promoter. To do this I performed pyrosequencing on 8 of the 11 CpGs located within the hypomethylated region previously defined by Leonard et al. (Figure 6.6A) and compared this to pyrosequencing data already available from the isolated GC B cells (Figure 6.6B). Figure 6.6C shows that relative to GC B cells, all 8 CpGs were hypomethylated in the HL cell lines.

Figure 6.6: Over-expression of BLIMP1β is accompanied by promoter hypomethylation in GC B cells and HL cells. A) Schematic illustration of the CpG sites within the PRDM1β promoter. The transcriptional start site (TSS) is indicated with an arrow and each CpG illustrated with a lollipop. B) Pyrosequencing of these 11 CpGs within the hypomethylated region in three GC B cell samples. C) Pyrosequencing of 8 CpGs within the hypomethylated region in three representative HL cell lines and from unmethylated (UC) and methylated controls (MC) (pyrosequencing was not informative for CpGs 6, 10, and 11). Compared to GC B cells (Figure 4) the BLIMP1β-specific promoter is hypomethylated in HL cells.

A



B

	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6	CpG 7	CpG 8	CpG 9	CpG 10	CpG 11
GC# 1	14%	12%	12%	16%	13%	16%	26%	24%	30%	29%	31%
GC# 2	12%	15%	13%	15%	15%	14%	24%	25%	31%	27%	31%
GC# 3	12%	13%	12%	15%	15%	17%	26%	22%	30%	27%	30%

C

	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6	CpG 7	CpG 8	CpG 9	CpG 10	CpG 11
KMH2	0%	11%	0%	0%	11%	-	10%	2%	9%	-	-
KMH2 EBV	0%	0%	10%	15%	0%	-	12%	4%	9%	-	-
L591	5%	0%	6%	0%	0%	-	0%	2%	0%	-	-
L428	5%	0%	0%	7%	0%	-	0%	2%	4%	-	-
UC	8%	8%	0%	12%	0%	-	12%	10%	15%	-	-
MC	90%	100%	91%	87%	85%	-	89%	96%	93%	-	-

6.2.5. Regulation of the BLIMP1 isoforms by LMP1

Finally, I explored if LMP1 could also regulate the expression of the BLIMP1β isoform in GC B cells. To do this, I transfected GC B cells with LMP1 as described previously (page 120). Figure 6.7 shows that even though the levels of BLIMP1β are very low in normal GC B cells, they were further decreased following LMP1 expression.

I next studied if LMP1 could regulate BLIMP1β in BL cell lines. To do this, I measured the levels of both BLIMP1α and BLIMP1β using RNA from the experiment described in chapter 5 (page 172) in which I had shown that LMP1 up-regulated BLIMP1 expression in BL2 and DG75 cells. Figure 6.8 shows that the mRNA levels of both BLIMP1α and BLIMP1β were increased following the expression of LMP1 in these BL cell lines. Consistent with this I showed that in luciferase reporter assays, the ectopic expression of LMP1 in BL2 cells increased the activity of both the BLIMP1α and BLIMP1β promoters.

Figure 6.7:. LMP1 down-regulates BLIMP1α and BLIMP1β isoforms in GC B cells. qRT-PCR analysis of the relative quantity of BLIMP1β in GC B cells isolated from two separate donors.

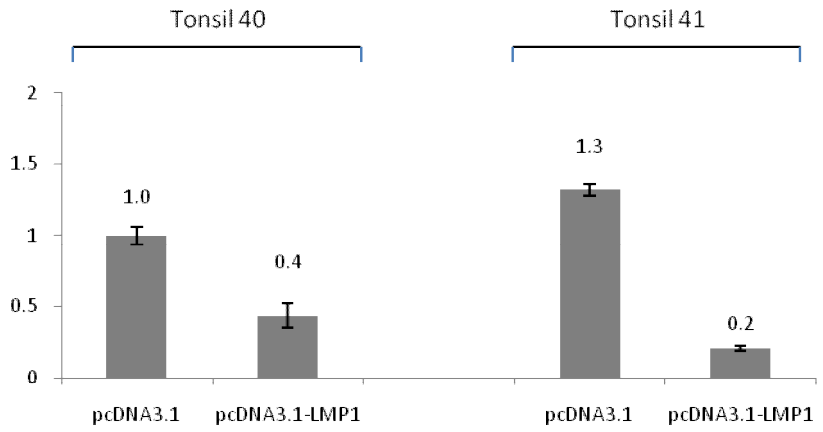
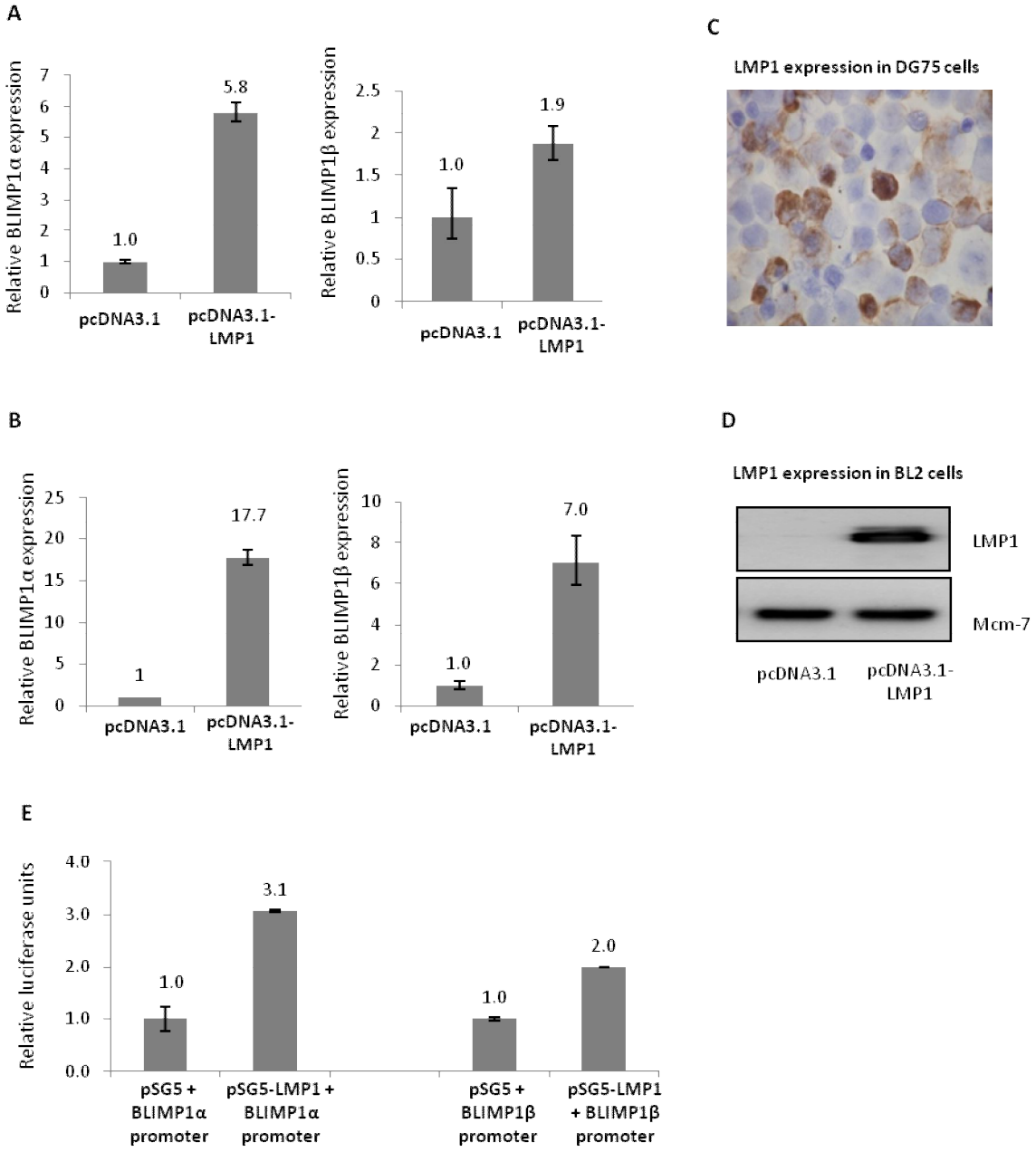


Figure 6.8: LMP1 up-regulates expression of BLIMP1α and BLIMP1β isoforms in BL cell lines. qRT-PCR analysis of DG75 (A) or BL2 (B) cells following transfection with LMP1 or control vector. LMP1 up-regulated transcriptional levels of both BLIMP1α and BLIMP1β isoforms. The ectopic expression of LMP1 in BL cell lines was confirmed by immunohistochemistry (C, DG75 cells) or by western blotting (D, BL2 cells). E) Luciferase assays were carried out to determine the effect of LMP1 on BLIMP1α or BLIMP1β promoter activity in BL2 cells. LMP1 expression activated both BLIMP1α or BLIMP1β promoter-mediated luciferase expression in BL2 cells. These results are representative of three independent experiments.



6.3. Discussion

I have shown here that the BLIMP1β isoform is up-regulated in EBV-transformed GC B cells and in HL cell lines. This could be important because previous studies have suggested that BLIMP1β may function to counteract the transcriptional repressive activities of the BLIMP1α isoform.

Although a dominant negative function for BLIMP1β is not yet firmly established, it is known that other truncated PRDM family members over-expressed in cancer cells can act as inhibitors of their respective full length isoform. For example, the effects of MDS1-EVI1, a PR domain containing form of the MDS1-EVI1 gene, can be overcome by over-expressing EVI1, the PR lacking isoform (Soderholm *et al.,* 1997). Furthermore, EVI1 has been shown to repress TGF-β signalling through interaction with Smad3, while MDS1-EVI1 augments the response to the growth inhibitory effect of TGF-β (Kurokawa *et al.,* 1998a; Kurokawa *et al.,* 1998b; Nucifora, 1997; Sood *et al.,* 1999). Moreover, the truncated form of the PRDM2 gene (RIZ2) which lacks the PR domain has been shown to inhibit transactivation activity of the oestrogen receptor by RIZ1, the full length product of PRDM2 (Steele-Perkins *et al.,* 2001).

For two main reasons the significance of the increased levels of BLIMP1β transcripts observed in both EBV transformed GC B cells and in HL cells remains unclear. First, although elevated, the levels of BLIMP1β mRNA still remained very low in the EBV transformed GC B cells and HL cell lines (for example they remained substantially lower than those observed in the U266 multiple myeloma cell line). Second, I have not studied BLIMP1β protein expression either in the EBV transformed GC B cells or in the HL cell

lines, in part due to the lack of suitable antibody reagents which can differentiate between the different BLIMP1 isoforms. Furthermore, previous studies have shown that BLIMP1β protein expression is virtually undetectable in DLBCL cell lines in which BLIMP1β mRNA levels are elevated (Tam *et al.,* 2008). It has also been shown that despite the relatively high levels of BLIMP1β mRNA in U266 cells, BLIMP1β protein is only weakly expressed in this cell line, suggesting that PRDM1β is not translated efficiently (Tam *et al.,* 2008). It should also be noted that the PRDM1 gene is subject to regulation by microRNAs which might further account for the discrepancy observed between the RNA and protein levels of BLIMP1 (Nie *et al.,*2008).

Transfection of GC B cells with LMP1 down-regulated BLIMP1β expression suggesting that another EBV gene(s) mediates the up-regulation of BLIMP1β observed in the EBV infected GC B cells. An alternative explanation for the up-regulation of BLIMP1β in the GC-derived LCLs is that this isoform is induced in proliferating cells. To investigate this possibility it will be necessary to study the expression of BLIMP1β in B cells which are induced into cell cycle by other stimuli for example by the addition of CD40 ligand and IL-4.

I observed that the increased expression of BLIMP1β in HL cell lines was accompanied by hypomethylation of the BLIMP1β-specific promoter. Previous work from our laboratory has shown that the BLIMP1β promoter is hypomethylated in the EBV-transformed GC B cells used in this study. Furthermore, it has also been shown that in DLBCL, the increased BLIMP1β mRNA levels are accompanied by hypomethylation of the BLIMP1β-specific promoter (Zhang *et al.,* 2010). The mechanism responsible for the hypomethylation of the BLIMP1β-specific promoter remains to be established but might involve the down-

regulation of the DNA methyltransferases, DNMT3B and DNMT1 which is observed in both EBV-infected GC B cells and in HL cell lines (Leonard *et al.,* unpublished data).

In experiments not presented in this thesis and performed in collaboration with Prof. Qian Tao (Chinese University of Hong Kong), the methylation status of the BLIMP1β-specific promoter has been studied in microdissected primary HRS cells isolated from seven cases of HL. We observed that the BLIMP1β promoter was completely unmethylated in three cases and only weakly methylated in a further three, while both strong methylated and unmethylated bands on MSP analysis were detected in normal GC B cells micro-dissected from reactive lymph nodes. These data show that hypomethylation of the BLIMP1β-specific promoter also occurs in primary HRS cells.

CHAPTER 7

FUTURE WORK

FUTURE WORK

Work presented in this thesis has suggested a number of areas for future study.

Although I have shown that the ectopic expression of BLIMP1α induces the virus lytic cycle in B cells, the mechanism responsible for this effect has yet to be established. In preliminary work not presented in this thesis, I have shown that the ectopic expression of BLIMP1α in GC-derived LCL, Akata BL cells and B95.8 cells increases XBP1 transcription. This could be important because XBP1 has been shown to bind to the BZLF1 promoter and induce its expression. To establish if this mechanism is involved in the induction of the viral lytic cycle by BLIMP1α it will be first necessary to show that BLIMP1α expression is followed by binding of XBP1 to the BZLF1 promoter.

Establishing that the induction of the virus lytic cycle by BLIMP1α is a consequence of plasma cell differentiation will be an important objective for future studies. In certain cellular environments, BLIMP1α can induce apoptosis. Therefore, an alternative explanation for my results is that the induction of the virus lytic cycle I observed occurs in response to this apoptosis. However, to determine if BLIMP1α -mediated viral replication is dependent upon plasma cell differentiation is challenging. This is because plasma cell differentiation takes several days to complete and because it is not possible *in vitro* to fully recapitulate the complexity of the signals received by a differentiating B cell *in vivo*. One approach might be to generate EBV-infected cell lines stably transduced with an inducible BLIMP1α vector or alternatively to attempt to induce plasma cell differentiation in EBV infected cells following their in vitro stimulation with cytokines such as IL-2 and IL-10. A further complication of these studies is that many of the in vitro models of plasma

cell differentiation use transformed B cells which might not accurately mirror the processes that occur during normal B cell differentiation.

During the course of this investigation I was able to confirm previous reports of low levels of BLIMP1α in HL and BL cells, suggesting that the loss of BLIMP1α expression contributes to a differentiation block in these lymphomas. Previous studies have shown that the ectopic expression of BLIMP1 in BL cells can induce many of the transcriptional changes characteristic of plasma cells even in a background of high C-MYC levels. However, it is not known if the ectopic expression of BLIMP1α can induce plasma cell differentiation in HL cells. This will be an important objective of future studies.

Although my observations show that BLIMP1α can regulate certain aspects of the virus life cycle. However, it is not known if BLIMP1α has a role in EBV-induced B cell immortalization. In preliminary data not presented in this thesis, I have shown that the infection of peripheral blood B cells with EBV is followed in the first few days of infection by the transient up-regulation of BLIMP1α mRNA which is followed soon after by its down-regulation. It is tempting to speculate that the up-regulation of BLIMP1α in newly infected B cells might contribute to the induction of BZLF1 expression and the virus replication which has shown to be important for efficient EBV-induced transformation. The use of recombinant viruses lacking the ability to express individual latent genes could be used to determine which genes regulate BLIMP1α expression in the early phases of B cell infection.

Finally, the role of the BLIMP1β isoform in normal B cell differentiation and its contribution to B cell lymphomagenesis remain enigmatic. In part, this is a consequence

of the lack of suitable antibody reagents which can detect the BLIMP1β protein. The generation of a monoclonal antibody which can recognize the region encompassing the three unique amino acids present in BLIMP1β will be required before meaningful studies of its expression in primary tumours can be undertaken. Only when this has been obtained can studies proceed to investigate the potential oncogenic activities of BLIMP1β in B cells.

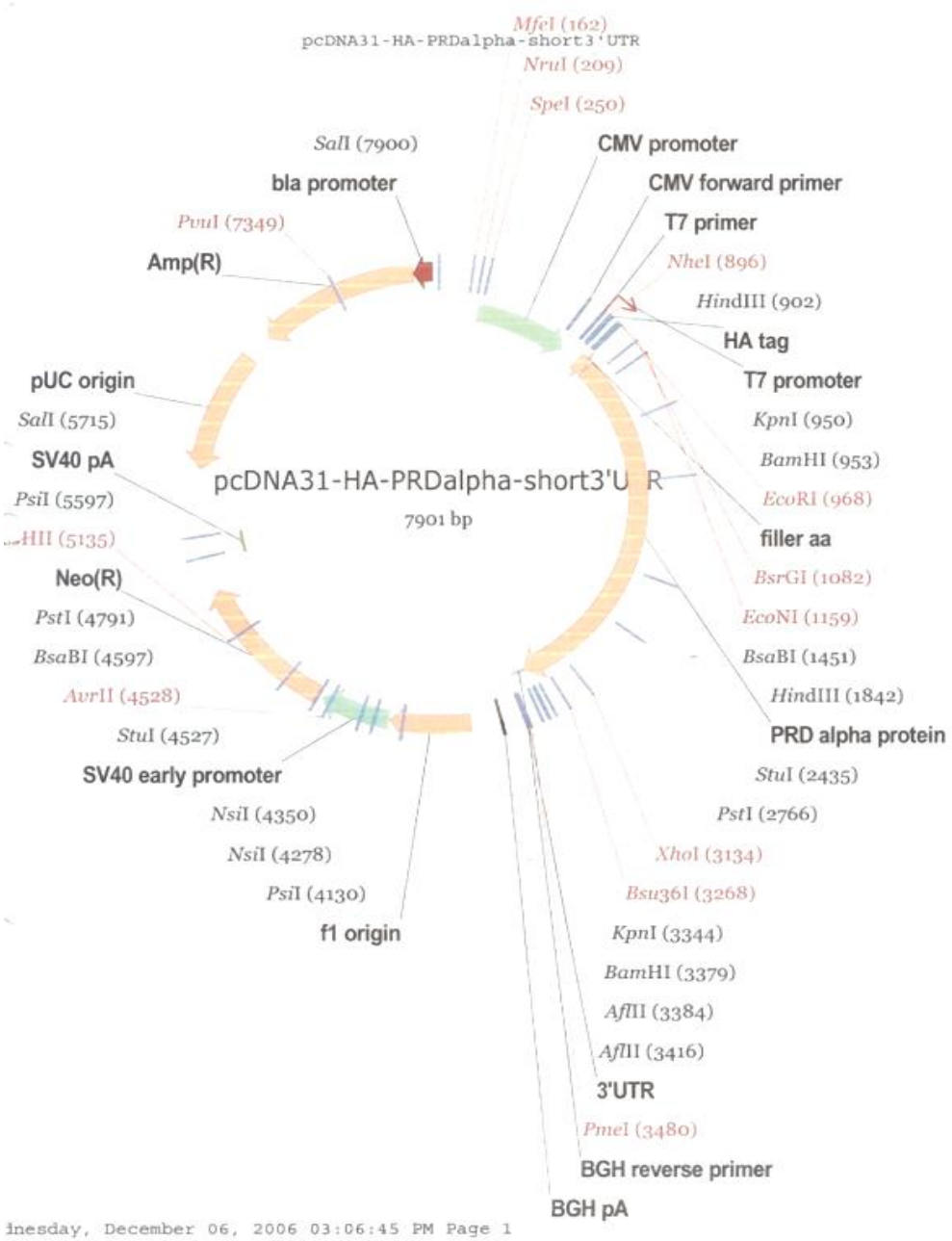
CHAPTER 8

APPENDICES

Appendix 1: Plasmids used in the study

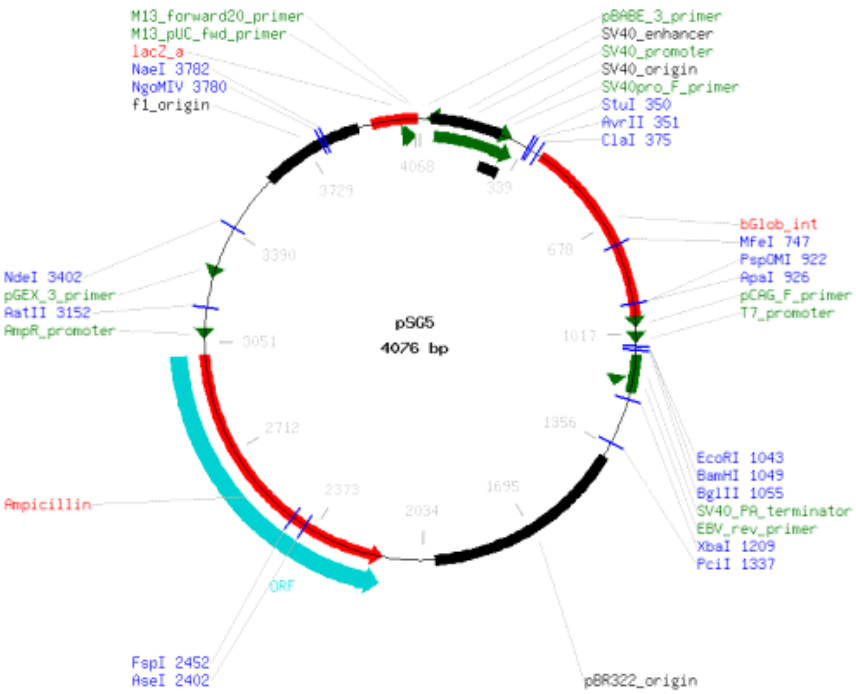
The ***pcDNA3.1-PRDM1α/BLIMP1α plasmid*** was kindly provided by Dr. Kenneth L. Wright (University of South Florida, Tampa, FL) and its map is displayed.

Map of PRDM1α/BLIMP1α plasmid

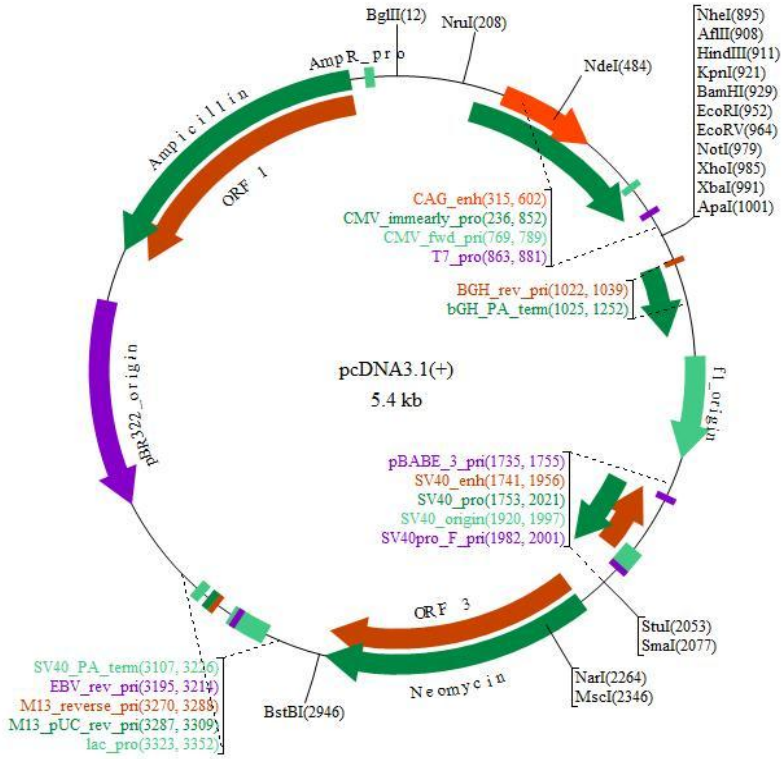


The ***pcDNA3.1 and pcDNA3.1-LMP1 plasmids*** were kindly provided by Dr. Georgia Kapatai (University of Birmingham, UK). The ***pSG5 and pSG5-LMP1 plasmids*** were kindly provided by Prof. Elliott D. Kieff (Brigham and Women's Hospital and Harvard Medical School, Boston, MA). I began working with pSG5 (map below) as a vector to express LMP1 and then switched to use pcDNA3.1 (map below) to express LMP1 or BLIMP1α in GC B cells. Using the same plasmid to express these genes ensured that differences in gene expression I observed after either transient transfection with LMP1 or BLIMP1α were not due to a plasmid effect.

Map of pSG5 plasmid



Map of pcDNA3.1plasmid

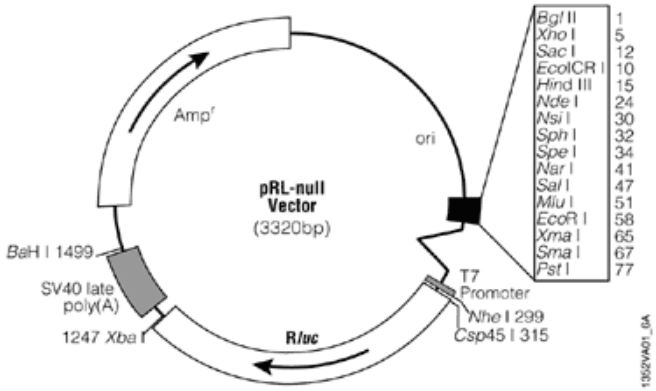


The ***pcDNA3.1-C-MYC plasmid*** was kindly provided by Prof. Georg W. Bornkamm (GSF-Institut fur Klinische Molekularbiologie und Tumorgenetik GSF-Forschungszentrum fur Umwelt und Gesundheit, Munich, Germany).

PRDM1α/BLIMP1α and PRDM1β/BLIMP1β promoter constructs were kindly provided by Dr. Kenneth L. Wright (University of South Florida, Tampa, FL) and were described previously (György *et al.,* 2003).

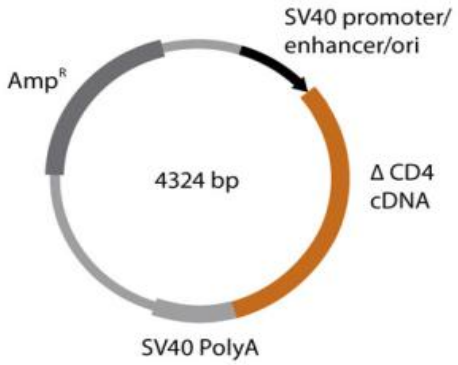
The ***pRL-null Vector*** (map bellow) was purchased from Promega UK Ltd. (Hampshire, UK). This vector contains cDNA encoding Renilla luciferase (Rluc) cloned from the anthozoan coelenterate Renilla reniformis (sea pansy).

Map of pRL-null Vector



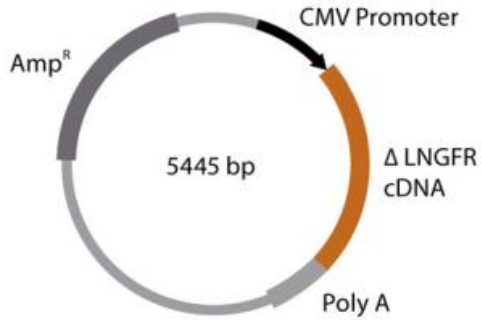
The ***pMACS CD4.1 vector*** (map bellow) uses the truncated human CD4 molecule which can be used in virtually all CD4-negative cell lines and primary cells as a marker to select transfected cells.

Map of pMACS CD4.1 vector



The ***pMACS LNGFR vector*** (map bellow) uses the truncated human low-affinity nerve growth factor receptor (LNGFR) molecule as a marker to select transfected cells. It can be used in LNGFR-negative cell lines and primary cells. The LNGFR molecule is expressed in the central and peripheral nervous system, on bone marrow fibroblasts, follicular dendritic cells, and some mesenchymal cells.

Map of LNGFR vector



Appendix 2: RT-PCR primers used in the study

Gene name	Forward Primer	Reverse Primer
GAPDH	5’-GGTGAAGGTCGGAGTCAACGGA-3’	5’-GAGGGATCTCGCTCCTGGAAGA-3’
LMP1	5’-AATTTGCACGGACAGGCATT-3’	5’-AAGGCCAAAAGCTGCCAGAT-3’

Appendix 3: qRT-PCR primers and probes used in the study

Gene name	Forward Primer	Reverse Primer
BLIMP1β	5’-CCGAACATGAAAAGACGATAAAACTGA-3’	5’-CCGTCAATGAAGTGGTGAAGCT-3’
LMP1	5’-AATTTGCACGGACAGGCATT-3’	5’-AAGGCCAAAAGCTGCCAGAT-3’
BZLF1	5’-ACGACGCACACGGAAACC-3’	5’-CTTGGCCCGGCATTTTCT-3’
BRLF1	5’-TTGGGCCATTCTCCGAAAC-3’	5’-TATAGGGCACGCGATGGAA-3’
BMLF1	5’-CCCGAACTAGCAGCATTTCCT-3’	5’-GACCGCTTCGAGTTCCAGAA-3’
BLNF2a	5’-TGGAGCGTGCTTTGCTAGAG-3’	5’-GGCCTGGTCTCCGTAGAAGAG-3’
BALF4	5’-CCAGCTTTCCTTTCCGAGTCT-3’	5’-ACACTGGATGTCCGAGGAGAA-3’

Gene name	Probe
BLIMP1β	5’-CTCTGGAATAGATCTTTTC-3’
LMP1	5’-TCCAGATACCTAAGACAAGTAAGCACCCGAAGAT-3’
BZLF1	5’-GCATTCCTCCAGCGATTCTGGCTGTT-3’
BRLF1	5’-AGACGGGCTGAGAATGCCGGC-3’
BMLF1	5’-AACGAGGATCCCGCAGAGAGCCA-3’
BLNF2a	5’-CCTCTGCCTGCGGCCTGCC-3’
BALF4	5’-TCCAGCCACGGCGACCTGTTC-3’

Appendix 4: Applied Biosystem Gene expression assays used in the study

Gene name	Gene expression assay number
BCL2A1	Hs00187845_m1
BCL6	Hs00277037_m1
CD20	Hs01585412_m1
CCL22	Hs99999075_m1
CIITA	Hs00172106_m1
RFX5	Hs00230841_m1
BLIMP1 (PRDM1) total	Hs00153357_m1
BLIMP1 (PRDM1) α isoform	Hs01068508_m1
IRF4	Hs01056534_m1
C-MYC	Hs99999003_m1
GAPDH	4310884E
β2m	4310886E

Appendix 5: Western blotting gels

Stacking gel (10%)	mL	**Separating gel (10%)**	mL
30% Polyacrylamide	0.83	30% Polyacrylamide	6.7
1M Tris(pH 6.8)	0.63	1.5M Tris(pH 8.8)	5
10% Ammonium persulfate	0.05	10% Ammonium persulfate	0.2
10% SDS	0.05	10% SDS	0.2
TEMED	0.005	TEMED	0.008
H_2O	3.4	H_2O	7.9
Total volume	5	Total volume	25

Appendix 6: Primary and secondary antibodies used in the study

Antigen	Species (clone)	Company & cat. no	Dilution
BLIMP1 (recognizes BLIMP1α isoform only)	Rabbit polyclonal (R21)	King gift of Dr.Reuben Tooze (St James's University Hospital, Leeds, UK)	1:1000
BLIMP1 (recognizes both BLIMP1α and BLIMP1β isoforms)	Mouse monoclonal	Kind gift of Prof. Teresa Marafioti (Oxford, UK)	neat
LMP1	Mouse monoclonal (CS1-4)	Dako	1:1000
C-MYC	Mouse monoclonal (9E10)	Santa Cruz Biotechnology, ING.	1:500
BZLF1	Mouse monoclonal (BZ-1) (culture supernatant)	University of Birmingham (Birmingham, UK)	1:50
VCA	Mouse monoclonal (L2)	Kind gift of Dr. Claire-Shannon Lowe (Birmingham, UK)	1:1000
gp350	Mouse hybridoma (72a.1)	Kind gift of Dr. Claire-Shannon Lowe (Birmingham, UK)	1:500
HA	Rabbit polyclonal (Y-11)	Santa Cruz Biotechnology (sc-805)	1:50
mcm-7	Mouse (M7931)	Sigma-Aldrich	1:2000
α-tubulin	Mouse	Sigma-Aldrich	1:1000
B-actin	Mouse monoclonal (c-2)	Santa Cruz (Sc-8432)	1:1000
Mouse IgG	Goat polyclonal (HRP)	Dako (P0447)	1:3000
Rabbit IgG	Goat polyclonal (HRP)	Dako (P0448)	1:1000
IgG antibody	Rabbit polyclonal	Insight Biotechnology Ltd	ChIP

BLIMP1 R21 is a purified rabbit IgG polyclonal antibody raised against GST fusion protein of full-length of human BLIMP1. This antibody was used in ChiP.

Appendix 7: Conditions used to electroporate individual cell lines

Cell line	°C	Washing media 1	Washing media 2	Electroporation media and volume	Pulse	Cultivation media
BL2	4 °C	RPM1 10%FCS 4°C	RPMI/10%FCS /10mM HEPES, 4°C	RPMI/10%FCS/25mM HEPES, 4°C, 250µl	250V 950µF	RPMI 10% FCS
Akata	RT	PBS RT	OptiMEM RT	OptiMEM, RT, 300µl	230V 975µF	RPMI 10% FCS
L428	4°C	RPM1 10%FCS 4°C	RPMI/10%FCS /10mM HEPES, 4°C	RPMI/10%FCS/25mM HEPES, 4°C, 500µl	250V 1350µF	RPMI 10% FCS
L591	4°C	RPM1 10%FCS 4°C	RPMI/10%FCS /10mM HEPES, 4°C	RPMI/10%FCS/25mM HEPES, 4°C, 500µl	250V 975µF	RPMI 10% FCS
OKU-LCL	RT	PBS RT	OptiMEM RT	OptiMEM, RT, 300µl	230V 975µF	RPMI 10% FCS
SAL-LCL	RT	PBS RT	OptiMEM RT	OptiMEM RT, 300µl	230V 975µF	RPMI 20% FCS
B95.8	RT	PBS RT	OptiMEM RT	OptiMEM RT, 300µl	230V 975µF	RPMI 20% FCS
SL1-LCL **SL3-LCL**	RT	PBS RT	OptiMEM RT	OptiMEM RT, 300µl	230V 975µF	RPMI 20% FCS
HK-LCL	RT	PBS RT	OptiMEM RT	OptiMEM RT, 300µl	230V 975µF	RPMI 20% FCS
PER213	RT	PBS RT	OptiMEM RT	OptiMEM RT, 300µl	230V 975µF	RPMI 20% FCS

OptiMEM® is a serum-free media which was purchased from Invitrogen Ltd., Paisley, UK.

RPMI/10%FCS/10mM HEPES contains RPMI 1640 (Sigma-Aldrich Ltd., Gillingham, UK) supplemented with 10% fetal calf serum, 2mM L-glutamine (all Invitrogen Ltd., Paisley, UK), 1% penicillin-streptomycin solution (Sigma-Aldrich Ltd., Gillingham, UK) and 25 mM HEPES (Sigma-Aldrich Ltd., Gillingham, UK).

RPMI/10%FCS/25mM HEPES contains RPMI 1640 (Sigma-Aldrich Ltd., Gillingham, UK) supplemented with 10% fetal calf serum, 2mM L-glutamine (all Invitrogen Ltd., Paisley, UK), 1% penicillin-streptomycin solution (Sigma-Aldrich Ltd., Gillingham, UK) and 25 mM HEPES (Sigma-Aldrich Ltd., Gillingham, UK).

Appendix 8: Primer sequences used in ChIP

primer	sequence
BZLF1 forward (region 1)	5'-TTGTGGTCAGTTCGTCCAAA-3'
BZLF1 reverse (region 1)	5'-GTCAGCCAAAGAGGATCAGG-3'
BZLF1 forward (region 2)	5'-GAGACTGGGAACAGCTGAGG-3'
BZLF1 reverse (region 2)	5'-GCCACCTTTGCTATCTTGG-3'
BZLF1 forward (region 3)	5'-GAAGCCACCCGATTCTTGTA-3'
BZLF1 reverse (region 3)	5'-TCCCAGTCTCCGAGATAACC-3'
Cp forward	5'-AAATGTTGGAGGGACCTAAGAGATG-3'
Cp reverse	5'-TGGCTTTAATTGTCATGTATGCTT-3'

All BZLF1 and Cp primers were designed using Primer 3 program. The BZLF1 primers cover the whole BZLF1 gene including a promoter. Cp primers cover an area within the Cp gene, upstream of the Cp promoter. The viral sequences were obtained from ENSMBL. The primers were kindly provided by Dr. Sarah Leonard (University of Birmingham, UK).

Appendix 9: Primer sequences used in pyrosequencing analysis

primer	sequence
PCR forward biotinylated primer (region 1)	5'-TAGGTTTGGTTAGTGA-3'
PCR reverse non biotinylated primer (region 1)	5'-CACTTTTATCTTTCCA-3'
Sequencing primer (region 1)	5'-TTTTATCAATTTTTCC-3'
PCR forward non biotinylated primer (region 2)	5'-GGTGGAGGATAGTTGA-3'
PCR reverse biotinylated primer (region 2)	5'-AAATAAACCAAATTCC-3'
Sequencing primer (region 2)	5'-TGTATAGTTGTTTGGG-3'

The BLIMP1β primers were designed for pyrosequencing using Biotage primer design software, this software in silico bisulphite converts the sequence so all primers (FWD, REV and SEQ) were then designed on the bisulphite modified DNA. Sequences were obtained from UCSC. The primers were kindly provided by Dr. Sarah Leonard (University of Birmingham, UK).

Appendix 10: List of genes up-regulated by BLIMP1α and LMP1 in GC B cells

	BLIMP1	LMP1	full name
LGALS1	increased	increased	Blimp1 target Shaffer,lectin, galactoside-binding, soluble, 1 (galectin 1)
RTN4	increased	increased	reticulon 4
EIF1	increased	increased	eukaryotic translation initiation factor 1
LAPTM4B	increased	increased	lysosomal associated protein transmembrane 4 beta
NSUN6	increased	increased	NOL1/NOP2/Sun domain family, member 6
DGAT2	increased	increased	diacylglycerol O-acyltransferase homolog 2
SLC7A11	increased	increased	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11
AKR1A1	increased	increased	aldo-keto reductase family 1, member A1 (aldehyde reductase)
BRI3 (I3)	increased	increased	brain protein I3
C6orf145	increased	increased	chromosome 6 open reading frame 145
CRIP1	increased	increased	cysteine-rich protein 1 (intestinal)
DNAJB1 (HSP40)	increased	increased	DnaJ (Hsp40) homolog, subfamily B, member 1
EIF4A1	increased	increased	eukaryotic translation initiation factor 4A, isoform 1
GADD45B	increased	increased	growth arrest and DNA-damage-inducible, beta
GALNT2	increased	increased	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2
HSPA1A	increased	increased	heat shock 70kDa protein 1A
HSPA9	increased	increased	heat shock 70kDa protein 9 (mortalin)
ID2 /// ID2B	increased	increased	inhibitor of DNA binding 2
ING3	increased	increased	inhibitor of growth family, member 3
KLHL21	increased	increased	kelch-like 21
PDXK	increased	increased	pyridoxal (pyridoxine, vitamin B6) kinase
PHACTR1	increased	increased	phosphatase and actin regulator 1
PPIF (cyclophilin E)	increased	increased	peptidylprolyl isomerase F (cyclophilin F)
PPP1R10	increased	increased	protein phosphatase 1, regulatory (inhibitor) subunit 10
PPP1R15A (GAD34)	increased	increased	protein phosphatase 1, regulatory (inhibitor) subunit 15A
PRO1073	increased	increased	PRO1073 protein
PTCD3	increased	increased	Pentatricopeptide repeat domain 3
QKI	increased	increased	quaking homolog, KH domain RNA binding
RPL10A	increased	increased	ribosomal protein L10a
SFRS8	increased	increased	splicing factor, arginine/serine-rich 8
SQSTM1 (p60,p62)	increased	increased	sequestosome 1
TCP1	increased	increased	chaperonin containing TCP1, subunit 6A (zeta 1)
TRIM73	increased	increased	tripartite motif-containing 73
ZFAND2A (AIRAP)	increased	increased	zinc finger, AN1-type domain 2A
ZNF260	increased	increased	zinc finger protein 260
ZNF420	increased	increased	zinc finger protein 420

Appendix 11: List of genes differentially regulated by BLIMP1α and LMP1 in GC B cells

	BLIMP1	LMP1	full name
IGHM (MU)	increased	decreased	immunoglobulin heavy constant mu
PTPN7	increased	decreased	protein tyrosine phosphatase, non-receptor type 7
C3orf37	increased	decreased	chromosome 3 open reading frame 37
FLJ20186	increased	decreased	hypothetical protein FLJ20186
RASSF6	increased	decreased	Ras association (RalGDS/AF-6) domain family 6
SAT1	increased	decreased	spermidine/spermine N1-acetyltransferase 1
ZBP1	increased	decreased	Z-DNA binding protein 1
CCDC88A	increased	decreased	coiled-coil domain containing 88A
CENTD2 (ARA	increased	decreased	centaurin, delta 2
EHBP1L1	increased	decreased	EH domain binding protein 1-like 1
GAPDH	increased	decreased	
IGHA1 (IgA)	increased	decreased	immunoglobulin heavy constant alpha 1
KIAA1618	increased	decreased	
KIAA1833	increased	decreased	hypothetical protein KIAA1833
LONP2	increased	decreased	lon peptidase 2, peroxisomal
MGC29506	increased	decreased	
MTG1 (GTP)	increased	decreased	mitochondrial GTPase 1 homolog (S. cerevisiae)
MYO9B	increased	decreased	myosin IXB
NLRP1	increased	decreased	NLR family, pyrin domain containing 1
OASL	increased	decreased	2'-5'-oligoadenylate synthetase-like
PCGF3 (RNF3	increased	decreased	polycomb group ring finger 3
PDCD4	increased	decreased	programmed cell death 4 (neoplastic transformation inhibitor)
PRIC285	increased	decreased	peroxisomal proliferator-activated receptor A interacting complex 285
PTPRS	increased	decreased	protein tyrosine phosphatase, receptor type, S
SLC44A2	increased	decreased	solute carrier family 44, member 2
SOX4	increased	decreased	SRY (sex determining region Y)-box 4
TMEM142B	increased	decreased	transmembrane protein 142B
WDR61	increased	decreased	WD repeat domain 61
C4orf34	decreased	increased	chromosome 4 open reading frame 34
COCH	decreased	increased	coagulation factor C homolog, cochlin (Limulus polyphemus)
LACTB	decreased	increased	lactamase, beta
ALCAM	decreased	increased	activated leukocyte cell adhesion molecule
ANKRD10	decreased	increased	ankyrin repeat domain 10
BCAT1	decreased	increased	branched chain aminotransferase 1, cytosolic
BCL2A1	decreased	increased	BCL2-related protein A1
CCL22	decreased	increased	chemokine (C-C motif) ligand 22
CD80	decreased	increased	CD80 molecule
CD86	decreased	increased	CD86 molecule
CEP135	decreased	increased	centrosomal protein 135kDa
CIITA	decreased	increased	class II, major histocompatibility complex, transactivator
DDEF1	decreased	increased	development and differentiation enhancing factor 1
FAM49A	decreased	increased	family with sequence similarity 49, member A
FNBP1	decreased	increased	formin binding protein 1
HECTD2	decreased	increased	HECT domain containing 2
IRF2BP2	decreased	increased	interferon regulatory factor 2 binding protein 2
KSR1	decreased	increased	kinase suppressor of ras 1
KYNU	decreased	increased	kynureninase (L-kynurenine hydrolase)
MCOLN2	decreased	increased	mucolipin 2
NAP1L1	decreased	increased	nucleosome assembly protein 1-like 1
NDE1	decreased	increased	nudE nuclear distribution gene E homolog 1 (A. nidulans)
PLAGL1 (ZAC,	decreased	increased	pleiomorphic adenoma gene-like 1
PRRG4	decreased	increased	proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)
PTGER4	decreased	increased	prostaglandin E receptor 4 (subtype EP4)
RFX5	decreased	increased	regulatory factor X, 5 (influences HLA class II expression)
RUFY3	decreased	increased	RUN and FYVE domain containing 3
TFDP1 (DP1)	decreased	increased	transcription factor Dp-1
TJP2	decreased	increased	tight junction protein 2 (zona occludens 2)

Appendix 12: List of genes down-regulated by BLIMP1α and LMP1 in GC B cells

	BLIMP1	LMP1	full name
ALOX5	decreased	decreased	arachidonate 5-lipoxygenase
ATP8A1	decreased	decreased	ATPase, aminophospholipid transporter (APLT), Class I, type 8A, member 1
BCL11A	decreased	decreased	B-cell CLL/lymphoma 11A (zinc finger protein)
ITPR2 (IP3R2	decreased	decreased	inositol 1,4,5-triphosphate receptor, type 2
LPP	decreased	decreased	LIM domain containing preferred translocation partner in lipoma
LRMP (JAW1	decreased	decreased	lymphoid-restricted membrane protein
MEF2C	decreased	decreased	myocyte enhancer factor 2C
MS4A1 (CD2(	decreased	decreased	membrane-spanning 4-domains, subfamily A, member 1 CD20
PRKCB1	decreased	decreased	protein kinase C, beta 1
STX7	decreased	decreased	syntaxin 7
SYPL1	decreased	decreased	synaptophysin-like 1
VNN2	decreased	decreased	vanin 2
ZNF85	decreased	decreased	zinc finger protein 85
CYP1B1	decreased	decreased	cytochrome P450, family 1, subfamily B, polypeptide 1
MAD2L1	decreased	decreased	MAD2 mitotic arrest deficient-like 1
PIGF	decreased	decreased	phosphatidylinositol glycan anchor biosynthesis, class F
SMARCA2	decreased	decreased	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2
B3GALNT1	decreased	decreased	beta-1,3-N-acetylgalactosaminyltransferase 1
BCL6	decreased	decreased	B-cell CLL/lymphoma 6 (zinc finger protein 51)
BRWD1	decreased	decreased	bromodomain and WD repeat domain containing 1
CASC5	decreased	decreased	cancer susceptibility candidate 5
DCK	decreased	decreased	deoxycytidine kinase
DHRS9	decreased	decreased	dehydrogenase/reductase (SDR family) member 9
DNAJC10	decreased	decreased	DnaJ (Hsp40) homolog, subfamily C, member 10
ELL3	decreased	decreased	elongation factor RNA polymerase II-like 3
ETS1	decreased	decreased	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)
FCRL1	decreased	decreased	Fc receptor-like 1
FCRL2	decreased	decreased	Fc receptor-like 2
HS2ST1	decreased	decreased	heparan sulfate 2-O-sulfotransferase 1
KLHL5	decreased	decreased	kelch-like 5 (Drosophila)
KLHL6	decreased	decreased	kelch-like 6 (Drosophila)
MAP4K4	decreased	decreased	mitogen-activated protein kinase kinase kinase kinase 4
MCTP2	decreased	decreased	multiple C2 domains, transmembrane 2
NAPSB	decreased	decreased	napsin B aspartic peptidase pseudogene
NCOA3 (TRAN	decreased	decreased	nuclear receptor coactivator 3
ANXA4	decreased	decreased	annexin A4
STAT1	decreased	decreased	signal transducer and activator of transcription 1, 91kDa
ACAA2	decreased	decreased	acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)
AKAP2	decreased	decreased	PALM2-AKAP2 protein
AMIGO2	decreased	decreased	adhesion molecule with Ig-like domain 2
ANAPC4	decreased	decreased	anaphase promoting complex subunit 4
AP2B1	decreased	decreased	adaptor-related protein complex 2, beta 1 subunit
ARMC1	decreased	decreased	armadillo repeat containing 1
ATAD2	decreased	decreased	ATPase family, AAA domain containing 2
BICD1	decreased	decreased	bicaudal D homolog 1 (Drosophila)
BMP7	decreased	decreased	bone morphogenetic protein 7
BMPR2	decreased	decreased	bone morphogenetic protein receptor, type II (serine/threonine kinase)
BPTF	decreased	decreased	bromodomain PHD finger transcription factor
C14orf142	decreased	decreased	chromosome 14 open reading frame 142
C14orf143	decreased	decreased	chromosome 14 open reading frame 143
C14orf145	decreased	decreased	chromosome 14 open reading frame 145
C15orf41	decreased	decreased	chromosome 15 open reading frame 41
C1orf80	decreased	decreased	chromosome 1 open reading frame 80
C20orf19	decreased	decreased	chromosome 20 open reading frame 19
C5orf33	decreased	decreased	chromosome 5 open reading frame 33
C9orf64	decreased	decreased	chromosome 9 open reading frame 64
CBX1	decreased	decreased	chromobox homolog 1 (HP1 beta homolog Drosophila)

	BLIMP1	LMP1	full name
CCDC109B	decreased	decreased	coiled-coil domain containing 109B
CCDC128	decreased	decreased	coiled-coil domain containing 128
CCDC131	decreased	decreased	coiled-coil domain containing 131
CCDC88A	decreased	decreased	coiled-coil domain containing 88A
CCDC98	decreased	decreased	coiled-coil domain containing 98
CCNG1 (cyclir	decreased	decreased	cyclin G1
CD164	decreased	decreased	CD164 molecule, sialomucin
CDC2 (CDK1)	decreased	decreased	cell division cycle 2, G1 to S and G2 to M
CDC7	decreased	decreased	cell division cycle 7 homolog (S. cerevisiae)
CDCA7	decreased	decreased	cell division cycle associated 7
CDV3	decreased	decreased	CDV3 homolog (mouse)
CLCC1	decreased	decreased	chloride channel CLIC-like 1
COMMD10	decreased	decreased	COMM domain containing 10
CSTF2	decreased	decreased	cleavage stimulation factor, 3' pre-RNA, subunit 2, 64kDa
DECR1	decreased	decreased	2,4-dienoyl CoA reductase 1, mitochondrial
DHFR	decreased	decreased	dihydrofolate reductase
DLD	decreased	decreased	dihydrolipoamide dehydrogenase
DMD	decreased	decreased	dystrophin
DOCK9	decreased	decreased	dedicator of cytokinesis 9
EBPL	decreased	decreased	emopamil binding protein-like
EGLN1	decreased	decreased	egl nine homolog 1 (C. elegans)
EIF4E2	decreased	decreased	eukaryotic translation initiation factor 4E family member 2
ENDOGL1	decreased	decreased	endonuclease G-like 1
ENPP5	decreased	decreased	ectonucleotide pyrophosphatase/phosphodiesterase 5 (putative function)
EXOC5	decreased	decreased	exocyst complex component 5
FAM129C	decreased	decreased	family with sequence similarity 129, member C
FAM135A	decreased	decreased	family with sequence similarity 135, member A
FAM72A	decreased	decreased	family with sequence similarity 72, member A
FAM82A	decreased	decreased	family with sequence similarity 82, member A
FAM91A2	decreased	decreased	family with sequence similarity 91, member A2
FBXO22	decreased	decreased	F-box protein 22
FBXO7	decreased	decreased	F-box protein 7
FCRLA	decreased	decreased	Fc receptor-like A
FLJ32312	decreased	decreased	coiled-coil domain containing 139
FLJ90709	decreased	decreased	hypothetical protein FLJ90709
FUNDC1	decreased	decreased	FUN14 domain containing 1
GALNT12	decreased	decreased	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12 (GalNAc-T12)
GMNN	decreased	decreased	geminin, DNA replication inhibitor
HAT1	decreased	decreased	histone acetyltransferase 1
HDAC4	decreased	decreased	histone deacetylase 4
HERC4	decreased	decreased	hect domain and RLD 4
HNRPD	decreased	decreased	heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)
HPS3	decreased	decreased	Hermansky-Pudlak syndrome 3
HSD17B6	decreased	decreased	hydroxysteroid (17-beta) dehydrogenase 6 homolog (mouse)
IDH1	decreased	decreased	isocitrate dehydrogenase 1 (NADP+), soluble
IFT80	decreased	decreased	intraflagellar transport 80 homolog (Chlamydomonas)
IGF2BP3	decreased	decreased	insulin-like growth factor 2 mRNA binding protein 3
IKZF1 (IKAROS	decreased	decreased	IKAROS family zinc finger 1 (Ikaros)
INPP5B	decreased	decreased	inositol polyphosphate-5-phosphatase, 75kDa
ITGB1 (CD29)	decreased	decreased	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
ITGB3BP	decreased	decreased	integrin beta 3 binding protein (beta3-endonexin)
ITPR1	decreased	decreased	inositol 1,4,5-triphosphate receptor, type 1
JAK2	decreased	decreased	Janus kinase 2 (a protein tyrosine kinase)
KIAA0672	decreased	decreased	Rho-type GTPase-activating protein RICH2
KIAA1815	decreased	decreased	endoplasmic reticulum metallopeptidase 1
KIAA1913	decreased	decreased	
KIF23	decreased	decreased	kinesin family member 23

	BLIMP1	LMP1	full name
KIF4A	decreased	decreased	kinesin family member 4A
KMO	decreased	decreased	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)
LAP3	decreased	decreased	leucine aminopeptidase 3
LMNB1	decreased	decreased	lamin B1
LOC116143	decreased	decreased	WD repeat domain 92
LOC220930	decreased	decreased	
LOC641845	decreased	decreased	
LOC731292 (	decreased	decreased	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)
LRBA	decreased	decreased	LPS-responsive vesicle trafficking, beach and anchor containing
LYN	decreased	decreased	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MAP4K5	decreased	decreased	mitogen-activated protein kinase kinase kinase kinase 5
MDM1	decreased	decreased	Mdm4, transformed 3T3 cell double minute 1, p53 binding protein (mouse)
MFAP3	decreased	decreased	microfibrillar-associated protein 3
MFN1	decreased	decreased	mitofusin 1
MIA3	decreased	decreased	
MKI67 (Ki67)	decreased	decreased	antigen identified by monoclonal antibody Ki-67
MNS1	decreased	decreased	meiosis-specific nuclear structural 1
MRE11A	decreased	decreased	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)
MSH2	decreased	decreased	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)
MUTED	decreased	decreased	muted homolog (mouse)
NCOA4	decreased	decreased	nuclear receptor coactivator 4
NFU1	decreased	decreased	NFU1 iron-sulfur cluster scaffold homolog (S. cerevisiae)
OCC-1	decreased	decreased	overexpressed in colon carcinoma-1
PARP15 (BAL	decreased	decreased	poly (ADP-ribose) polymerase family, member 15
PGM2	decreased	decreased	phosphoglucomutase 2
PHF14	decreased	decreased	PHD finger protein 14
PPP2R3C	decreased	decreased	protein phosphatase 2 (formerly 2A), regulatory subunit B", gamma
PPP2R5E	decreased	decreased	protein phosphatase 2, regulatory subunit B', epsilon isoform
PRKAR2B	decreased	decreased	protein kinase, cAMP-dependent, regulatory, type II, beta
PRUNE	decreased	decreased	prune homolog (Drosophila)
PTEN	decreased	decreased	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)
PTK2	decreased	decreased	PTK2 protein tyrosine kinase 2
RAB14	decreased	decreased	RAB14, member RAS oncogene family
RAB18	decreased	decreased	RAB18, member RAS oncogene family
RAC1	decreased	decreased	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
RANBP6	decreased	decreased	RAN binding protein 6
RASA1	decreased	decreased	RAS p21 protein activator (GTPase activating protein) 1
RBM35B	decreased	decreased	RNA binding motif protein 35B
RECQL5	decreased	decreased	RecQ protein-like 5
RFC1 (A1)	decreased	decreased	replication factor C (activator 1) 1, 145kDa
RGS16	decreased	decreased	regulator of G-protein signalling 16
RP2	decreased	decreased	retinitis pigmentosa 2 (X-linked recessive)
RRM1	decreased	decreased	ribonucleotide reductase M1 polypeptide
RTCD1	decreased	decreased	RNA terminal phosphate cyclase domain 1
SCP2	decreased	decreased	sterol carrier protein 2
SEC23IP	decreased	decreased	SEC23 interacting protein
SERPINB1	decreased	decreased	serpin peptidase inhibitor, clade B (ovalbumin), member 1
SETD2 (HIF-1)	decreased	decreased	SET domain containing 2
SETX	decreased	decreased	senataxin
SH3BGRL	decreased	decreased	SH3 domain binding glutamic acid-rich protein like
SKAP2	decreased	decreased	src kinase associated phosphoprotein 2
SLC7A6 (LAT3	decreased	decreased	solute carrier family 7 (cationic amino acid transporter, y+ system), member 6
SNTB2	decreased	decreased	syntrophin, beta 2 (dystrophin-associated protein A1, 59kDa, basic component 2)
SNX10	decreased	decreased	sorting nexin 10
SNX3	decreased	decreased	sorting nexin 3
SOAT1	decreased	decreased	sterol O-acyltransferase (acyl-Coenzyme A: cholesterol acyltransferase) 1
TBC1D5	decreased	decreased	TBC1 domain family, member 5

	BLIMP1	LMP1	full name
TCEAL8	decreased	decreased	transcription elongation factor A (SII)-like 8
TCF19	decreased	decreased	transcription factor 19 (SC1)
TCP11L1	decreased	decreased	t-complex 11 (mouse)-like 1
TMF1	decreased	decreased	TATA element modulatory factor 1
TNFSF10	decreased	decreased	tumor necrosis factor (ligand) superfamily, member 10
TOP2A (Apo-2	decreased	decreased	topoisomerase (DNA) II alpha 170kDa
TOPBP1	decreased	decreased	topoisomerase (DNA) II binding protein 1
TPK1	decreased	decreased	thiamin pyrophosphokinase 1
TRIM5	decreased	decreased	tripartite motif-containing 5
TRIOBP	decreased	decreased	TRIO and F-actin binding protein
TYMS	decreased	decreased	thymidylate synthetase
UBE1L2	decreased	decreased	ubiquitin-activating enzyme E1-like 2
UCHL5	decreased	decreased	ubiquitin carboxyl-terminal hydrolase L5
VTA1	decreased	decreased	Vps20-associated 1 homolog (S. cerevisiae)
WDHD1	decreased	decreased	WD repeat and HMG-box DNA binding protein 1
WDR32	decreased	decreased	WD repeat domain 32
WDR41	decreased	decreased	WD repeat domain 41
WIPF1	decreased	decreased	WAS/WASL interacting protein family, member 1
XRN1	decreased	decreased	5'-3' exoribonuclease 1
ZMYND8	decreased	decreased	zinc finger, MYND-type containing 8
ZNF397	decreased	decreased	zinc finger protein 397
ZNF404	decreased	decreased	zinc finger protein 404

CHAPTER 9

LIST OF REFERENCES

REFERENCES

Adler B, Schaadt E, Kempkes B, Zimber-Strobl U, Baier B, Bornkamm GW. (2002) Control of Epstein-Barr virus reactivation by activated CD40 and viral latent membrane protein 1. *Proc Natl Acad Sci USA* **99**:437-442.

Aldinucci D, Lorenzon D, Olivo K, Rapanà B, Gattei V. (2004) Interactions between tissue fibroblasts in lymph nodes and Hodgkin/Reed–Sternberg cells. *Leuk. Lymphoma* **45**:1731-1739.

Aldinucci D, Lorenzon D, Cattaruzza L, Pinto A, Gloghini A, Carbone A, Colombatti A. (2008) Expression of CCR5 receptors on Reed-Sternberg cells and Hodgkin lymphoma cell lines: involvement of CCL5/Rantes in tumor cell growth and microenvironmental interactions. *Int. J. Cancer* **122:**769-776.

Alfieri C, Birkenbach M, Kieff E. (1991) Early events in Epstein-Barr virus infection of human B lymphocytes. *Virology* **181**:595-608.

Allan GJ, Inman GJ, Parker BD, Rowe DT, Farrell PJ. (1992) Cell growth effects of Epstein-Barr virus leader protein. *J Gen Virol.* **73**:1547-1551.

Allday MJ, Crawford DH. (1988) Role of epithelium in EBV persistence and pathogenesis of B-cell tumours. *Lancet.* **1**:855-857.

Allday MJ, Farrell PJ. (1994) Epstein-Barr virus nuclear antigen EBNA3C/6 expression maintains the level of latent membrane protein 1 in G1-arrested cells. *J Virol.* **68**:3491-3498.

Allen CD, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, Cyster JG. (2004) Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunl.* **5**:943-952.

Allen CD, Okada T, Tang HL, Cyster JG. (2007) Imaging of germinal center selection events during affinity maturation. *Science* **31**:528-531.

Allen MD, Young LS, Dawson CW (2005). The Epstein-Barr virus-encoded LMP2A and LMP2B proteins promote epithelial cell spreading and motility. *J Virol* .**79**:1789-1802.

Altmann M, Hammerschmidt W. (2005) Epstein-Barr virus provides a new paradigm: a requirement for the immediate inhibition of apoptosis. *PLoS Biol.* **3**:e404.

Anagnostopoulos I, Herbst H, Niedobitek G, Stein H. (1989) Demonstration of monoclonal EBV genomes in Hodgkin's disease and Ki-1-positive anaplastic large cell lymphoma by combined Southern blot and in situ hybridization. *Blood* **74**:810-816.

Anagnostopoulos I, Hummel M, Kreschel C, Stein H. (1995) Morphology, immunophenotype, and distribution of latently and/or productively Epstein-Barr virus-infected cells in acute infectious mononucleosis: implications for the interindividual infection route of Epstein-Barr virus. *Blood* **85**:744-750.

Anastasiadou E, Vaeth S, Cuomo L, Boccellato F, Vincenti S, Cirone M, Presutti C, Junker S, Winberg G, Frati L, Wade PA, Faggioni A, Trivedi P. (2009) Epstein-Barr virus infection leads to partial phenotypic reversion of terminally differentiated malignant B cells. *Cancer Letters* **284**: 165-174.

Ancelin K, Lange UC, Hajkova P, Schneider R, Bannister AJ, Kouzarides T, Surani MA. (2006) Blimp1 associates with Prmt5 and directs histone arginine methylation in mouse germ cells. *Nat. Cell Biol.* **8**:623-630.

Anderton E, Yee J, Smith P, Crook T, White RE, Allday MJ. (2008) Two Epstein-Barr virus (EBV) oncoproteins cooperate to repress expression of the proapoptotic tumour-suppressor Bim: clues to the pathogenesis of Burkitt's lymphoma. *Oncogene* **27**:421-433.

Angelin-Duclos C, Cattoretti G, Lin KI, Calame K. (2000) Commitment of B lymphocytes to a plasma cell fate is associated with Blimp-1 expression in vivo. *J. Immunol.* **165**:5462-5471.

Arpin C, Déchanet J, Van Kooten C, Merville P, Grouard G, Brière F, Banchereau J, Liu YJ. (1995) Generation of memory B cells and plasma cells in vitro. *Science* **268**:720-722.

Artavanis-Tsakonas S, Matsuno K, Fortini ME. (1995) Notch signaling. *Science* **268**:225-232.

Atkinson PG, Coope HJ, Rowe M, Ley SC (2003). Latent membrane protein 1 of Epstein-Barr virus stimulates processing of NF-kappa B2 p100 to p52. *J Biol Chem.* **278**:51134-51142.

Avery DT, Ellyard JI, Mackay F, Corcoran LM, Hodgkin PD, Tangye SG. (2005) Increased expression of CD27 on activated human memory B cells correlates with their commitment to the plasma cell lineage. *J. Immunol.* **174**:4034-4042.

Babcock GJ, Decker LL, Volk M, Thorley-Lawson DA. (1998) EBV persistence in memory B cells in vivo. *Immunity* **9**:395-404.

Babcock GJ, Hochberg D, Thorley-Lawson AD. (2000) The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell. *Immunity* **13**:497-506.

Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, Hatfull G, Hudson GS, Satchwell SC, Séguin C, Tuffnell PS, Barrell PG. (1984) DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature* **310**:207-211.

Bargou RC, Emmerich F, Krappmann D, Bommert K, Mapara MY, Arnold W, Royer HD, Grinstein E, Greiner A, Scheidereit C, Dorken B. (1997) Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J Clin Invest.* **100**:2961-2969.

Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, Löwenberg B, Delwel R. (2003) Low expression of MDS1-EVI1-like-1 (MEL1) and EVI1-like-1 (EL1) genes in favorable-risk acute myeloid leukemia. *Exp Hematol.* **31**:1066-1072.

Basso K, Klein U, Niu H, Stolovitzky GA, Tu Y, Califano A, Cattoretti G, Dalla-Favera R. (2004) Tracking CD40 signaling during germinal center development. *Blood* **104**:4088-4096.

Baumforth KR, Flavell JR, Reynolds GM, Davies G, Pettit TR, Wei W, Morgan S, Stankovic T, Kishi Y, Arai H, Nowakova M, Pratt G, Aoki J, Wakelam MJ, Young LS, Murray PG. (2005) Induction of autotaxin by the Epstein-Barr virus promotes the growth and survival of Hodgkin lymphoma cells. *Blood* **106**:2138-2146.

Baumforth KR, Birgersdotter A, Reynolds GM, Wei W, Kapatai G, Flavell JR, Kalk E, Piper K, Lee S, Machado L, Hadley K, Sundblad A, Sjoberg J, Bjorkholm M, Porwit AA, Yap LF, Teo S, Grundy RG, Young LS, Ernberg I, Woodman CB, Murray PG. (2008) Expression of the Epstein-Barr virus-encoded Epstein-Barr virus nuclear antigen 1 in Hodgkin's lymphoma cells mediates Up-regulation of CCL20 and the migration of regulatory T cells. *Am J Pathol.* **173**:195-204.

Baus D, Pfitzner E. (2006) Specific function of STAT3, SOCS1, and SOCS3 in the regulation of proliferation and survival of classical Hodgkin lymphoma cells. *Int. J. Cancer.* **118:** 1404-1413.

Bechtel D, Kurth J, Unkel C, Kuppers R. (2005) Transformation of BCR-deficient germinal-center B cells by EBV supports a major role of the virus in the pathogenesis of Hodgkin and posttransplantation lymphomas. *Blood* **106**:4345-4350.

Bell J, Gray D. (2003) Antigen-capturing cells can masquerade as memory B cells. *J. Exp. Med.* **197**:1233-1244.

Bellows DS, Howell M, Pearson C, Hazlewood SA, Hardwick JM. (2002) Epstein-Barr virus BALF1 is a BCL-2-like antagonist of the herpesvirus antiapoptotic BCL-2 proteins. *J Virol.* **76**:2469-2479.

Ben-Bassat H, Goldblum N, Mitrani S, Goldblum T, Yoffey JM, Cohen MM, Bentwich Z, Ramot B, Klein E, Klein G. (1977) Establishment in continuous culture of a new type of lymphocyte from a "Burkitt like" malignant lymphoma (line D.G.-75). *Int J Cancer.* **19**:27-33.

Ben-Sasson SA, Klein G. (1981) Activation of the Epstein-Barr virus genome by 5-aza-cytidine in latently infected human lymphoid lines. *Int J Cancer* **28**:131-135.

Berberich I, Shu GL, Clark EA. (1994) Cross-linking CD40 on B cells rapidly activates nuclear factor-kappa B. *J Immunol.* **153**:4357-4366.

Bereshchenko OR, Gu W, Dalla-Favera R. (2002) Acetylation inactivates the transcriptional repressor BCL6. *Nat Genet.* **32**:606-613.

Bernard MP, Phipp RP. (2010) Inhibition of cyclooxygenase-2 impairs the expression of essential plasma cell transcription factors and human B-lymphocyte differentiation *Immunology* **129**:87-96.

Bhende PM, Dickerson SJ, Sun X, Feng WH, Kenney SC. (2007) X-box-binding protein 1 activates lytic Epstein-Barr virus gene expression in combination with protein kinase D. *J Virol.* **81**:7363-7370.

Biggin M, Bodescot M, Perricaudet M, Farrell P. (1987) Epstein-Barr virus gene expression in P3HR1-superinfected Raji cells. *J Virol.* **61**:3120-3132.

Bishop GA, Hostager BS. (2001) Signaling by CD40 and its mimics in B cell activation. *Immunol Res.* **24**:97-109.

Blackman MA, Tigges MA, Minie ME, Koshland ME. (1986) A model system for peptide hormone action in differentiation:interleukin 2 induces a B lymphoma to transcribe the J chain gene. *Cell* **46**:609-617.

Blake SM, Eliopoulos AG, Dawson CW, Young LS (2001). The transmembrane domains of the EBV-encoded latent membrane protein 1 (LMP1) variant CAO regulate enhanced signalling activity. *Virology* **282**:278-287.

Blink EJ, Light A, Kallies A, Nutt SL, Hodgkin PD, Tarlinton DM. (2005) Early appearance of germinal center derived memory B cells and plasma cells in blood after primary immunization. *J. Exp. Med.* **201**:545-554.

Bodescot M, Chambraud B, Farrell P, Perricaudet M. (1984) Spliced RNA from the IR1-U2 region of Epstein-Barr virus: presence of an open reading frame for a repetitive polypeptide. *EMBO J.* **3**:1913-1917.

Booth K, Burkitt DP, Bassett DJ, Cooke RA, Biddulph J. (1967)Burkitt lymphoma in Papua, New Guinea. *Br J Cancer.* **21**:657-664.

Borza CM, Hutt-Fletcher LM. (2002) Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus. *Nat Med.* **8**:594-599.

Brauninger A, Schmitz R, Bechtel D, Renne C, Hansmann ML, Kuppers R (2006). Molecular biology of Hodgkin's and Reed/Sternberg cells in Hodgkin's lymphoma. *Int J Cancer* **118**: 1853-1861.

Brennan P, Floettmann JE, Mehl A, Jones M, Rowe M (2001). Mechanism of action of a novel latent membrane protein-1 dominant negative. *J Biol Chem.* **276**:1195-1203.

Brodeur SR, Cheng G, Baltimore D, Thorley-Lawson DA. (1997) Localization of the major NF-kappaB-activating site and the sole TRAF3 binding site of LMP-1 defines two distinct signaling motifs. *J Biol Chem.* **272**:19777-19784.

Brooks L, Yao QY, Rickinson AB, Young LS. (1992) Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. *J Virol.* **66**:2689-2697.

Bross L, Fukita Y, McBlane F, Démollière C, Rajewsky K, Jacobs H. (2000) DNA double-strand breaks in immunoglobulin genes undergoing somatic hypermutation. *Immunity* **13**:589-597.

Brune V, Tiacci E, Pfeil I, Döring C, Eckerle S, van Noesel CJ, Klapper W, Falini B, von Heydebreck A, Metzler D, Bräuninger A, Hansmann ML, Küppers R. (2008) Origin and pathogenesis of nodular lymphocyte-predominant Hodgkin lymphoma as revealed by global gene expression analysis. *J Exp Med.* **205**:2251-2268.

Buettner M, Greiner A, Avramidou A, Jäck HM, Niedobitek G. (2005) Evidence of abortive plasma cell differentiation in Hodgkin and Reed-Sternberg cells of classical Hodgkin lymphoma. *Hematol Oncol*. **23**:127-132.

Burkitt D. A sarcoma involving the jaws in African children. (1958) *Br J Surg.* **46**:218-223.

Burkitt DP. (1961) Observations on the geography of malignant lymphoma. *East Afr Med J.* **38**:511-514.

Cabannes E, Khan G, Aillet F, Jarrett RF, Hay RT. (1999) Mutations in the IκBα gene in Hodgkin's disease suggest a tumour suppressor role for IκBα. *Oncogene* **18:**3063-3070.

Cahir-McFarland ED, Carter K, Rosenwald A, Giltnane JM, Henrickson SE, Staudt LM, Kieff E. (2004) Role of NF-kappa B in cell survival and transcription of latent membrane protein 1-expressing or Epstein-Barr virus latency III-infected cells. *J Virol.* **78**: 4108-4119.

Calado DP, Zhang B, Srinivasan L, Sasaki Y, Seagal J, Unitt C, Rodig S, Kutok J, Tarakhovsky A, Schmidt-Supprian M, Rajewsky K. (2010) Constitutive canonical NF-κB activation cooperates with disruption of BLIMP1 in the pathogenesis of activated B cell-like diffuse large cell lymphoma. *Cancer Cell* **18**:580-589.

Calame KL, Lin KI, Tunyaplin C. (2003) Regulatory mechanisms that determine the development and function of plasma cells. *Annu Rev Immunol.* **21**:205-230.

Calame K. (2006). Transcription factors that regulate memory in humoral responses. *Immunol. Rev.* **211**:269-279.

Calame K. (2008) Activation-dependent induction of Blimp-1. *Curr Opin Immunol.* **20**:259-264.

Caldwell RG, Wilson JB, Anderson SJ, Longnecker R. (1998) Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. *Immunity* **9**:405-411.

Caldwell RG, Brown RC, Longnecker R. (2000) Epstein-Barr virus LMP2A-induced B-cell survival in two unique classes of EmuLMP2A transgenic mice. *J Virol.* **74**:1101-1113.

Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* **415**:92-96.

Callard RE, Herbert J, Smith SH, Armitage RJ, Costelloe KE. (1995) CD40 cross-linking inhibits specific antibody production by human B cells. *Int Immunol.* **7**:1809-1815.

Camacho SA, Kosco-Vilbois MH, Berek C. (1998) The dynamic structure of the germinal center. *Immunol. Today* **19**:511-514.

Canaan A, Haviv I, Urban AE, Schulz VP, Hartman S, Zhang Z, Palejev D, Deisseroth AB, Lacy J, Snyder M, Gerstein M, Weissman SM. (2009) EBNA1 regulates cellular gene expression by binding cellular promoters. *Proc Natl Acad Sci U S A* **106**:22421-22426.

Carbone A, Gloghini A, Gruss HJ, Pinto A. (1995) CD40 ligand is constitutively expressed in a subset of T cell lymphomas and on the microenvironmental reactive T cells of follicular lymphomas and Hodgkin's disease. *Am J Pathol.* **147**:912-922.

Casali P, Zan H. (2004) Class switching and Myc translocation: how does DNA break? *Nat Immunol.* **5**:1101-1103.

Casola S, Otipoby KL, Alimzhanov M, Humme S, Uyttersprot N, Kutok JL, Carroll MC, Rajewsky K. (2004) B cell receptor signal strength determines B cell fate. *Nat Immunol.* **5**:317-27.

Castigli E, Wilson SA, Scott S, Dedeoglu F, Xu S, Lam KP, Bram RJ, Jabara H, Geha RS. (2005) TACI and BAFF R mediate isotype switching in B cells. *J. Exp. Med.* **201**:35-39.

Cattoretti G, Angelin-Duclos C, Shaknovich R, Zhou H, Wang D, Alobeid B. (2005b) PRDM1/Blimp-1 is expressed in human B-lymphocytes committed to the plasma cell lineage. *J. Pathol.* **206**:76-86.

Cattoretti G, Pasqualucci L, Ballon G, Tam W, Nandula SV, Shen Q, Mo T, Murty VV, Dalla-Favera R. (2005a) Deregulated BCL6 expression recapitulates the pathogenesis of human diffuse large B cell lymphomas in mice. *Cancer Cell.* **7**:445-455.

Caven TH, Sturgill JL, Conrad DH. (2007) BCR ligation antagonizes the IL-21 enhancement of anti-CD40/IL-4 plasma cell differentiation and IgE production found in low density human B cell cultures. *Cell Immunol.* **247**:49-58.

Chadwick RB, Jiang GL, Bennington GA, Yuan B, Johnson CK, Stevens MW, Niemann TH, Peltomaki P, Huang S, de la Chapelle A. (2000) Candidate tumor suppressor RIZ is frequently involved in colorectal carcinogenesis. *Proc Natl Acad Sci U S A* **97**:2662-2667.

Chaganti S, Bell AI, Pastor NB, Milner AE, Drayson M, Gordon J, Rickinson AB (2005). Epstein-Barr virus infection in vitro can rescue germinal center B cells with inactivated immunoglobulin genes. *Blood* **106**:4249-4252.

Chan WC. (1999) Cellular origin of nodular lymphocyte-predominant Hodgkin's lymphoma: immunophenotypic and molecular studies. *Semin Hematol.* **36**:242-252.

Chang DW, Claassen GF, Hann SR, Cole MD. (2000) The c-Myc transactivation domain is a direct modulator of apoptotic versus proliferative signals. *Mol Cell Biol.* **20**:4309-4319.

Chaudhuri J, Tian M, Khuong C, Chua K, Pinaud E, Alt FW. (2003) Transcription-targeted DNA deamination by the AID antibody diversification enzyme. *Nature* **422**:726-730.

Chemnitz JM, Eggle D, Driesen J, Classen S, Riley JL, Debey-Pascher S, Beyer M, Popov A, Zander T, Schultze JL. (2007) RNA fingerprints provide direct evidence for the inhibitory role of TGFbeta and PD-1 on CD4+ T cells in Hodgkin lymphoma. *Blood* **110**:3226-3233.

Chen F, Zou JZ, di Renzo L, Winberg G, Hu LF, Klein E, Klein G, Ernberg I. (1995) A subpopulation of normal B cells latently infected with Epstein-Barr virus resembles Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP1. *J Virol.* **69**:3752-3758.

Chen H, Lee JM, Zong Y, Borowitz M, Ng MH, Ambinder RF, Hayward SD (2001a). Linkage between STAT regulation and Epstein-Barr virus gene expression in tumors. *J Virol* **75**: 2929-2937.

Cheng PC, Brown BK, Song W, Pierce SK. (2001b) Translocation of the B cell antigen receptor into lipid rafts reveals a novel step in signaling. *J. Immunol.* **166**:3693-3701.

Chen-Kiang S. (1995) Regulation of terminal differentiation of human B-cells by IL-6. *Curr. Top. Microbiol. Immunol.* **194**:189-198.

Choe J, Kim HS, Zhang X, Armitage RJ, Choi YS. (1996) Cellular and molecular factors that regulate the differentiation and apoptosis of germinal center B cells. Anti-Ig down-regulates Fas expression of CD40 ligand-stimulated germinal center B cells and inhibits Fas-mediated apoptosis. *J Immunol.* **157**:1006-1016.

Choe J, Choi YS. (1998) IL-10 interrupts memory B cell expansion in the germinal center by inducing differentiation into plasma cells. *Eur. J. Immunol.* **28**:508-515.

Clybouw C, McHichi B, Mouhamad S, Auffredou MT, Bourgeade MF, Sharma S, Leca G, Vazquez A. (2005) EBV infection of human B lymphocytes leads to down-regulation of Bim expression: relationship to resistance to apoptosis. *J Immunol.* **175**:2968-2973.

Cobaleda C, Schebesta A, Delogu A, Busslinger M. (2007) Pax5: the guardian of B cell identity and function. *Nat Immunol*. **8**:463-470.

Cohen JH, Revillard JP, Magaud JP, Lenoir G, Vuillaume M, Manel AM, Vincent C, Bryon PA. (1987) B-cell maturation stages of Burkitt's lymphoma cell lines according to Epstein-Barr virus status and type of chromosome translocation. *J Natl Cancer Inst.* **78**:235-242.

Cohen JI, Wang F, Mannick J, Kieff E. (1989) Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. *Proc Natl Acad Sci U S A* **86**:9558-9562.

Coller HA, Grandori C, Tamayo P, Colbert T, Lander ES, Eisenman RN, Golub TR. (2000) Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. *Proc Natl Acad Sci U S A* **97**:3260-3265.

Corcoran LM, Hasbold J, Dietrich W, Hawkins E, Kallies A, Nutt SL, Tarlinton DM, Matthias P, Hodgkin PD. (2005) Differential requirement for OBF-1 during antibody-secreting cell differentiation. *J Exp Med.* **201**:1385-1396.

Cordier M, Calender A, Billaud M, Zimber U, Rousselet G, Pavlish O, Banchereau J, Tursz T, Bornkamm G, Lenoir GM. (1990) Stable transfection of Epstein-Barr virus (EBV) nuclear antigen 2 in lymphoma cells containing the EBV P3HR1 genome induces expression of B-cell activation molecules CD21 and CD23. *J Virol.* **64**:1002-1013.

Correa P, O'Conor GT. (1971) Epidemiologic patterns of Hodgkin's disease. *Int J Cancer.* **8**:192-201.

Countryman J, Miller G. (1985) Activation of expression of latent Epstein-Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. *Proc Natl Acad Sci U S A.* **82**:4085-4589.

Courts C, Montesinos-Rongen M, Brunn A, Bug S, Siemer D, Hans V, Blümcke I, Klapper W, Schaller C, Wiestler OD, Küppers R, Siebert R, Deckert M. (2008) Recurrent inactivation of the PRDM1 gene in primary central nervous system lymphoma. *J Neuropathol Exp Neurol.* **67**:720-727.

Crawford DH, Ando I. (1986) EB virus induction is associated with B-cell maturation. *Immunology* **59**:405-409.

Crossen PE, Morrison MJ, Rodley P, Cochrane J, Morris CM. (1999) Identification of amplified genes in a patient with acute myeloid leukemia and double minute chromosomes. *Cancer Genet Cytogenet.* **113**:126-133.

Cuenco GM, Nucifora G, Ren R. (2000) Human AML1/MDS1/EVI1 fusion protein induces an acute myelogenous leukemia (AML) in mice: a model for human AML. *Proc Natl Acad Sci U S A* **97**:1760-17665.

Curran RC, Jones EL. (1978) Hodgkin's disease: an immunohistochemical and histological study. *J Pathol.* **125**:39-51.

Cyster JG, Ansel KM, Reif K, Ekland EH, Hyman PL, Tang HL, Luther SA, Ngo VN. (2000) Follicular stromal cells and lymphocyte homing to follicles. *Immunol Rev.* **176**:181-193.

Davison AJ. (2002) Evolution of the herpesviruses. *Vet Microbiol.* **86**:69-88.

Dawson CW, Tramountanis G, Eliopoulos AG, Young LS. (2003) Epstein-Barr virus latent membrane protein 1 (LMP1) activates the phosphatidylinositol 3-kinase/Akt pathway to promote cell survival and induce actin filament remodeling. *J Biol Chem.* **278**:3694-3704.

de Groot RP, Coffer PJ, Koenderman L. (1998) Regulation of proliferation, differentiation and survival by the IL-3/IL-5/GM-CSF receptor family. *Cell Signal.* **10**:619-628.

de Vinuesa CG, Cook MC, Ball J, Drew M, Sunners Y, Cascalho M, Wabl M, Klaus GG, MacLennan IC. (2000) Germinal centers without T cells. *J Exp Med.* **191**:485-494.

Deacon EM, Pallesen G, Niedobitek G, Crocker J, Brooks L, Rickinson AB, Young LS. (1993) Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells. *J Exp Med.* **177**:339-349.

Decaussin G, Leclerc V, Ooka T. (1995) The lytic cycle of Epstein-Barr virus in the nonproducer Raji line can be rescued by the expression of a 135-kilodalton protein encoded by the BALF2 open reading frame. *J Virol.* **69**:7309-7314.

Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. (1997) Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science* **276**:589-592.

Desai S, Bolick SC, Maurin M, Wright KL. (2009) PU.1 regulates positive regulatory domain I-binding factor 1/Blimp-1 transcription in lymphoma cells. *J Immunol.* **183**:5778-5787.

de-Thé G, Geser A, Day NE, Tukei PM, Williams EH, Beri DP, Smith PG, Dean AG, Bronkamm GW, Feorino P, Henle W. (1978) Epidemiological evidence for causal relationship between Epstein-Barr virus and Burkitt's lymphoma from Ugandan prospective study. *Nature* **274**:756-761.

Di Noia JM, Neuberger MS. (2007) Molecular mechanisms of antibody somatic hypermutation. *Annu. Rev. Biochem.* **76**:1-22.

Dickerson SK, Market E, Besmer E, Papavasiliou FN. (2003) AID mediates hypermutation by deaminating single stranded DNA. *J. Exp. Med.* **197:** 1291-1296.

Diehl SA, Schmidlin H, Nagasawa M, van Haren SD, Kwakkenbos MJ, Yasuda E, Beaumont T, Scheeren FA, Spits H. (2008) STAT3-Mediated Up-Regulation of BLIMP1 Is Coordinated with BCL6 Down-Regulation to Control Human Plasma Cell Differentiation. *J Immunol.* **180**: 4805-4815.

Diehl V, Kirchner HH, Burrichter H, Stein H, Fonatsch C, Gerdes J, Schaadt M, Heit W, Uchanska-Ziegler B, Ziegler A, Heintz F, Sueno K. (1982) Characteristics of Hodgkin's disease-derived cell lines. *Cancer Treatment Reports.* **66**:615-632.

Dillon SC, Zhang X,Trievel RC, Cheng X. (2005) The SET-domain protein superfamily: protein lysine methyltransferases. *Genome Biol.* **6**:227.

Dolcetti R, Boiocchi M, Gloghini A, Carbone A. (2001) Pathogenetic and histogenetic features of HIV-associated Hodgkin's disease. *Eur J Cancer.* **37**:1276-1287.

Doody GM, Stephenson S, Tooze RM. (2006) BLIMP-1 is a target of cellular stress and downstream of the unfolded protein response. *Eur. J. Immunol.* **36**:1572-1582.

Drexler HG. (1992) Recent results on the biology of Hodgkin and Reed-Sternberg cells. I. Biopsy material. *Leuk Lymphoma* **8**:283-313.

Driver DJ, McHeyzer-Williams LJ, Cool M, Stetson DB, McHeyzer-Williams MG. (2001) Development and maintenance of a B220- memory B cell compartment. *J. Immunol.* **167**: 1393-1405.

Dukers DF, van Galen JC, Giroth C, Jansen P, Sewalt RG, Otte AP, Kluin-Nelemans HC, Meijer CJ, Raaphorst FM. (2004) Unique polycomb gene expression pattern in Hodgkin's lymphoma and Hodgkin's lymphoma-derived cell lines. *Am J Pathol.* **164**:873-881.

Durandy A. (2003) Activation-induced cytidine deaminase: a dual role in class-switch recombination and somatic hypermutation. *Eur J Immunol.* **33**:2069-2073.

Dutton A, O'Neil JD, Milner AE, Reynolds GM, Starczynski J, Crocker J, Young LS, Murray PG. (2004) Expression of the cellular FLICE-inhibitory protein (c-FLIP) protects Hodgkin's lymphoma cells from autonomous Fas-mediated death. *Proc Natl Acad Sci U S A* **101**:6611-6616.

Dutton A, Reynolds GM, Dawson CW, Young LS, Murray PG. (2005) Constitutive activation of phosphatidyl-inositide 3 kinase contributes to the survival of Hodgkin's lymphoma cells through a mechanism involving Akt kinase and mTOR. *J Pathol.* **205**:498-506.

Ebell MH. (2004) Epstein-Barr virus infectious mononucleosis. *Am Fam Physician* **70**:1279-1287.

Edson CM, Thorley-Lawson DA. (1981) Epstein-Barr virus membrane antigens: characterization, distribution, and strain differences. *J Virol.* **39**:172-184.

Eilers M. (1999) Control of cell proliferation by Myc family genes. *Mol Cells.* **9**:1-6.

Eliopoulos AG, Stack M, Dawson CW, Kaye KM, Hodgkin L, Sihota S, Rowe M, Young LS. (1997) Epstein-Barr virus-encoded LMP1 and CD40 mediate IL-6 production in epithelial cells via an NF-kappaB pathway involving TNF receptor-associated factors. *Oncogene* **14**:2899-28916.

Eliopoulos AG, Gallagher NJ, Blake SM, Dawson CW, Young LS. (1999) Activation of the p38 mitogen-activated protein kinase pathway by Epstein-Barr virus-encoded latent membrane protein 1 coregulates interleukin-6 and interleukin-8 production. *J Biol Chem.* **274**:16085-16096.

Emmerich F, Meiser M, Hummel M, Demel G, Foss HD, Jundt F, Mathas S, Krappmann D, Scheidereit C, Stein H, Dörken B. (1999) Overexpression of I kappa B alpha without inhibition of NF-κB activity and mutations in the I kappa B alpha gene in Reed-Sternberg cells. *Blood* **94**:3129-3134.

Endres R, Alimzhanov MB, Plitz T, Fütterer A, Kosco-Vilbois MH, Nedospasov SA, Rajewsky K, Pfeffer K. (1999) Mature follicular dendritic cell networks depend on expression of lymphotoxin beta receptor by radioresistant stromal cells and of lymphotoxin beta and tumor necrosis factor by B cells. *J Exp Med.* **189**:159-168.

Ettinger R, Sims GP, Fairhurst AM, Robbins R, da Silva YS, Spolski R, Leonard WJ, Lipsky PE. (2005) IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J. Immunol.* **175**:7867-7879.

Fairfax KA, Corcoran LM, Pridans C, Huntington ND, Kallies A, Nutt SL, Tarlinton DM. (2007) Different kinetics of blimp-1 induction in B cell subsets revealed by reporter gene. *J Immunol.* **178**:4104-4111.

Fearon DT, Manders PM, Wagner SD. (2002) Bcl-6 uncouples B lymphocyte proliferation from differentiation. *Adv. Exp. Med. Biol.* **512**:21-28.

Feederle R, Kost M, Baumann M, Janz A, Drouet E, Hammerschmidt W, Delecluse HJ. (2000) The Epstein-Barr virus lytic program is controlled by the co-operative functions of two transactivators. *EMBO J.* **19**:3080-3089.

Fernández de Mattos S, Essafi A, Soeiro I, Pietersen AM, Birkenkamp KU, Edwards CS, Martino A, Nelson BH, Francis JM, Jones MC, Brosens JJ, Coffer PJ, Lam EW. (2004) FoxO3a and BCR-ABL regulate cyclin D2 transcription through a STAT5/BCL6-dependent mechanism. *Mol Cell Biol.* **24**:10058-10071.

Fingeroth JD, Weis JJ, Tedder TF, Strominger JL, Biro PA, Fearon DT. (1984) Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. *Proc Natl Acad Sci U S A* **81**:4510-4514.

Fischer M, Juremalm M, Olsson N, Backlin C, Sundström C, Nilsson K, Enblad G, Nilsson G. (2003) Expression of CCL5/RANTES by Hodgkin and Reed–Sternberg cells and its possible role in the recruitment of mast cells into lymphomatous tissue. *Int. J. Cancer* **107:**197-201.

Fiumara P, Snell V, Li Y, Mukhopadhyay A, Younes M, Gillenwater AM, Cabanillas F, Aggarwal BB, Younes A. (2001) Functional expression of receptor activator of nuclear factor kappaB in Hodgkin disease cell lines. *Blood* **98**:2784-2790.

Fleisher G, Henle W, Henle G, Lennette ET, Biggar RJ. (1979) Primary infection with Epstein-Barr virus in infants in the United States: clinical and serologic observations. *J Infect Dis.* **139**:553-558.

Floettmann JE, Ward K, Rickinson AB, Rowe M. (1996) Cytostatic effect of Epstein-Barr virus latent membrane protein-1 analyzed using tetracycline-regulated expression in B cell lines. *Virology* 223:29-40.

Fornek JL, Tygrett LT, Waldschmidt TJ, Poli V, Rickert RC, Kansas GS. (2006) Critical role for Stat3 in T-dependent terminal differentiation of IgG B cells. *Blood* **107**:1085-1091.

Fox CP, Haigh TA, Taylor GS, Long HM, Lee SP, Shannon-Lowe C, O'Connor S, Bollard CM, Iqbal J, Chan WC, Rickinson AB, Bell AI, Rowe M. (2010) A novel latent membrane 2 transcript expressed in Epstein-Barr virus-positive NK- and T-cell lymphoproliferative disease encodes a target for cellular immunotherapy. *Blood* **116**:3695-704.

Frank DA, Robertson MJ, Bonni A, Ritz J, Greenberg ME. (1995) Interleukin 2 signaling involves the phosphorylation of Stat proteins. *Proc. Natl. Acad. Sci. USA* **92**:7779-7783.

Fruehling S, Longnecker R. (1997) The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BCR-mediated signal transduction *Virology.* **235**:241-251.

Fujita N, Jaye DL, Geigerman C, Akyildiz A, Mooney MR, Boss JM, Wade PA. (2004) MTA3 and the Mi-2/NuRD complex regulate cell fate during B lymphocyte differentiation. *Cell* **119**:75-86.

Gahn TA, Sugden B. (1995) An EBNA-1-dependent enhancer acts from a distance of 10 kilobase pairs to increase expression of the Epstein-Barr virus LMP gene. *J Virol.* **69**:2633-2636.

Gandhi MK, Moll G, Smith C, Dua U, Lambley E, Ramuz O, Gill D, Marlton P, Seymour JF, Khanna R. (2007) Galectin-1 mediated suppression of Epstein-Barr virus specific T-cell immunity in classic Hodgkin lymphoma. *Blood* **110**:1326-1329.

Garcia JF, Roncador G, García JF, Sánz AI, Maestre L, Lucas E, Montes-Moreno S, Fernandez Victoria R, Martinez-Torrecuadrara JL, Marafioti T, Mason DY, Piris MA. (2006) PRDM1/BLIMP-1 expression in multiple B and T-cell lymphoma. *Haematologica* **91**:467-474.

Garcia-Bates TM, Baglole CJ, Bernard MP, Murant TI, Simpson-Haidaris PJ, Phipps RP. (2009) Peroxisome proliferator-activated receptor gamma ligands enhance human B cell antibody production and differentiation. *J Immunol.* **183**:6903-6912.

García-Cosío M, Santón A, Martín P, Camarasa N, Montalbán C, García JF, Bellas C. (2004) Analysis of transcription factor OCT.1, OCT.2 and BOB.1 expression using tissue arrays in classical Hodgkin's lymphoma. *Mod Pathol.* **17**:1531-1538.

Genestier L, Taillardet M, Mondiere P, Gheit H, Bella C, Defrance T. (2007) TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. *J Immunol.* **178**:7779-7786.

Georgakis GV, Li Y, Rassidakis GZ, Medeiros LJ, Mills GB, Younes A. (2006) Inhibition of the phosphatidylinositol-3 kinase/Akt promotes G1 cell cycle arrest and apoptosis in Hodgkin lymphoma. *Br. J. Haematol.* **132:**503-511.

Geser A, de The G, Lenoir G, Day NE, Williams EH. (1982) Final case reporting from the Ugandan prospective study of the relationship between EBV and Burkitt's lymphoma. *Int J Cance.* **29**:397-400.

Gires O, Zimber-Strobl U, Gonnella R, Ueffing M, Marschall G, Zeidler R, Pich D, Hammerschmidt W. (1997) Latent membrane protein 1 of Epstein–Barr virus mimics a constitutively active receptor molecule. *EMBO J.* **16**:6131-6140.

Gires O, Kohlhuber F, Kilger E, Baumann M, Kieser A, Kaiser C, Zeidler R, Scheffer B, Ueffing M, Hammerschmidt W. (1999) Latent membrane protein 1 of Epstein-Barr virus interacts with JAK3 and activates STAT proteins. *EMBO J.* **18**:3064-3073.

Given D, Kieff E. DNA of Epstein-Barr virus. (1979) VI. Mapping of the internal tandem reiteration. *J Virol.* **31**:315-324.

Glaser SL, Lin RJ, Stewart SL, Ambinder RF, Jarrett RF, Brousset P, Pallesen G, Gulley ML, Khan G, O'Grady J, Hummel M, Preciado MV, Knecht H, Chan JK, Claviez A. (1997) Epstein-Barr virus-associated Hodgkin's disease: epidemiologic characteristics in international data. *Int J Cancer.* **70**:375-382.

Gonda H, Sugai M, Nambu Y, Katakai T, Agata Y, Mori KJ, Yokota Y, Shimizu A. (2003) The balance between Pax5 and Id2 activities is the key to AID gene expression. *J. Exp. Med.* **198**: 1427-1437.

González-García I, Ocaña E, Jiménez-Gómez G, Campos-Caro A, Brieva JA. (2006) Immunization-induced perturbation of human blood plasma cell pool: progressive maturation, IL-6 responsiveness, and high PRDI-BF1/BLIMP1 expression are critical distinctions between antigen-specific and nonspecific plasma cells. *J Immunol.* **176**:4042-4050.

Goormachtigh G, Ouk TS, Mougel A, Tranchand-Bunel D, Masy E, Le Clorennec C, Feuillard J, Bornkamm GW, Auriault C, Manet E, Fafeur V, Adriaenssens E, Coll J (2006). Autoactivation of the Epstein-Barr Virus Oncogenic Protein LMP1 during Type II Latency through Opposite Roles of the NF-κB and JNK Signaling Pathways. *J Virol.* **80**:7382-7393.

Goossens T, Klein U, Küppers R. (1998) Frequent occurrence of deletions and duplications during somatic hypermutation: implications for oncogene translocations and heavy chain disease. *Proc Natl Acad Sci U S A* **95**:2463-2468.

Gossen M, Bujard H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A.* **89**:5547-5551.

Gradoville L, Grogan E, Taylor N, Miller G. (1990) Differences in the extent of activation of Epstein-Barr virus replicative gene expression among four nonproducer cell lines stably transformed by oriP/BZLF1 plasmids. *Virolog.* **178**:345-354.

Gregory CD, Murray RJ, Edwards CF, Rickinson AB. (1988) Downregulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's lymphoma underlies tumor cell escape from virus-specific T cell surveillance. *J Exp Med.* **167**:1811-1824.

Gregory CD, Rowe M, Rickinson AB. (1990) Different Epstein-Barr virus-B cell interactions in phenotypically distinct clones of a Burkitt's lymphoma cell line. *J Gen Virol.* **71**:1481-1495.

Grossman SR, Johannsen E, Tong X, Yalamanchili R, Kieff E. (1994) The Epstein-Barr virus nuclear antigen 2 transactivator is directed to response elements by the J kappa recombination signal binding protein. *Proc Natl Acad Sci U S A* **91**:7568-7572.

Gruhne B, Sompallae R, Masucci MG. (2009) Three Epstein-Barr virus latency proteins independently promote genomic instability by inducing DNA damage, inhibiting DNA repair and inactivating cell cycle checkpoints. *Oncogene* **28**:3997-4008.

Grumont RJ, Gerondakis S. (2000) Rel induces interferon regulatory factor 4 (IRF-4) expression in lymphocytes: modulation of interferon-regulated gene expression by rel/nuclear factor kappaB. *J Exp Med.* **191**:1281-1292.

Gutiérrez NC, Ocio EM, de Las Rivas J, Maiso P, Delgado M, Fermiñán E, Arcos MJ, Sánchez ML, Hernández JM, San Miguel JF. (2007) Gene expression profiling of B lymphocytes and plasma cells from Waldenström's macroglobulinemia: comparison with expression patterns of the same cell counterparts from chronic lymphocytic leukemia, multiple myeloma and normal individuals. *Leukemia.* **21**:541-549.

Györy I, Fejér G, Ghosh N, Seto E, Wright KL. (2003) Identification of a functionally impaired positive regulatory domain I binding factor 1 transcription repressor in myeloma cell lines. *J Immunol.* **170**:125-133.

Györy I, Wu J, Fejér G, Seto E, Wright KL. (2004) PRDI-BF1 recruits the histone H3 methyltransferase G9a in transcriptional silencing. *Nat. Immunol.* **5**:299-308.

Hammerschmidt W, Sugden B. (1988) Identification and characterization of oriLyt, a lytic origin of DNA replication of Epstein-Barr virus. *Cell* **55**:427-433.

Hammerschmidt W, Sugden B. (1989) Genetic analysis of immortalizing functions of Epstein-Barr virus in human B lymphocytes. *Nature* **340**:393-397.

Hancz A, Hérincs Z, Neer Z, Sármay G, Koncz G. (2008) Integration of signals mediated by B-cell receptor, B-cell activating factor of the tumor necrosis factor family (BAFF) and Fas (CD95). *Immunol Lett.* **116**:211-217.

Hangaishi A, Kurokawa M. (2010) Blimp-1 is a tumor suppressor gene in lymphoid malignancies. *Int J Hematol.* **91**:46-53.

Harada S, Kieff E. (1997) Epstein-Barr virus nuclear protein LP stimulates EBNA-2 acidic domain-mediated transcriptional activation. *J Virol.* **71**:6611-6618.

Hardwick JM, Lieberman PM, Hayward SD. (1988) A new Epstein-Barr virus transactivator, R, induces expression of a cytoplasmic early antigen. *J Virol.* **62**:2274-84.

Hardy RR, Hayakawa K. (2001) B cell development pathways. *Annu Rev Immunol.* **19**:595-621.

Harris NL, Jaffe ES, Stein H, Banks PM, Chan JKC, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC, Grogan TM, Isaacson PG, Knowles DM, Mason DY, Muller-Hermelink H, Pileri SA, Piris NA, Ralfkiaer E, Warnke RA. (1994) A Revised European-American Classification of Lymphoid Neoplasms: A proposal from the International Lymphoma Study Group. *Blood* **84**:1361-1392.

Hasbold J, Corcoran LM, Tarlinton DM, Tangye SG, Hodgkin PD. (2004) Evidence from the generation of immunoglobulin G-secreting cells that stochastic mechanisms regulate lymphocyte differentiation. *Nat Immunol.* **5**:55-63.

Hauser AE, Junt T, Mempel TR, Sneddon MW, Kleinstein SH, Henrickson SE, von Andrian UH, Shlomchik MJ, Haberman AM. (2007) Definition of germinal-center B cell migration in vivo reveals predominant intrazonal circulation patterns *Immunity* **26**:655-667.

Hayden MS, Ghosh S. (2004) Signaling to NF-kappaB. *Genes Dev.* **18**:2195-2224.

Hayward SD, Kieff E. (1977) DNA of Epstein-Barr virus. II. Comparison of the molecular weights of restriction endonuclease fragments of the DNA of Epstein-Barr virus strains and identification of end fragments of the B95-8 strain. *J Virol.* **23**:421-429.

He B, Raab-Traub N, Casali P, Cerutti A. (2003) EBV-encoded latent membrane protein 1 cooperates with BAFF/BLyS and APRIL to induce T cell-independent Ig heavy chain class switching. *J Immunol.* **171**:5215-5224.

He L, Yu JX, Liu L, Buyse IM, Wang MS, Yang QC, Nakagawara A, Brodeur GM, Shi YE, Huang S. (1998) RIZ1, but not the alternative RIZ2 product of the same gene, is underexpressed in breast cancer, and forced RIZ1 expression causes G2-M cell cycle arrest and/or apoptosis. *Cancer Res.* **58**:4238-4244.

Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L (1998) Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J.* **334**:297-314.

Hemann MT, Bric A, Teruya-Feldstein J, Herbst A, Nilsson JA, Cordon-Cardo C, Cleveland JL, Tansey WP, Lowe SW. (2005) Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants. *Nature* **436**:807-811.

Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**:740-745.

Henderson S, Rowe M, Gregory C, Croom-Carter D, Wang F, Longnecker R, Kieff E, Rickinson A. (1991) Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. *Cell* **65**:1107-1115.

Henle G, Henle W, Clifford P, Diehl V, Kafuko GW, Kirya BG, Klein G, Morrow RH, Munube GM, Pike P, Tukei PM, Ziegler JL. (1969) Antibodies to Epstein-Barr virus in Burkitt's lymphoma and control groups. *J Natl Cancer Inst.* **43**:1147-1157.

Henle W, Henle G, Zajac BA, Pearson G, Waubke R, Scriba M. (1970) Differential reactivity of human serums with early antigens induced by Epstein-Barr virus. *Science* **169**:188190.

Henle W, Henle G (1979). The virus as the etiologic agent of infectious mononucleosis. In: *The Epstein-Barr Virus.* Eds: Achong BG, Springer, Berlin; pp 297-320.

Herbst H, Foss HD, Samol J, Araujo I, Klotzbach H, Krause H, Agathanggelou A, Niedobitek G, Stein H. (1996) Frequent expression of interleukin-10 by Epstein-Barr virus-harboring tumor cells of Hodgkin's disease. *Blood* **87**:2918-2929.

Herrold RE, Marchini A, Fruehling S, Longnecker R. (1996) Glycoprotein 110, the Epstein-Barr virus homolog of herpes simplex virus glycoprotein B, is essential for Epstein-Barr virus replication in vivo. *J Virol.* **70**:2049-2054.

Hertel CB, Zhou XG, Hamilton-Dutoit SJ, Junker S. (2002) Loss of B cell identity correlates with loss of B cell-specific transcription factors in Hodgkin/Reed-Sternberg cells of classical Hodgkin lymphoma. *Oncogene* **21**:4908-4920.

Hertle ML, Popp C, Petermann S, Maier S, Kremmer E, Lang R, Mages J, Kempkes B. (2009) Differential gene expression patterns of EBV infected EBNA-3A positive and negative human B lymphocytes. *PLoS Pathog.* **5**:e1000506.

Herzenberg LA. (2000) B-1 cells: the lineage question revisited. *Immunol Rev.* **175**:9-22.

Hess J, Laumen H, Muller KB, Wirth T. (1998) Molecular genetics of the germinal center reaction. *J Cell Physiol.* **177**:525-534.

Hickabottom M, Parker GA, Freemont P, Crook T, Allday MJ. (2002) Two nonconsensus sites in the Epstein-Barr virus oncoprotein EBNA3A cooperate to bind the co-repressor carboxyl-terminal-binding protein (CtBP). *J Biol Chem.* **277**:47197-47204.

Hiriart E, Bardouillet L, Manet E, Gruffat H, Penin F, Montserret R, Farjot G, Sergeant A (2003). A region of the Epstein-Barr virus (EBV) mRNA export factor EB2 containing an arginine-rich motif mediates direct binding to RNA. *J Biol Chem.* **278**:37790-37798.

Hislop AD, Taylor GS, Sauce D, Rickinson AB. (2007) Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annu Rev Immunol.* **25**: 587-617.

Hodgkin T (1832). On some morbid appearances of the absorbent glands and spleen. *Med Chir Trans* **17**:68-114.

Högerkorp CM, Borrebaeck CA. (2006) The human CD77- B cell population represents a heterogeneous subset of cells comprising centroblasts, centrocytes, and plasmablasts, prompting phenotypical revision. *J Immunol.* **177**: 4341-4349.

Holley-Guthrie EA, Seaman WT, Bhende P, Merchant JL, Kenney SC. (2005) The Epstein-Barr virus protein BMRF1 activates gastrin transcription. *J Virol.* **79**:745-755.

Hömig-Hölzel C, Hojer C, Rastelli J, Casola S, Strobl LJ, Müller W, Quintanilla-Martinez L, Gewies A, Ruland J, Rajewsky K, Zimber-Strobl U. (2008) Constitutive CD40 signaling in B cells selectively activates the noncanonical NF-kappaB pathway and promotes lymphomagenesis. *J Exp Med.* **205**:1317-1329.

Hong M, Murai Y, Kutsuna T, Takahashi H, Nomoto K, Cheng CM, Ishizawa S, Zhao QL, Ogawa R, Harmon BV, Tsuneyama K, Takano Y. (2006) Suppression of Epstein-Barr nuclear antigen 1 (EBNA1) by RNA interference inhibits proliferation of EBV-positive Burkitt's lymphoma cells. *J Cancer Res Clin Oncol.* **132**:1-8.

Horikawa K, Takatsu K. (2006) Interleukin-5 regulates genes involved in B-cell terminal maturation. *Immunology* **118**:497-508.

Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol.* **162**:3749-3752.

Hsieh JJ, Nofziger DE, Weinmaster G, Hayward SD. (1997) Epstein-Barr virus immortalization: Notch2 interacts with CBF1 and blocks differentiation. *J Virol.* **71**:1938-1945

Hu BT, Lee SC, Marin E, Ryan DH, Insel RA. (1997) Telomerase is up-regulated in human germinal center B cells in vivo and can be re-expressed in memory B cells activated in vitro. *J Immunol.* **159**:1068-1071.

Huang S. (1994) Blimp-1 is the murine homolog of the human transcriptional repressor PRDI-BF1. *Cell.* **78**:9.

Huang S, Shao G, Liu L. (1998) The PR domain of the Rb-binding zinc finger protein RIZ1 is a protein binding interface and is related to the SET domain functioning in chromatin-mediated gene expression. *J. Biol. Chem.* **273**:15933-15939.

Huang S. (1999) The retinoblastoma protein-interacting zinc finger gene RIZ in 1p36-linked cancers. *Front Biosci*. **4**:D528-D532.

Hudewentz J, Bornkamm GW, Zur Hausen H. (1980) Effect of the diterpene ester TPA on Epstein-Barr virus antigen- and DNA synthesis in producer and nonproducer cell lines. *Virology* **100**:175-178.

Huen DS, Henderson SA, Croom-Carter D, Rowe M. (1995) The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-kappa B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain. *Oncogene* **10**:549-560.

Humme S, Reisbach G, Feederle R, Delecluse HJ, Bousset K, Hammerschmidt W, Schepers A. (2003) The EBV nuclear antigen 1 (EBNA1) enhances B cell immortalization several thousandfold. *Proc Natl Acad Sci U S A* **100**:10989-10994.

Hyland L, Sangster M, Sealy R, Coleclough C. (1994) Respiratory virus infection of mice provokes a permanent humoral immune response. *J Virol* **68**:6083-6086.

Ikeda M, Ikeda A, Longan LC, Longnecker R (2000). The Epstein-Barr virus latent membrane protein 2A PY motif recruits WW domain-containing ubiquitin-protein ligases. *Virology* **268**: 178-191.

Ikeda M, Ikeda A, Longnecker R. (2001) PY motifs of Epstein-Barr virus LMP2A regulate protein stability and phosphorylation of LMP2A-associated proteins. *J. Virol.* **75**:5711-5718.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**:249-264.

Izumi KM, Kieff ED. (1997) The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to mediate B lymphocyte growth transformation and activate NF-kappaB. *Proc Natl Acad Sci USA* **94**:12592-12597.

Izumi KM, Cahir McFarland ED, Ting AT, Riley EA, Seed B, Kieff ED. (1999) The Epstein-Barr virus oncoprotein latent membrane protein 1 engages the tumor necrosis factor receptor-associated proteins TRADD and receptor-interacting protein (RIP) but does not induce apoptosis or require RIP for NF-kappaB activation. *Mol Cell Biol.* **19**:5759-5767.

Jackson A, Carrara P, Duke V, Sinclair P, Papaioannou M, Harrison CJ, Foroni L. (2000) Deletion of 6q16-q21 in human lymphoid malignancies: a mapping and deletion analysis. *Cancer Res.* **60**:2775-2779.

Jaffe ES, Harris NL, Stein H, Vardiman JW (2001). Hodgkin lymphoma. World Health Organization classification of tumours. In: *Pathology and genetics of tumours of haematopoietic and lymphoid tissues.* Eds: IARC Press, Washington, DC; 2001; pp 237-253.

Jayachandra S, Low KG, Thlick AE, Yu J, Ling PD, Chang Y, Moore PS. (1999) Three unrelated viral transforming proteins (vIRF, EBNA2, and E1A) induce the MYC oncogene through the interferon-responsive PRF element by using different transcription coadaptors. *Proc Natl Acad Sci U S A.* **96**:11566-11571.

Jiang WQ, Wendel-Hansen V, Lundkvist A, Ringertz N, Klein G, Rosen A. (1991) Intranuclear distribution of Epstein-Barr virus-encoded nuclear antigens EBNA-1, -2, -3 and -5. *J Cell Sci.* **99**:497-502.

Johannsen E, Koh E, Mosialos G, Tong X, Kieff E, Grossman SR. (1995) Epstein-Barr virus nuclear protein 2 transactivation of the latent membrane protein 1 promoter is mediated by J and PU.1. *J Virol.* **69**:253-262.

Johannsen E, Luftig M, Chase MR, Weicksel S, Cahir-McFarland E, Illanes D, Sarracino D, Kieff E. (2004) Proteins of purified Epstein-Barr virus. *Proc Natl Acad Sci USA* **101**:6286-16291.

John SA, Clements JL, Russell LM, Garrett-Sinha LA. (2007) Ets-1 regulates plasma cell differentiation by interfering with the activity of the transcription factor Blimp-1. *J Biol Chem.* **283**:951-962.

John SA, Garrett-Sinha LA. (2009) Blimp1: a conserved transcriptional repressor critical for differentiation of many tissues. *Exp Cell Res.* **315**:1077-1084.

Johnson K, Shapiro-Shelef M, Tunyaplin C, Calame K. (2005) Regulatory events in early and late B-cell differentiation. *Mol Immunol.* **42**:749-761.

Johnson SA, Pleiman CM, Pao L, Schneringer J, Hippen K, Cambier JC. (1995) Phosphorylated immunoreceptor signaling motifs (ITAMs) exhibit unique abilities to bind and activate Lyn and Syk tyrosine kinases. *J. Immunol.* **155**:4596-4603.

Joos S, Granzow M, Holtgreve-Grez H, Siebert R, Harder L, Martín-Subero JI, Wolf J, Adamowicz M, Barth TF, Lichter P, Jauch A. Hodgkin's lymphoma cell lines are characterized by frequent aberrations on chromosomes 2p and 9p including REL and JAK2. *Int J Cancer.* **103**:489-495.

Jundt F, Anagnostopoulos I, Bommert K, Emmerich F, Müller G, Foss HD, Royer HD, Stein H, Dörken B. (1999) Hodgkin/Reed–Sternberg cells induce fibroblasts to secrete eotaxin, a potent chemoattractant for T cells and eosinophils. *Blood* **94**:2065-2071.

Jundt F, Kley K, Anagnostopoulos I, Schulze Pröbsting K, Greiner A, Mathas S, Scheidereit C, Wirth T, Stein H, Dörken B. (2002) Loss of PU.1 expression is associated with defective immunoglobulin transcription in Hodgkin and Reed-Sternberg cells of classical Hodgkin disease. *Blood* **99**:3060-3062.

Jundt F, Acikgöz O, Kwon SH, Schwarzer R, Anagnostopoulos I, Wiesner B, Mathas S, Hummel M, Stein H, Reichardt HM, Dörken B (2008) Aberrant expression of Notch1 interferes with the B-lymphoid phenotype of neoplastic B cells in classical Hodgkin lymphoma. *Leukemia* **22**:1587-1594.

Jungnickel B, Staratschek-Jox A, Brauninger A, Spieker T, Wolf J, Diehl V, Hansmann ML, Rajewsky K, Kuppers R. (2000) Clonal deleterious mutations in the IkappaBalpha gene in the malignant cells in Hodgkin's lymphoma. *J Exp Med.* **191**: 395-402.

Juszczynski P, Ouyang J, Monti S, Rodig SJ, Takeyama K, Abramson J, Chen W, Kutok JL, Rabinovich GA, Shipp MA. (2007) The AP1-dependent secretion of galectin-1 by Reed Sternberg cells fosters immune privilege in classical Hodgkin lymphoma. *Proc. Natl Acad. Sci. USA* **104**: 13134-13139.

Kabashima K, Haynes NM, Xu Y, Nutt SL, Allende ML, Proia RL, Cyster JG. (2006) Plasma cell S1P1 expression determines secondary lymphoid organ retention versus bone marrow tropism. *J Exp Med.* **203**:2683-2690.

Kadin ME, Stites DP, Levy R, Warnke R. (1978) Exogenous immunoglobulin and the macrophage origin of Reed-Sternberg cells in Hodgkin's disease. *N Engl J Med.* **299**:1208-1214.

Kaiser C, Laux G, Eick D, Jochner N, Bornkamm GW, Kempkes B. (1999) The proto-oncogene c-myc is a direct target gene of Epstein-Barr virus nuclear antigen 2. *J Virol.* **73**:4481-4484.

Kalla M, Schmeinck A, Bergbauer M, Pich D, Hammerschmidt W. (2010) AP-1 homolog BZLF1 of Epstein-Barr virus has two essential functions dependent on the epigenetic state of the viral genome. *Proc Natl Acad Sci U S A.* **107**:850-855.

Kallies A, Hasbold J, Tarlinton DM, Dietrich W, Corcoran LM, Hodgkin PD, Nutt SL. (2004) Plasma cell ontogeny defined by quantitative changes in blimp-1 expression. *J Exp Med.* **200**:967-977.

Kallies A, Hasbold J, Fairfax K, Pridans C, Emslie D, McKenzie BS, Lew AM, Corcoran LM, Hodgkin PD, Tarlinton DM, Nutt SL. (2007) Initiation of plasma-cell differentiation is independent of the transcription factor Blimp-1. *Immunity.* **26**:555-566.

Kamesaki H, Fukuhara S, Tatsumi E, Uchino H, Yamabe H, Miwa H, Shirakawa S, Hatanaka M, Honjo T. (1986) Cytochemical, immunologic, chromosomal, and molecular genetic analysis of a novel cell line derived from Hodgkin's disease. *Blood.* **68**:285-292.

Kang MS, Hung SC, Kieff E. (2001) Epstein-Barr virus nuclear antigen 1 activates transcription from episomal but not integrated DNA and does not alter lymphocyte growth. *Proc Natl Acad Sci U S A.* **98**:15233-15238.

Kang MS, Lu H, Yasui T, Sharpe A, Warren H, Cahir-McFarland E, Bronson R, Hung SC, Kieff E. (2005) Epstein-Barr virus nuclear antigen 1 does not induce lymphoma in transgenic FVB mice. *Proc Natl Acad Sci USA.* **102**:820-825.

Kang MS, Soni V, Bronson R, Kieff E. (2008) Epstein-Barr virus nuclear antigen 1 does not cause lymphoma in C57BL/6J mice. *J Virol.* **82**:4180-4183.

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

Kapatai G, Murray P. (2007) Contribution of the Epstein Barr virus to the molecular pathogenesis of Hodgkin lymphoma. *J Clin Pathol.* **60**:1342-1349.

Kapp U, Düx A, Schell-Frederick E, Banik N, Hummel M, Mücke S, Fonatsch C, Bullerdiek J, Gottstein C, Engert A, et al. (1994) Disseminated growth of Hodgkin's derived cell lines L540 and L540cy in immune-deficient SCID mice. *Ann. Oncol.* **5**:121-126.

Kaul R, Murakami M, Choudhuri T, Robertson ES. (2007) Epstein-Barr virus latent nuclear antigens can induce metastasis in a nude mouse model. *J Virol.* **81**:10352-10361.

Kaye KM, Izumi KM, Kieff E. (1993) Epstein–Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. *Proc Natl Acad Sci USA.* **90**:9150-9154.

Kaykas A, Worringer K, Sugden B. (2001) CD40 and LMP-1 both signal from lipid rafts but LMP-1 assembles a distinct, more efficient signaling complex. *EMBO J.* **20**:2641-2654.

Kearney JF, Cooper MD, Lawton AR. (1976) B lymphocyte differentiation induced by lipopolysaccharide. III. Suppression of B cell maturation by antimouse immunoglobulin antibodies. *J. Immunol.* **116**:1664-1668.

Keller AD, Maniatis T. (1991) Identification and characterization of a novel repressor of beta-interferon gene expression. *Genes Dev.* **5**:868-879.

Keller AD, Maniatis T. (1992) Only two of the five zinc fingers of the eukaryotic transcriptional repressor PRDI-BF1 are required for sequence-specific DNA binding. *Mol Cell Biol.* **12**:1940-1949.

Kelly G, Bell A, Rickinson A. (2002) Epstein-Barr virus-associated Burkitt lymphomagenesis selects for downregulation of the nuclear antigen EBNA2. *Nat Med.* **8**:1098-1104.

Kelly GL, Milner AE, Baldwin GS, Bell AI, Rickinson AB. (2006) Three restricted forms of Epstein-Barr virus latency counteracting apoptosis in c-myc-expressing Burkitt lymphoma cells. *Proc Natl Acad Sci U S A.* **103**:14935-14940.

Kelly GL, Long HM, Stylianou J, Thomas WA, Leese A, Bell AI, Bornkamm GW, Mautner J, Rickinson AB, Rowe M. (2009) An Epstein-Barr virus anti-apoptotic protein constitutively expressed in transformed cells and implicated in burkitt lymphomagenesis: the Wp/BHRF1 link. *PLoS Pathog.* **5**:e1000341.

Kennedy G, Komano J, Sugden B. (2003) Epstein-Barr virus provides a survival factor to Burkitt's lymphomas. *Proc Natl Acad Sci U S A.* **100**:14269-14274.

Kieff E. In: *Fields Virology*. Eds: Knipe DM, Howley PM, Lippincott Williams and Wilkins, Philadelphia; 1996; pp 2343-2396.

Kieff E, Rickinson AB. In: *Fields Virology*. Eds: Knipe DM, Howley PM, Lippincott Williams and Wilkins, Philadelphia; 2001; pp 2511-2574.

Kieff E, Rickinson AB. Epstein-Barr virus and its replication. In: *Fields Virology.* Eds: Knipe DM, Howley PM, Lippincott Williams and Wilkins, Philadelphia; 2007; pp. 2603-2654.

Kieser A, Kilger E, Gires O, Ueffing M, Kolch W, Hammerschmidt W. (1997) Epstein-Barr virus latent membrane protein-1 triggers AP-1 activity via the c-Jun N-terminal kinase cascade. *EMBO J.* **16**:6478-6485.

Kilger E, Kieser A, Baumann M, Hammerschmidt W. (1998) Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. *EMBO J.* **17**:1700-1709.

King W, Dambaugh T, Heller M, Dowling J, Kieff E. (1982) Epstein-Barr virus DNA XII. A variable region of the Epstein-Barr virus genome is included in the P3HR-1 deletion. *J Virol* **43**:979-986.

Klein G, Dombos L, Gothosokar B. (1972) Sensitivity of Epstein-Barr virus (EBV) producer and non-producer human lymphoblastoid cell lines to superinfection of with EB-virus. *Int. J. Cancer* **10**:44–57.

Klein G, Sugden B, Leibold W, Menezes J. (1974) Infection of EBV-genome-negative and -positive human lymphoblastoid cell lines with biologically different preparations of EBV. *Intervirology.* **3**:232-244.

Klein U, Tu Y, Stolovitzky GA, Keller JL, Haddad J Jr, Miljkovic V, Cattoretti G, Califano A, Dalla-Favera R. (2003) Transcriptional analysis of the B cell germinal center reaction. *Proc Natl Acad Sci U S A.* **100**:2639-2644.

Klein U, Casola S, Cattoretti G, Shen Q, Lia M, Mo T, Ludwig T, Rajewsky K, Dalla-Favera R. (2006) IRF4 controls plasma cell differentiation and class switch recombination. *Nature Immunol.* **7**:773-782.

Klein U, Dalla-Favera R. (2008) Germinal centres: role in B-cell physiology and malignancy. *Nat Rev Immunol.* **8**:22-33.

Knight JS, Sharma N, Kalman DE, Robertson ES. (2004) A cyclin-binding motif within the amino-terminal homology domain of EBNA3C binds cyclin A and modulates cyclin A-dependent kinase activity in Epstein-Barr virus-infected cells. *J Virol.* **78**:12857-12867.

Knight JS, Sharma N, Robertson ES. (2005) SCFSkp2 complex targeted by Epstein-Barr virus essential nuclear antigen. *Mol Cell Biol.* **25**:1749-1763.

Knödel M, Kuss AW, Lindemann D, Berberich I, Schimpl A. (1999) Reversal of Blimp-1-mediated apoptosis by A1, a member of the Bcl-2 family. *Eur J Immunol.* **29**:2988-2998.

Knödel M, Kuss AW, Berberich I, Schimpl A. (2001) Blimp-1 overexpression abrogates IL-4- and CD40-mediated suppression of terminal B cell differentiation but arrests isotype switching. *Eur. J. Immunol.* **31**:1972-1980.

Komano J, Sugiura M, Takada K. (1998) Epstein-Barr virus contributes to the malignant phenotype and to apoptosis resistance in Burkitt's lymphoma cell line Akata. *J Virol.* **72**: 9150-9156.

Kosco-Vilbois MH, Bonnefoy JY, Chvatchko Y. (1997) The physiology of murine germinal center reactions. *Immunol. Rev.* **156**:127-136.

Kube D, Vockerodt M, Weber O, Hell K, Wolf J, Haier B, Grasser FA, Muller-Lantzsch N, Kieff E, Diehl V, Tesch H (1999). Expression of epstein-barr virus nuclear antigen 1 is associated with enhanced expression of CD25 in the Hodgkin cell line L428. *J Virol.* **73**:1630-1636.

Kube D, Holtick U, Vockerodt M, Ahmadi T, Haier B, Behrmann I, Heinrich PC, Diehl V, Tesch H. (2001) STAT3 is constitutively activated in Hodgkin cell lines. *Blood* **98**:762-770.

Kudoh A, Fujita M, Kiyono T, Kuzushima K, Sugaya Y, Izuta S, Nishiyama Y, Tsurumi T (2003). Reactivation of lytic replication from B cells latently infected with Epstein-Barr virus occurs with high S-phase cyclin-dependent kinase activity while inhibiting cellular DNA replication. *J Virol.* **77**:851-861.

Kulwichit W, Edwards RH, Davenport EM, Baskar JF, Godfrey V, Raab-Traub N. (1998) Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. *Proc Natl Acad Sci U S A.* **95**:11963-11968.

Kuo TC, Calame KL. (2004). B lymphocyte-induced maturation protein (Blimp)-1, IFN regulatory factor (IRF)-1, and IRF-2 can bind to the same regulatory sites. *J. Immunol.* **173**:5556-5563.

Kuo TC, Shaffer AL, Haddad J Jr, Choi YS, Staudt LM, Calame K. (2007) Repression of BCL-6 is required for the formation of human memory B cells *in vitro*. *J. Exp. Med.* **204**:819-830.

Kuppers R, Rajewsky K, Zhao M, Simons G, Laumann R, Fischer R, Hansmann ML. (1994) Hodgkin disease: Hodgkin and Reed-Sternberg cells picked from histological sections show clonal immunoglobulin gene rearrangements and appear to be derived from B cells at various stages of development. *Proc Natl Acad Sci USA.* **91**:10962-10966.

Kuppers R, Klein U, Schwering I, Distler V, Brauninger A, Cattoretti G, Tu Y, Stolovitzky GA, Califano A, Hansmann ML, Dalla-Favera R. (2003) Identification of Hodgkin and Reed-Sternberg cell-specific genes by gene expression profiling. *J Clin Invest.* **111**:529-537.

Kuppers R, Hansmann M. (2005) The Hodgkin and Reed/Sternberg cell. *Int J Biochem Cell Biol.* 37:511-517.

Küppers R. (2009) Molecular biology of Hodgkin lymphoma. *Hematology Am Soc Hematol Educ Program.* 491-496.

Kurokawa M, Mitani K, Imai Y, Ogawa S, Yazaki Y, Hirai H. (1998a) The t(3;21) fusion product, AML1/Evi-1, interacts with Smad3 and blocks transforming growth factor-beta-mediated growth inhibition of myeloid cells. *Blood* **92**:4003-4012.

Kurokawa M, Mitani K, Irie K, Matsuyama T, Takahashi T, Chiba S, Yazaki Y, Matsumoto K, Hirai H. (1998b) The oncoprotein Evi-1 represses TGF-beta signalling by inhibiting Smad3. *Nature* **394**:92-96.

Kurosaki T. (1999) Genetic analysis of B cell antigen receptor signaling. *Annu. Rev. Immunol.* **17**:555-592.

Kwon H, Thierry-Mieg D, Thierry-Mieg J, Kim HP, Oh J, Tunyaplin C, Carotta S, Donovan CE, Goldman ML, Tailor P, Ozato K, Levy DE, Nutt SL, Calame K, Leonard WJ. (2009) Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional cooperation of STAT3 and IRF4 transcription factors. *Immunity* **31**:941-952.

Laherty CD, Hu HM, Opipari AW, Wang F, Dixit VM. (1992) The Epstein-Barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor kappa B. *J Biol Chem.* **267**:24157-24160.

Laichalk LL, Hochberg D, Babcock GJ, Freeman RB, Thorley-Lawson DA (2002). The dispersal of mucosal memory B cells: evidence from persistent EBV infection. *Immunity* **16**:745-754.

Laichalk LL, Thorley-Lawson DA. (2005) Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. *J. Virol.* **79**:1296-1307.

Lalmanach-Girard AC, Chiles TC, Parker DC, Rothstein TL. (1993) T cell-dependent induction of NF-kappa B in B cells. *J Exp Med.* **177**:1215-1219.

Lam N, Sugden B. (2003) CD40 and its viral mimic, LMP1: similar means to different ends. *Cell Signal.* **15**:9-16.

Landais E, Saulquin X, Houssaint E (2005). The human T cell immune response to Epstein-Barr virus. *Int J Dev Biol* **49**:285-292.

Larson ED, Maizels N. (2004) Transcription-coupled mutagenesis by the DNA deaminase AID. *Genome Biol.* **5**:211.

Laux G, Perricaudet M, Farrell PJ. (1988) A spliced Epstein-Barr virus gene expressed in immortalized lymphocytes is created by circularization of the linear viral genome. *EMBO J.* **7**:769-774.

Laux G, Economou A, Farrell PJ. (1989) The terminal protein gene 2 of Epstein-Barr virus is transcribed from a bidirectional latent promoter region. *J Gen Virol.* 70:3079-3084.

Lee CH, Melchers M, Wang H, Torrey TA, Slota R, Qi CF, Kim JY, Lugar P, Kong HJ, Farrington L, van der Zouwen B, Zhou JX, Lougaris V, Lipsky PE, Grammer AC, Morse HC 3rd. (2006) Regulation of the germinal center gene program by interferon (IFN) regulatory factor 8/IFN consensus sequence-binding protein. *J. Exp. Med.* **203**:63-72.

Leonard WJ, Spolski R. (2005) Interleukin-21: a modulator of lymphoid proliferation, apoptosis, and differentiation. *Nat. Rev. Immunol*. **5**:688-698.

Leung CS, Haigh TA, Mackay LK, Rickinson AB, Taylor GS. (2010) Nuclear location of an endogenously expressed antigen, EBNA1, restricts access to macroautophagy and the range of CD4 epitope display. *Proc Natl Acad Sci U S A.* **107**:2165-2170.

Levitskaya J, Coram M, Levitsky V, Imreh S, Steigerwald-Mullen PM, Klein G, Kurilla MG, Masucci MG. (1995) Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature* **375**:685-688.

Li Z, Van Calcar S, Qu C, Cavenee WK, Zhang MQ, Ren B. (2003) A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. *Proc Natl Acad Sci U S A.* **100**:8164-8169.

Lieber MR, Yu K, Raghavan SC. (2006) Roles of nonhomologous DNA end joining, V(D)J recombination, and class switch recombination in chromosomal translocations. *DNA Repair (Amst).* **5**:1234-1245.

Liebowitz D, Wang D, Kieff E. (1986) Orientation and patching of the latent infection membrane protein encoded by Epstein-Barr virus. *J Virol.* **58**: 233-237.

Lien C, Fang CM, Huso D, Livak F, Lu R, Pitha PM. (2010) Critical role of IRF-5 in regulation of B-cell differentiation. *Proc Natl Acad Sci U S A.* **107**:4664-4668.

Lin J, Lwin T, Zhao JJ, Tam W, Choi YS, Moscinski LC, Dalton WS, Sotomayor EM, Wright KL, Tao J. (2011) Follicular dendritic cell-induced microRNA-mediated upregulation of PRDM1 and downregulation of BCL-6 in non-Hodgkin's B-cell lymphomas. *Leukemia* **25**:145-152.

Lin KI, Lin Y, Calame K. (2000) Repression of c-myc is necessary but not sufficient for terminal differentiation of B lymphocytes in vitro. *Mol Cell Biol.* **20**:8684-8695.

Lin KI, Angelin-Duclos C, Kuo TC, Calame K. (2002) Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. *Mol Cell Biol.* **22**:4771-4780.

Lin KI, Tunyaplin C, Calame K. (2003) Transcriptional regulatory cascades controlling plasma cell differentiation. *Immunol Rev.* **194**:19-28.

Lin KI, Kao YY, Kuo HK, Yang WB, Chou A, Lin HH, Yu AL, Wong CH. (2006) Reishi polysaccharides induce immunoglobulin production through the TLR4/TLR2-mediated induction of transcription factor Blimp-1. *J. Biol. Chem.* **281**:24111-24123.

Lin L, Gerth AJ, Peng SL. (2004) Active inhibition of plasma cell development in resting B cells by microphthalmia-associated transcription factor. *J. Exp. Med.* **200**:115-122.

Lin Y, Wong K, Calame K. (1997) Repression of c-myc transcription by Blimp-1, an inducer of terminal B cell differentiation. *Science* **276**:596-599.

Lindstrom MS, Wiman KG. (2002) Role of genetic and epigenetic changes in Burkitt lymphoma. *Semin Cancer Biol.* **12**:381-387.

Linehan LA, Warren WD, Thompson PA, Grusby MJ, Berton MT. (1998) STAT6 is required for IL-4-induced germline Ig gene transcription and switch recombination. *J Immunol.* **161**:302-310.

Liu YJ, Mason DY, Johnson GD, Abbot S, Gregory CD, Hardie DL, Gordon J, MacLennan IC. (1991) Germinal center cells express bcl-2 protein after activation by signals which prevent their entry into apoptosis. *Eur J Immunol.* **21**:1905-1910.

Liu YY, Leboeuf C, Shi JY, Li JM, Wang L, Shen Y, Garcia JF, Shen ZX, Chen Z, Janin A, Chen SJ, Zhao WL. (2007) Rituximab plus CHOP (R-CHOP) overcomes PRDM1-associated resistance to chemotherapy in patients with diffuse large B-cell lymphoma. *Blood* **110**: 339-344.

Longnecker R, Kieff E. (1990) A second Epstein-Barr virus membrane protein (LMP2) is expressed in latent infection and colocalizes with LMP1. *J Virol.* **64**:2319-2326.

Lupton S, Levine AJ. (1985) Mapping genetic elements of Epstein- Barr virus that facilitate extrachromosomal persistence of Epstein-Barr virus-derived plasmids in human cells. *Mol. Cell. Biol.* **5**:2533-2542.

Lyss G, Knorre A, Schmidt TJ, Pahl HL, Merfort I. (1998) The anti inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-kappaB by directly targeting p65. *J. Biol. Chem.* **273**:33508-33516.

MacLennan IC, Liu YJ, Oldfield S, Zhang J, Lane PJ. (1990) The evolution of B-cell clones *Curr Top Microbiol Immunol.* **159**:37-63.

MacLennan IC. (1994) Germinal centers. *Annu. Rev. Immunol.* **12**:117-139.

MacMahon B. (1966) Epidemiology of Hodgkin's disease. *Cancer Res.* **26**:1189-1201.

Magrath I. (1990) The pathogenesis of Burkitt's lymphoma. *Adv Cancer Res.* **55**:133-270.

Mancao C, Altmann M, Jungnickel B, Hammerschmidt W. (2005) Rescue of "crippled" germinal center B cells from apoptosis by Epstein-Barr virus. *Blood* **106**:4339-4344.

Mancao C, Hammerschmidt W. (2007) Epstein-Barr virus latent membrane protein 2A is a B-cell receptor mimic and essential for B-cell survival. *Blood* **110**:3715-3721.

Mandelbaum J, Bhagat G, Tang H, Mo T, Brahmachary M, Shen Q, Chadburn A, Rajewsky K, Tarakhovsky A, Pasqualucci L, Dalla-Favera R. (2010) BLIMP1 is a tumor suppressor gene frequently disrupted in activated B cell-like diffuse large B cell lymphoma. *Cancer Cell* **18**:568-579.

Manis JP, Gu Y, Lansford R, Sonoda E, Ferrini R, Davidson L, Rajewsky K, Alt FW. (1998) Ku70 is required for late B cell development and immunoglobulin heavy chain class switching. *J Exp Med.* **187**:2081-2089.

Manz RA, Thiel A, Radbruch A. (1997) Lifetime of plasma cells in the bone marrow. *Nature* **388**:133-134.

Manz RA, Lohning M, Cassese G, Thiel A, Radbruch A. (1998) Survival of long-lived plasma cells is independent of antigen. *Int. Immunol.* **10**:1703-1711.

Manz RA, Arce S, Cassese G, Hauser AE, Hiepe F, Radbruch A. (2002) Humoral immunity and long-lived plasma cells. *Curr. Opin. Immunol.* **14**:517-521.

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

Marechal V, Dehee A, Chikhi-Brachet R, Piolot T, Coppey-Moisan M, Nicolas JC. (1999) Mapping EBNA-1 domains involved in binding to metaphase chromosomes. *J. Virol.* **73**:4385-4392.

Marr-Belvin AK, Carville AK, Fahey MA, Boisvert K, Klumpp SA, Ohashi M, Wang F, O'Neil SP, Westmoreland SV. (2008) Rhesus lymphocryptovirus type 1-associated B-cell nasal lymphoma in SIV-infected rhesus macaques. *Vet Pathol.* **45**:914-921.

Marshall NA, Christie LE, Munro LR, Culligan DJ, Johnston PW, Barker RN, Vickers MA. (2004) Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood* **103**:1755-1762.

Martin F, Oliver AM, Kearney JF. (2001) Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* **14**:617-629.

Martin F, Kearney JF. (2002) Marginal-zone B cells. *Nat Rev Immunol.* **2**:323-335.

Martinez-Valdez H, Guret C, de Bouteiller O, Fugier I, Banchereau J, Liu YJ. (1996) Human germinal center B cells express the apoptosis-inducing genes *Fas*, *c-myc*, *P53*, and *Bax* but not the survival gene *bcl-2*. *J. Exp. Med.* **183**:971–977.

Martins G, Calame K. (2008) Regulation and functions of Blimp-1 in T and B lymphocytes. *Annu Rev Immunol.* **26**:133-169.

Mason DY, Banks PM, Chan J, Cleary ML, Delsol G, de Wolf Peeters C, Falini B, Gatter K, Grogan TM, Harris NL. (1994) Nodular lymphocyte predominance Hodgkin's disease. A distinct clinicopathological entity. *Am J Surg Pathol.* **18**:526-530.

Mathas S, Hinz M, Anagnostopoulos I, Krappmann D, Lietz A, Jundt F, Bommert K, Mechta-Grigoriou F, Stein H, Dörken B, Scheidereit C. (2002) Aberrantly expressed c-Jun and JunB are a hallmark of Hodgkin lymphoma cells, stimulate proliferation and synergize with NF-kappa B. *EMBO J.* **21**:4104-4113.

Mathas S, Lietz A, Anagnostopoulos I, Hummel F, Wiesner B, Janz M, Jundt F, Hirsch B, Jöhrens-Leder K, Vornlocher HP, Bommert K, Stein H, Dörken B. (2004) C-Flip mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. *J Exp Med.* **199**:1041-1052.

Mathas S, Janz M, Hummel F, Hummel M, Wollert-Wulf B, Lusatis S, Anagnostopoulos I, Lietz A, Sigvardsson M, Jundt F, Jöhrens K, Bommert K, Stein H, Dörken B. (2006) Intrinsic inhibition of transcription factor E2A by HLH proteins ABF-1 and Id2 mediates reprogramming of neoplastic B cells in Hodgkin lymphoma. *Nat Immunol.* **7**:207-215.

McAdam AJ, Greenwald RJ, Levin MA, Chernova T, Malenkovich N, Ling V, Freeman GJ, Sharpe AH. (2001) ICOS is critical for CD40-mediated antibody class switching. *Nature* **409**:102-105.

McHeyzer-Williams LJ, Cool M, McHeyzer-Williams MG. (2000) Antigen-specific B cell memory: expression and replenishment of a novel B220- memory b cell compartment. *J. Exp. Med.* **191**:1149–1166.

Messika EJ, Lu PS, Sung YJ, Yao T, Chi JT, Chien YH, Davis MM. (1998) Differential effect of B lymphocyte-induced maturation protein (Blimp-1) expression on cell fate during B cell development. *J. Exp. Med.* **188**:515-525.

Meyer RM, Ambinder RF, Stroobants S. (2004) Hodgkin's lymphoma: evolving concepts with implications for practice. *Hematology Am Soc Hematol Educ Program. 2004:184-202.*

Miller CL, Lee JH, Kieff E, Longnecker R. (1994) An integral membrane protein (LMP2) blocks reactivation of Epstein-Barr virus from latency following surface immunoglobulin crosslinking. *Proc Natl Acad Sci USA.* **91**:772-776.

Miller CL, Burkhardt AL, Lee JH, Stealey B, Longnecker R, Bolen JB, Kieff E. (1995) Integral membrane protein 2 of Epstein-Barr virus regulates reactivation from latency through dominant negative effects on protein-tyrosine kinases. *Immunity* **2**:155-166.

Miller G, Rabson M, Heston L. (1984) Epstein-Barr virus with heterogeneous DNA disrupts latency. *J Virol.* **50**:174-182.

Minges Wols HA, Underhill GH, Kansas GS, Witte PL. (2002) The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. *J Immunol.* **169**:4213-4221.

Mochizuki N, Shimizu S, Nagasawa T, Tanaka H, Taniwaki M, Yokota J, Morishita K. (2000) A novel gene, *MEL1*, mapped to 1p36.3 is highly homologous to the *MDS1/EVI1* gene and is transcriptionally activated in t(1;3)(p36;q21)-positive leukemia cells. *Blood* **96**:3209-3214.

Mock BA, Liu L, LePaslier D, Huang S. (1996) The B-lymphocyte maturation promoting transcription factor BLIMP1/PRDI-BF1 maps to D6S447 on human chromosome 6q21-q22.1 and the syntenic region of mouse chromosome 10. *Genomics* **37**:24-28.

Moorthy RK, Thorley-Lawson DA. (1993) All three domains of the Epstein-Barr virus-encoded latent membrane protein LMP-1 are required for transformation of rat-1 fibroblasts. *J Virol.* **67**:1638-1646.

Mora-Lopez F, Reales E, Brieva JA, Campos-Caro. (2007) A: Human BSAP and BLIMP1 conform an autoregulatory feedback loop. *Blood* **110**:3150-3157.

Morgan DG, Niederman JC, Miller G, Smith HW, Dowaliby JM. (1979) Site of Epstein-Barr virus replication in the oropharynx. *Lancet.* **2**:154-1157.

Morgan MA, Magnusdottir E, Kuo TC, Tunyaplin C, Harper J, Arnold SJ, Calame K, Robertson EJ, Bikoff EK. (2009) Blimp-1/Prdm1 alternative promoter usage during mouse development and plasma cell differentiation. *Mol Cell Biol.* **29**:5813-5827.

Moriggl R, Topham DJ, Teglund S, Sexl V, McKay C, Wang D, Hoffmeyer A, van Deursen J, Sangster MY, Bunting KD, Grosveld GC, Ihle JN. (1999) Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity* **10**:249-259.

Morishita K. (2007) Leukemogenesis of the EVI1/MEL1 gene family. *Int J Hematol.* **85**:279-286.

Mosialos G, Birkenbach M, Yalamanchili R, VanArsdale T, Ware C, Kieff E. (1995) The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell* **80**:389-399.

Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. (2000) Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102**:553-563.

Murray PG, Young LS, Rowe M, Crocker J. (1992) Immunohistochemical demonstration of the Epstein-Barr virus-encoded latent membrane protein in paraffin sections of Hodgkin's disease. *J Pathol.* **166**:1-5.

Murray PG, Young LS (2001). Epstein-Barr virus infection: basis of malignancy and potential for therapy. *Expert Rev Mol Med.* **2001**:1-20.

Muto A, Tashiro S, Nakajima O, Hoshino H, Takahashi S, Sakoda E, Ikebe D, Yamamoto M, Igarashi K. (2004) The transcriptional programme of antibody class switching involves the repressor Bach2. *Nature.* **429**:566-571.

Muto A, Ochiai K, Kimura Y, Itoh-Nakadai A, Calame KL, Ikebe D, Tashiro S, Igarashi K. (2010) Bach2 represses plasma cell gene regulatory network in B cells to promote antibody class switch. *EMBO J.* **29**:4048-4061.

Nakagomi H, Dolcetti R, Bejarano MT, Pisa P, Kiessling R, Masucci MG. (1994) The Epstein-Barr virus latent membrane protein-1 (LMP1) induces interleukin-10 production in Burkitt lymphoma lines. *Int J Cancer.* **57**:240-244.

Natkunam Y, Hsi ED, Aoun P, Zhao S, Elson P, Pohlman B, Naushad H, Bast M, Levy R, Lossos IS. (2007) Expression of the human germinal center-associated lymphoma (HGAL) protein identifies a subset of classic Hodgkin lymphoma of germinal center derivation and improved survival. *Blood* **109**:298-305.

Nemerow GR, Mold C, Schwend VK, Tollefson V, Cooper NR. (1987) Identification of gp350 as the viral glycoprotein mediating attachment of Epstein–Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complment fragment C3d. *J Virol.* **61**:1416-1420.

Nera KP, Kohonen P, Narvi E, Peippo A, Mustonen L, Terho P, Koskela K, Buerstedde JM, Lassila O. (2006) Loss of Pax5 promotes plasma cell differentiation. *Immunity* **24**:283-293.

Neri A, Barriga F, Inghirami G, Knowles DM, Neequaye J, Magrath IT, Dalla-Favera R. (1991) Epstein-Barr virus infection precedes clonal expansion in Burkitt's and acquired immunodeficiency syndrome-associated lymphoma. *Blood* **77**:1092-1095.

Neuberger MS, Di Noia JM, Beale RC, Williams GT, Yang Z, Rada C. (2005) Somatic hypermutation at A.T pairs: polymerase error versus dUTP incorporation. *Nat Rev Immunol.* **5**:171-178.

Newcom SR, Gu L. (1995) Transforming growth factor beta 1 messenger RNA in Reed-Sternberg cells in nodular sclerosing Hodgkin's disease. *J Clin Pathol.* **48**:160-163.

Nie K, Gomez M, Landgraf P, Garcia JF, Liu Y, Tan LH, Chadburn A, Tuschl T, Knowles DM, Tam W. (2008) MicroRNA-mediated down-regulation of PRDM1/Blimp-1 in Hodgkin/Reed-Sternberg cells: a potential pathogenetic lesion in Hodgkin lymphomas. *Am J Pathol.* **173**:242-252.

Nie K, Zhang T, Allawi H, Gomez M, Liu Y, Chadburn A, Wang YL, Knowles DM, Tam W. (2010) Epigenetic down-regulation of the tumor suppressor gene PRDM1/Blimp-1 in diffuse large B cell lymphomas: a potential role of the microRNA let-7. *Am J Pathol.* **177**:1470-1479.

Niedobitek G, Agathanggelou A, Herbst H, Whitehead L, Wright DH, Young LS. (1997) Epstein-Barr virus (EBV) infection in infectious mononucleosis: virus latency, replication and phenotype of EBV-infected cells. *J. Pathol.* **182**:151-159.

Niedobitek G, Agathanggelou A, Steven N, Young LS. (2000) Epstein-Barr virus (EBV) in infectious mononucleosis: detection of the virus in tonsillar B lymphocytes but not in desquamated oropharyngeal epithelial cells. *Mol Pathol.* **53**:37-42.

Nilsson K, Bennich H, Johansson SG, Pontén J. (1970) Established immunoglobulin producing myeloma (IgE) and lymphoblastoid (IgG) cell lines from an IgE myeloma patient. *Clin Exp Immunol.* **7**:477-489.

Nitsche F, Bell A, Rickinson A. (1997) Epstein-Barr virus leader protein enhances EBNA-2-mediated transactivation of latent membrane protein 1 expression: a role for the W1W2 repeat domain. *J Virol.* **71**:6619-6628.

Niu H, Ye BH, Dalla-Favera R. (1998) Antigen receptor signaling induces MAP kinase-mediated phosphorylation and degradation of the BCL-6 transcription factor. *Genes Dev.* **12**: 1953-1961.

Niu H, Cattoretti G, Dalla-Favera R. (2003) BCL6 controls the expression of the B7-1/CD80 costimulatory receptor in germinal center B cells. *J. Exp. Med.* **198**:211-221.

Nonkwelo C, Skinner J, Bell A, Rickinson A, Sample J. (1996) Transcription start sites downstream of the Epstein-Barr virus (EBV) Fp promoter in early-passage Burkitt lymphoma cells define a fourth promoter for expression of the EBV EBNA-1 protein. *J Virol.* **70**:623-627.

Nucifora G. (1997) The EVI1 gene in myeloid leukemia. *Leukemia* **11**:2022-2031.

Ocaña E, González-García I, Gutiérrez NC, Mora-López F, Brieva JA, Campos-Caro A. (2006) The expression of PRDI-BF1 beta isoform in multiple myeloma plasma cells. *Haematologica* **91**:1579-1580.

Ochiai K, Katoh Y, Ikura T, Hoshikawa Y, Noda T, Karasuyama H, Tashiro S, Muto A, Igarashi K. (2006) Plasmacytic transcription factor Blimp-1 is repressed by Bach2 in B cells. *J. Biol. Chem.* **281**:38226-38234.

O'Connor BP, Raman VS, Erickson LD, Cook WJ, Weaver LK, Ahonen C, Lin LL, Mantchev GT, Bram RJ, Noelle RJ. (2004) BCMA is essential for the survival of long-lived bone marrow plasma cells. *J. Exp. Med.* **199**:91-98.

Okkenhaug K, Ali K, Vanhaesebroeck B. (2007) Antigen receptor signalling: a distinctive role for the p110delta isoform of PI3K. *Trends Immunol.* **28**:80-87.

Omori SA, Cato MH, Anzelon-Mills A, Puri KD, Shapiro-Shelef M, Calame K, Rickert RC. (2006) Regulation of class-switch recombination and plasma cell differentiation by phosphatidylinositol 3-kinase signaling. *Immunity* **25**:545-557.

O'Neil JD, Owen TJ, Wood VH, Date KL, Valentine R, Chukwuma MB, Arrand JR, Dawson CW, Young LS. (2008) Epstein-Barr virus-encoded EBNA1 modulates the AP-1 transcription factor pathway in nasopharyngeal carcinoma cells and enhances angiogenesis in vitro. *J Gen Virol.* **89**:2833-2842.

Owen TJ, O'Neil JD, Dawson CW, Hu C, Chen X, Yao Y, Wood VH, Mitchell LE, White RJ, Young LS, Arrand JR. (2010) Epstein-Barr virus-encoded EBNA1 enhances RNA polymerase III-dependent EBER expression through induction of EBER-associated cellular transcription factors. *Mol Cancer.* **9**:241.

Ozaki K, Spolski R, Ettinger R, Kim HP, Wang G, Qi CF, Hwu P, Shaffer DJ, Akilesh S, Roopenian DC, Morse HC 3rd, Lipsky PE, Leonard WJ. (2004) Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J. Immunol.* **173**:5361-5371.

Pajic A, Staege MS, Dudziak D, Schuhmacher M, Spitkovsky D, Eissner G, Brielmeier M, Polack A, Bornkamm GW (2001). Antagonistic effects of c-myc and Epstein-Barr virus latent genes on the phenotype of human B cells. *Int J Cancer* **93**:810-816.

Pallesen G, Hamilton-Dutoit SJ, Rowe M, Young LS. (1991) Expression of Epstein-Barr virus latent gene products in tumour cells of Hodgkin's disease. *Lancet.* **337**:320-322.

Panagopoulos D, Victoratos P, Alexiou M, Kollias G, Mosialos G. (2004) Comparative Analysis of signal Transduction by CD40 and the EBV oncoprotein LMP1 in vivo. *J Virol.* **78**:13253-13261.

Papavasiliou FN, Schatz DG. (2000) Cell-cycle-regulated DNA double-stranded breaks in somatic hypermutation of immunoglobulin genes. *Nature* **408**:216-221.

Parekh S, Polo JM, Shaknovich R, Juszczynski P, Lev P, Ranuncolo SM, Yin Y, Klein U, Cattoretti G, Dalla Favera R, Shipp MA, Melnick A. (2007) BCL6 programs lymphoma cells

for survival and differentiation through distinct biochemical mechanisms. *Blood* **110**:2067-2074.

Parker GA, Crook T, Bain M, Sara EA, Farrell PJ, Allday MJ. (1996) Epstein-Barr virus nuclear antigen (EBNA)3C is an immortalizing oncoprotein with similar properties to adenovirus E1A and papillomavirus E7. Oncogene 13:2541-2549.

Pasare C, Medzhitov R. (2005) Control of B-cell responses by Toll-like receptors. *Nature* **438**:364-368.

Pasqualucci L, Compagno M, Houldsworth J, Monti S, Grunn A, Nandula SV, Aster JC, Murty VV, Shipp MA, Dalla-Favera R. (2006) Inactivation of the PRDM1/BLIMP1 gene in diffuse large B cell lymphoma. *J Exp Med.* **203**:311-317.

Paus D, Phan TG, Chan TD, Gardam S, Basten A, Brink R. (2006) Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J. Exp. Med.* **203**:1081-1091.

Petti L, Sample C, Kieff E. (1990) Subnuclear localization and phosphorylation of Epstein-Barr virus latent infection nuclear proteins. *Virology* **176**:563-574.

Phan RT, Dalla-Favera R. (2004) The BCL6 protooncogene suppresses p53 expression in germinal centre B cells. *Nature* **432**:635-639.

Phan RT, Saito M, Basso K, Niu H, Dalla-Favera R. (2005) BCL6 interacts with the transcription factor Miz-1 to suppress the cyclin-dependent kinase inhibitor p21 and cell cycle arrest in germinal center B cells. *Nature Immunol.* **6**:1054-1060.

Phan RT, Saito M, Kitagawa Y, Means A, Dalla-Favera R. (2007) Genotoxic stress regulates expression of the BCL6 proto-oncogene in germinal center B cells. *Nature Immunol.* **8**:1132-1139.

Phan TG, Paus D, Chan TD, Turner ML, Nutt SL, Basten A, Brink R. (2006) High affinity germinal center B cells are actively selected into the plasma cell compartment. *J. Exp. Med.* **203**:2419-2424.

Pinto A, Gattei V, Zagonel V, Aldinucci D, Degan M, De Iuliis A, Rossi FM, Tassan Mazzocco F, Godeas C, Rupolo M, Poletto D, Gloghini A, Carbone A, Gruss HJ. (1998) Hodgkin's disease: a disorder of dysregulated cellular cross-talk. *Biotherapy* **10**:309-320.

Piskurich JF, Lin KI, Lin Y, Wang Y, Ting JP, Calame K. (2000) BLIMP-I mediates extinction of major histocompatibility class II transactivator expression in plasma cells. *Nat. Immunol.* **1**:526-532.

Pleiman CM, Abrams C, Gauen LT, Bedzyk W, Jongstra J, Shaw AS, Cambier JC. (1994) Distinct p53/56lyn and p59fyn domains associate with nonphosphorylated and phosphorylated Ig-alpha. *Proc Natl Acad Sci U S A.* **91**:4268-4672.

Polack A, Delius H, Zimber U, Bornkamm GW. (1984) Two deletions in the Epstein-Barr virus genome of the Burkitt lymphoma nonproducer line Raji. *Virology* **133**:146-157.

Polack A, Hörtnagel K, Pajic A, Christoph B, Baier B, Falk M, Mautner J, Geltinger C, Bornkamm GW, Kempkes B. (1996) c-myc activation renders proliferation of Epstein-Barr virus (EBV)-transformed cells independent of EBV nuclear antigen 2 and latent membrane protein 1. *Proc Natl Acad Sci U S A.* **93**:10411-10416.

Polo JM, Dell'Oso T, Ranuncolo SM, Cerchietti L, Beck D, Da Silva GF, Prive GG, Licht JD, Melnick A. (2004) Specific peptide interference reveals BCL6 transcriptional and oncogenic mechanisms in B-cell lymphoma cells. *Nat Med.* **10**:1329-1335.

Portis T, Dyck P, Longnecker R. (2003) Epstein-Barr Virus (EBV) LMP2A induces alterations in gene transcription similar to those observed in Reed-Sternberg cells of Hodgkin lymphoma. *Blood* **102**:4166-4178.

Prince S, Keating S, Fielding C, Brennan P, Floettmann E, Rowe M. (2003) Latent membrane protein 1 inhibits Epstein-Barr virus lytic cycle induction and progress via different mechanisms. *J Virol.* **77**: 5000-5007.

Raaphorst FM, van Kemenade FJ, Blokzijl T, Fieret E, Hamer KM, Satijn DP, Otte AP, Meijer CJ. (2000) Coexpression of BMI-1 and EZH2 polycomb group genes in Reed-Sternberg cells of Hodgkin's disease. *Am J Pathol.* **157**:709-715.

Radkov SA, Touitou R, Brehm A, Rowe M, West M, Kouzarides T, Allday MJ. Epstein-Barr virus nuclear antigen 3C interacts with histone deacetylase to repress transcription. *J Virol.* **73**:5688-5697.

Ragoczy T, Miller G. (1999) Role of the epstein-barr virus RTA protein in activation of distinct classes of viral lytic cycle genes. *J Virol.* **73**:9858-9866.

Rajewsky K (1996) Clonal selection and learning in the antibody system. *Nature* **381**:751-758.

Ramiro AR, Stavropoulos P, Jankovic M, Nussenzweig MC. (2003) Transcription enhances AID mediated cytidine deamination by exposing single stranded DNA on the non-template strand. *Nature Immunol.* **4**:452–456.

Randall TD, Heath AW, Santos-Argumedo L, Howard MC, Weissman IL, Lund FE. (1998) Arrest of B lymphocyte terminal differentiation by CD40 signaling: mechanism for lack of antibody-secreting cells in germinal centers. *Immunity* **8**:733-742.

Ranuncolo SM, Polo JM, Dierov J, Singer M, Kuo T, Greally J, Green R, Carroll M, Melnick A. (2007) Bcl-6 mediates the germinal center B cell phenotype and lymphomagenesis through transcriptional repression of the DNA-damage sensor ATR. *Nat Immunol.* **8**:705-714.

Rastelli J, Hömig-Hölzel C, Seagal J, Müller W, Hermann AC, Rajewsky K, Zimber-Strobl U. (2008) LMP1 signaling can replace CD40 signaling in B cells in vivo and has unique features of inducing class-switch recombination to IgG1. *Blood* **111**:1448-1455.

Razzouk BI, Srinivas S, Sample CE, Singh V, Sixbey JW. (1996) Epstein-Barr Virus DNA recombination and loss in sporadic Burkitt's lymphoma. *J Infect Dis.* **173**:529-535.

Re D, Hofmann A, Wolf J, Diehl V, Staratschek-Jox A. (2000) Cultivated H-RS cells are resistant to CD95L-mediated apoptosis despite expression of wild-type CD95. *Exp Hematol.* **28**:31-35.

Re D, Müschen M, Ahmadi T, Wickenhauser C, Staratschek-Jox A, Holtick U, Diehl V, Wolf J. (2001) Oct-2 and Bob-1 deficiency in Hodgkin and Reed Sternberg cells. *Cancer Res.* **61**:2080-2084.

Rechsteiner MP, Berger C, Weber M, Sigrist JA, Nadal D, Bernasconi M. (2007) Silencing of latent membrane protein 2B reduces susceptibility to activation of lytic Epstein-Barr virus in Burkitt's lymphoma Akata cells. *J Gen Virol.* **88**:1454-1459.

Reed DM. (1902) On the pathological changes in Hodgkin's disease with special reference to its relation to tuberculosis. *Johns Hopkins Hosp Rep.* **10**:133-196.

Reimold AM, Ponath PD, Li YS, Hardy RR, David CS, Strominger JL, Glimcher LH. (1996) Transcription factor B cell lineage-specific activator protein regulates the gene for human X-box binding protein 1. *J Exp Med.* **183**:393-401.

Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, Gravallese EM, Friend D, Grusby MJ, Alt F, Glimcher LH. (2001) Plasma cell differentiation requires the transcription factor XBP-1. *Nature* **412**: 300-307.

Reljic R, Wagner SD, Peakman LJ, Fearon DT. (2000) Suppression of signal transducer and activator of transcription 3-dependent B lymphocyte terminal differentiation by BCL-6. *J. Exp. Med.* **192**:1841-1848.

Ren B, Chee KJ, Kim TH, Maniatis T. (1999) PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. *Genes Dev.* **13**:125-137.

Renne C, Willenbrock K, Kuppers R, Hansmann ML, Brauninger A. (2005) Autocrine- and paracrine-activated receptor tyrosine kinases in classic Hodgkin lymphoma. *Blood* **105**:4051-4059.

Renné C, Martin-Subero JI, Eickernjäger M, Hansmann ML, Küppers R, Siebert R, Bräuninger. (2006) Aberrant expression of ID2, a suppressor of B-cell-specific gene expression, in Hodgkin's lymphoma. *Am J Pathol.* **169**:655-664.

Revy P, Muto T, Levy Y, Geissmann F, Plebani A, Sanal O, Catalan N, Forveille M, Dufourcq-Labelouse R, Gennery A, Tezcan I, Ersoy F, Kayserili H, Ugazio AG, Brousse N, Muramatsu M, Notarangelo LD, Kinoshita K, Honjo T, Fischer A, Durandy A. (2000) Activation-induced

cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* **102**:565-575.

Rickinson AB, Kieff E. In: *Fields Virology*. Eds: Knipe DM, Howley PM, Lippincott Williams and Wilkins, Philadelphia; 2001; pp 2575-2627.

Riley JK, Takeda K, Akira S, Schreiber RD. (1999) Interleukin-10 receptor signaling through the JAK-STAT pathway: requirement for two distinct receptor-derived signals for anti-inflammatory action. *J. Biol. Chem.* **274**:16513-16521.

Rinkenberger JL, Wallin JJ, Johnson KW, Koshland ME. (1996) An interleukin-2 signal relieves BSAP (Pax5)-mediated repression of the immunoglobulin J chain gene. *Immunity* **5**:377-386.

Roberts ML, Cooper NR. (1998) Activation of a ras-MAPK-dependent pathway by Epstein-Barr virus latent membrane protein 1 is essential for cellular transformation. *Virology* **240**:93-99.

Robertson KD, Ambinder RF. (1997) Mapping promoter regions that are hypersensitive to methylation-mediated inhibition of transcription: application of the methylation cassette assay to the Epstein-Barr virus major latency promoter. *J Virol.* **71**:6445-6454.

Rooney CM, Rowe DT, Ragot T, Farrell PJ. (1989) The spliced BZLF1 gene of Epstein-Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. *J Virol.* **63**:3109-3116.

Rooney CM, Brimmell M, Buschle M, Allan G, Farrell PJ, Kolman JL. (1992) Host cell and EBNA-2 regulation of Epstein-Barr virus latent-cycle promoter activity in B lymphocytes. *J Virol.* **66**:496-504.

Rothstein TL. (2000) Inducible resistance to Fas-mediated apoptosis in B cells. *Cell Res.* **10**:245-266.

Roughan JE, Thorley-Lawson DA. (2009) The intersection of Epstein-Barr virus with the germinal center. *J Virol.* **83**: 3968-3976.

Rousset F, Garcia E, Defrance T, Péronne C, Vezzio N, Hsu DH, Kastelein R, Moore KW, Banchereau J. (1992) Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc. Natl. Acad. Sci. USA* **89**:1890-1893.

Rovedo M, Longnecker R. (2007) Epstein Barr Virus Latent Membrane Protein 2B (LMP2B) Modulates LMP2A Activity. *J Virol.* **81**: 84-94.

Rovigatti U, Watson DK, Yunis JJ. (1986) Amplification and rearrangement of Hu-ets-1 in leukemia and lymphoma with involvement of 11q23. *Science* **232**:398-400.

Rowe M, Rowe DT, Gregory CD, Young LS, Farrell PJ, Rupani H, Rickinson AB. (1987). Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. *EMBO J.* **6**:2743-2751.

Rowe M, Lear AL, Croom-Carter D, Davies AH, Rickinson AB. (1992) Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. *J Virol.* **66**:122-131.

Rowe M, Glaunsinger B, van Leeuwen D, Zuo J, Sweetman D, Ganem D, Middeldorp J, Wiertz EJHJ, Ressing ME. (2007) Host shutoff during productive Epstein–Barr virus infection is mediated by BGLF5 and may contribute to immune evasion. *Proc Natl Acad Sci USA* **104**:3366-3371.

Rowe M, Kelly GL, Bell AI, Rickinson AB. (2009) Burkitt's lymphoma: the Rosetta Stone deciphering Epstein-Barr virus biology. *Semin Cancer Biol.* **19**:377-388.

Ruf IK, Rhyne PW, Yang C, Cleveland JL, Sample JT. (2000) Epstein-Barr virus small RNAs potentiate tumorigenicity of Burkitt lymphoma cells independently of an effect on apoptosis. *J Virol.* **74**:10223-10228.

Rui L, Healy JI, Blasioli J, Goodnow CC. (2006) ERK signaling is a molecular switch integrating opposing inputs from B cell receptor and T cell cytokines to control TLR4-driven plasma cell differentiation. *J. Immunol.* **177**:5337-5346.

Ruprecht CR, Lanzavecchia A. (2006) Toll-like receptor stimulation as a third signal required for activation of human naive B cells. *Eur J Immunol.* **36**:810-816.

Ryan EP, Pollock SJ, Murant TI, Bernstein SH, Felgar RE, Phipps RP. (2005) Activated human B lymphocytes express cyclooxygenase-2 and cyclooxygenase inhibitors attenuate antibody production. *J Immunol.* **174**:2619–2626.

Sait SN, Qadir MU, Conroy JM, Matsui S, Nowak NJ, Baer MR. (2002) Double minute chromosomes in acute myeloid leukemia and myelodysplastic syndrome: identification of new amplification regions by fluorescence in situ hybridization and spectral karyotyping. *Genes Chromosomes Cancer.* **34**:42-47.

Saito M, Gao J, Basso K, Kitagawa Y, Smith PM, Bhagat G, Pernis A, Pasqualucci L, Dalla-Favera R. (2007) A signaling pathway mediating downregulation of BCL6 in germinal center B cells is blocked by BCL6 gene alterations in B cell lymphoma. *Cancer Cell.* **3**:280-92.

Sakai T, Honjo T. (1997) [Transcriptional activity of EBNA2 through RBP-J]. *Nippon Rinsho* **55**:293-298.

Sample J, Liebowitz D, Kieff E. (1989) Two related Epstein-Barr virus membrane proteins are encoded by separate genes. *J Virol.* **63**:933-937.

Sánchez-Beato M, Sánchez E, García JF, Pérez-Rosado A, Montoya MC, Fraga M, Artiga MJ, Navarrete M, Abraira V, Morente M, Esteller M, Koseki H, Vidal M, Piris MA. (2004) Abnormal PcG protein expression in Hodgkin's lymphoma. Relation with E2F6 and NFkappaB transcription factors. *J Pathol.* **204**:528-537.

Sasaki O, Meguro K, Tohmiya Y, Funato T, Shibahara S, Sasaki T. (2002) Altered expression of retinoblastoma protein-interacting zinc finger gene, RIZ, in human leukaemia. *Br J Haematol.* **119**:940-948.

Satpathy S, Shenoy GN, Kaw S, Vaidya T, Bal V, Rath S, George A. (2010) Inhibition of terminal differentiation of B cells mediated by CD27 and CD40 involves signaling through JNK. *J Immunol.* **185**:6499-6507.

Savitsky D, Calame K. (2006) B-1 B lymphocytes require Blimp-1 for immunoglobulin secretion. *J. Exp. Med.* **203**:2305-14.

Sayegh CE, Quong MW, Agata Y, Murre C. (2003) E-proteins directly regulate expression of activationinduced deaminase in mature B cells. *Nature Immunol.* **4**:586-593.

Schaadt E, Baier B, Mautner J, Bornkamm GW, Adler B. (2005) Epstein-Barr virus latent membrane protein 2A mimics B-cell receptor-dependent virus reactivation. *J Gen Virol.* **86**:551-559.

Schaadt M, Diehl V, Stein H, Fonatsch C, Kirchner HH. (1980) Two neoplastic cell lines with unique features derived from Hodgkin's disease. *Int J Cancer.* **26**:723-731.

Schebesta A, McManus S, Salvagiotto G, Delogu A, Busslinger GA, Busslinger M. (2007) Transcription factor Pax5 activates the chromatin of key genes involved in B cell signaling, adhesion, migration, and immune function. *Immunity* **27**:49-63.

Scheeren FA, Naspetti M, Diehl S, Schotte R, Nagasawa M, Wijnands E, Gimeno R, Vyth-Dreese FA, Blom B, Spits H. (2005) STAT5 regulates the self-renewal capacity and differentiation of human memory B cells and controls Bcl-6 expression. *Nat. Immunol.* **6**:303-313.

Schlager S, Speck SH, Woisetschlager M. (1996) Transcription of the Epstein-Barr virus nuclear antigen 1 (EBNA1) gene occurs before induction of the BCR2 (Cp) EBNA gene promoter during the initial stages of infection in B cells. *J Virol.* **70**:3561-3570.

Schliephake DE, Schimpl A. (1996). Blimp-1 overcomes the block in IgM secretion in lipopolysaccharide/antimu F(ab')2-costimulated B lymphocytes. *Eur. J. Immunol.* **26**:268-71.

Schmidt D, Nayak A, Schumann JE, Schimpl A, Berberich I, Berberich-Siebelt F. (2008) Blimp-1Deltaexon7: a naturally occurring Blimp-1 deletion mutant with auto-regulatory potential. *Exp Cell Res.* **314**:3614-3627.

Schmitz R, Stanelle J, Hansmann ML, Küppers R. (2009) Pathogenesis of classical and lymphocyte-predominant Hodgkin lymphoma. *Annu Rev Pathol.* **4**:151-174.

Schneider EM, Torlakovic E, Stühler A, Diehl V, Tesch H, Giebel B. (2004) The early transcription factor GATA-2 is expressed in classical Hodgkin's lymphoma. *J Pathol.* **204**:538-545.

Schwering I, Brauninger A, Klein U, Jungnickel B, Tinguely M, Diehl V, Hansmann ML, Dalla-Favera R, Rajewsky K, Kuppers R (2003). Loss of the B-lineage-specific gene expression program in Hodgkin and Reed-Sternberg cells of Hodgkin lymphoma. *Blood* **101**:1505-1512.

Schwickert TA, Lindquist RL, Shakhar G, Livshits G, Skokos D, Kosco-Vilbois MH, Dustin ML, Nussenzweig MC. (2007) In vivo imaging of germinal centres reveals a dynamic open structure. *Nature* **446**:83-87.

Sciammas R, Davis MM. (2004) Modular nature of Blimp-1 in the regulation of gene expression during B cell maturation. *J Immunol.* **172**:5427-5440.

Sciammas R, Shaffer AL, Schatz JH, Zhao H, Staudt LM, Singh H. (2006) Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity* **25**:225-236.

Sears J, Ujihara M, Wong S, Ott C, Middeldorp J, Aiyar A. (2004) The amino terminus of Epstein-Barr Virus (EBV) nuclear antigen 1 contains AT hooks that facilitate the replication and partitioning of latent EBV genomes by tethering them to cellular chromosomes. *J. Virol.* **78**:11487-11505.

Seki M, Gearhart PJ, Wood RD. (2005) DNA polymerases and somatic hypermutation of immunoglobulin genes. *EMBO Rep.* **6**:1143-1148.

Shaffer AL, Yu X, He Y, Boldrick J, Chan EP, Staudt LM. (2000) BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. *Immunity* **13**: 199-212.

Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A, Giltnane JM, Yang L, Zhao H, Calame K, Staudt LM. (2002) Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* **17**:51-62.

Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, Lee AH, Qian SB, Zhao H, Yu X, Yang L, Tan BK, Rosenwald A, Hurt EM, Petroulakis E, Sonenberg N, Yewdell JW, Calame K, Glimcher LH, Staudt LM. (2004) XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity* **21:** 81-93.

Shaffer AL, Wright G, Yang L, Powell J, Ngo V, Lamy L, Lam LT, Davis RE, Staudt LM. (2006) A library of gene expression signatures to illuminate normal and pathological lymphoid biology. *Immunol Rev.* **210**:67-85.

Shah KM, Stewart SE, Wei W, Woodman CB, O'Neil JD, Dawson CW, Young LS. (2009) The EBV-encoded latent membrane proteins, LMP2A and LMP2B, limit the actions of interferon by targeting interferon receptors for degradation. *Oncogene* **28**:3903-3914.

Shannon-Lowe C, Adland E, Bell AI, Delecluse HJ, Rickinson AB, Rowe M. (2009) Features distinguishing Epstein-Barr virus infections of epithelial cells and B cells: viral genome expression, genome maintenance, and genome amplification. *J Virol.* **83**:7749-7760.

Shapiro-Shelef M, Lin KI, McHeyzer-Williams LJ, Liao J, McHeyzer-Williams MG, Calame K. (2003) Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity* **19**:607-620.

Shapiro-Shelef M, Lin KI, Savitsky D, Liao J, Calame K. (2005) Blimp-1 is required for maintenance of long-lived plasma cells in the bone marrow. *J. Exp. Med.* **202**:1471-1476.

Sheu LF, Chen A, Meng CL, Ho KC, Lee WH, Leu FJ, Chao CF. (1996) Enhanced malignant progression of nasopharyngeal carcinoma cells mediated by the expression of Epstein-Barr nuclear antigen 1 in vivo. *J Pathol.* **180**:243-248.

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA, Shi Y. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119**:941-953.

Shuai K, Liu B. (2003) Regulation of JAK-STAT signalling in the immune system. *Nat. Rev. Immunol.* **3**:900-911.

Silacci P, Mottet A, Steimle V, Reith W, Mach B. (1994) Developmental extinction of major histocompatibility complex class II gene expression in plasmocytes is mediated by silencing of the transactivator gene CIITA. *J Exp Med.* **180**:1329-1336.

Sinclair AJ, Palmero I, Peters G, Farrell PJ. (1994) EBNA-2 and EBNA-LP cooperate to cause G0 to G1 transition during immortalization of resting human B lymphocytes by Epstein-Barr virus. *EMBO J.* **13**:3321-3328.

Sinclair AJ, Farrell PJ. (1995) Host cell requirements for efficient infection of quiescent primary B lymphocytes by Epstein-Barr virus. *J Virol.* **69**:5461-5468.

Singh M, Birshtein BK. (1993) NF-HB (BSAP) is a repressor of the murine immunoglobulin heavy-chain 3' alpha enhancer at early stages of B-cell differentiation. *Mol Cell Biol.* **13**:3611-3622.

Sivachandran N, Sarkari F, Frappier L. (2008) Epstein-Barr nuclear antigen 1 contributes to nasopharyngeal carcinoma through disruption of PML nuclear bodies. *PLoS Pathog.* **4**:e1000170.

Sixbey JW, Nedrud JG, Raab-Traub N, Hanes RA, Pagano JS. (1984) Epstein-Barr virus replication in oropharyngeal epithelial cells. *N Engl J Med.* **310**:1225-1230.

Skinnider BF, Elia AJ, Gascoyne RD, Patterson B, Trumper L, Kapp U, Mak TW. (2002) Signal transducer and activator of transcription 6 is frequently activated in Hodgkin and Reed-Sternberg cells of Hodgkin lymphoma. *Blood* **99**:618-626.

Skinnider BF, Mak TW. (2002) The role of cytokines in classical Hodgkin lymphoma. *Blood* **99**:4283-4297.

Slifka MK, Matloubian M, Ahmed R. (1995) Bone marrow is a major site of long-term antibody production after acute viral infection. *J. Virol.* **69**:1895-1902.

Smith MA, Maurin M, Cho HI, Becknell B, Freud AG, Yu J, Wei S, Djeu J, Celis E, Caligiuri MA, Wright KL. (2010) PRDM1/Blimp-1 controls effector cytokine production in human NK cells. *J Immunol.* **185**:6058-6067.

Soderholm J, Kobayashi H, Mathieu C, Rowley JD, Nucifora G. (1997) The leukemia-associated gene MDS1/EVI1 is a new type of GATA-binding transactivator. *Leukemia* **11**:352-358.

Sood R, Talwar-Trikha A, Chakrabarti SR, Nucifora G. (1999) MDS1/EVI1 enhances TGF-beta1 signaling and strengthens its growth-inhibitory effect but the leukemia-associated fusion protein AML1/MDS1/EVI1, product of the t(3;21), abrogates growth-inhibition in response to TGF-beta1. *Leukemia* **13**:348-357.

Souza TA, Stollar BD, Sullivan JL, Luzuriaga K, Thorley-Lawson DA. (2007) Influence of EBV on the peripheral blood memory B cell compartment. *J Immunol.* **179**:3153-3160.

Speck SH, Strominger JL. (1985) Analysis of the transcript encoding the latent Epstein-Barr virus nuclear antigen I: a potentially polycistronic message generated by long-range splicing of several exons. *Proc Natl Acad Sci U S A.* **82**:8305-8309.

Steele-Perkins G, Fang W, Yang XH, Van Gele M, Carling T, Gu J, Buyse IM, Fletcher JA, Liu J, Bronson R, Chadwick RB, de la Chapelle A, Zhang X, Speleman F, Huang S. (2001) Tumor formation and inactivation of RIZ1, an Rb-binding member of a nuclear proteinmethyltransferase superfamily. *Genes Dev*. **15**:2250-2262.

Stein H, Uchánska-Ziegler B, Gerdes J, Ziegler A, Wernet P. (1982) Hodgkin and Sternberg-Reed cells contain antigens specific to late cells of granulopoiesis. *Int J Cancer.* **29**:283-290.

Stephenson LM, Miletic AV, Kloeppel T, Kusin S, Swat W. (2006) Vav proteins regulate the plasma cell program and secretory Ig production. *J Immunol.* **177**:8620-8625.

Sternberg C. (1898) Über eine eigenartige unter dem Bilde der Pseudoleukämie verlaufenden Tuberculose des lymphatischen Apparates. *Z Heilkunde* **19**:21-90.

Stevenson D, Charalambous C, Wilson JB. (2005) Epstein-Barr virus latent membrane protein 1 (CAO) up-regulates VEGF and TGF alpha concomitant with hyperlasia, with subsequent up-regulation of p16 and MMP9. *Cancer Res.* **65**:8826-8835.

Stoler MH, Nichols GE, Symbula M, Weiss LM. (1995) Lymphocyte predominance Hodgkin's disease. Evidence for a kappa light chain-restricted monotypic B-cell neoplasm. *Am J Pathol*. **146**:812-818.

Strobl LJ, Hofelmayr H, Marschall G, Brielmeier M, Bornkamm GW, Zimber-Strobl U. (2000) Activated Notch1 modulates gene expression in B cells similarly to Epstein-Barr viral nuclear antigen 2. *J Virol.* **74**:1727-1735.

Stunz LL, Busch LK, Munroe ME, Sigmund CD, Tygrett LT, Waldschmidt TJ, Bishop GA. (2004) Expression of the cytoplasmic tail of LMP1 in mice induces hyperactivation of B lymphocytes and disordered lymphoid architecture. *Immunity* **21**:255-266.

Su GH, Chen HM, Muthusamy N, Garrett-Sinha LA, Baunoch D, Tenen DG, Simon MC. (1997). Defective B cell receptor-mediated responses in mice lacking the Ets protein, Spi-B. *EMBO J.* **16:**7118-7129.

Su ST, Ying HY, Chiu YK, Lin FR, Chen MY, Lin KI. (2009) Involvement of histone demethylase LSD1 in Blimp-1-mediated gene repression during plasma cell differentiation. *Mol Cell Biol.* **29**:1421-1431.

Sugano N, Chen W, Roberts ML, Cooper NR. (1997) Epstein-Barr virus binding to CD21 activates the initial viral promoter via NF-kappaB induction. *J Exp Med.* **186**:731-737.

Sun CC, Thorley-Lawson DA. (2007) Plasma cell-specific transcription factor XBP-1 binds to and transactivates the Epstein-Barr virus BZLF1 promoter. *J Virol.* **81**:13566-13577.

Swanson-Mungerson MA, Caldwell RG, Bultema R, Longnecker R. (2005) Epstein-Barr virus LMP2A alters in vivo and in vitro models of B-cell anergy, but not deletion, in response to autoantigen. *J Virol.* **79**:7355-7362.

Tafuri A, Shahinian A, Bladt F, Yoshinaga SK, Jordana M, Wakeham A, Boucher LM, Bouchard D, Chan VS, Duncan G, Odermatt B, Ho A, Itie A, Horan T, Whoriskey JS, Pawson T, Penninger JM, Ohashi PS, Mak TW. (2001) ICOS is essential for effective T-helper-cell responses. *Nature* **409**:105-109.

Takada K, Shimizu N, Sakuma S, Ono Y. (1986) Trans activation of the latent Epstein-Barr virus (EBV) genome after transfection of the EBV DNA fragment. *J. Virol.* **57**:1016-1022.

Takada K, Ono Y. (1989) Synchronous and sequential activation of latently infected Epstein-Barr virus genomes. *J Virol.* **63**:445-449.

Tam W, Gomez M, Chadburn A, Lee JW, Chan WC, Knowles DM. (2006) Mutational analysis of PRDM1 indicates a tumor-suppressor role in diffuse large B-cell lymphomas. *Blood* **107**:4090-4100.

Tanner J, Weis J, Fearon D, Whang Y, Kieff E. (1987) Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. *Cell* **50**:203-213.

Thelander EF, Ichimura K, Corcoran M, Barbany G, Nordgren A, Heyman M, Berglund M, Mungall A, Rosenquist R, Collins VP, Grandér D, Larsson C, Lagercrantz S. (2008) Characterization of 6q deletions in mature B cell lymphomas and childhood acute lymphoblastic leukemia. *Leuk Lymphoma.* **49**:477-487.

Thomas RK, Kallenborn A, Wickenhauser C. (2002) Constitutive expression of c-FLIP in Hodgkin and Reed-Sternberg cells. *Am J Pathol.* **160**:1521-1528.

Thorley-Lawson DA, Poodry CA. (1982) Identification and isolation of the main component (gp350-gp220) of Epstein-Barr virus responsible for generating neutralizing antibodies in vivo. *J Virol.* **43**:730-736.

Thorley-Lawson DA. (2001) Epstein-Barr virus: exploiting the immune system. *Nat Rev Immunol.* **1**:75-82.

Thorley-Lawson DA, Gross A. (2004) Persistence of the Epstein-Barr virus and the origins of associated lymphomas. *N Engl J Med.* **350**:1328-1337.

Toellner KM, Gulbranson-Judge A, Taylor DR, Sze DM, MacLennan IC. (1996) Immunoglobulin switch transcript production in vivo related to the site and time of antigen-specific B cell activation. *J. Exp. Med.* **183**:2303-2312.

Tomkinson B, Robertson E, Kieff E. (1993) Epstein-Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation. *J Virol.* **67**:2014-2025.

Tooze RM, Stephenson S, Doody GM. (2006) Repression of IFN-gamma induction of class II transactivator: a role for PRDM1/Blimp-1 in regulation of cytokine signaling. *J Immunol.* **177**:4584-4593.

Torlakovic E, Tierens A, Dang HD, Delabie J. (2001) The transcription factor PU.1, necessary for B-cell development is expressed in lymphocyte predominance, but not classical Hodgkin's disease. *Am J Pathol.* **159**:1807-1814.

Torrisi MR, Cirone M, Pavan A, Zompetta C, Barile G, Frati L, Faggioni A. (1989) Localization of Epstein-Barr virus envelope glycoproteins on the inner nuclear membrane of virus-producing cells. *J Virol.* **63**:828-832.

Tsai CN, Lee CM, Chien CK, Kuo SC, Chang YS. (1999) Additive effect of Sp1 and Sp3 in regulation of the ED-L1E promoter of the EBV LMP 1 gene in human epithelial cells. *Virology* **261**:288-294.

Tsang SF, Wang F, Izumi KM, Kieff E. (1991) Delineation of the cis-acting element mediating EBNA-2 transactivation of latent infection membrane protein expression. *J Virol.* **65**:6765-6771.

Tsimbouri P, Drotar ME, Coy JL, Wilson JB. (2002) bcl-xL and RAG genes are induced and the response to IL-2 enhanced in EmuEBNA-1 transgenic mouse lymphocytes. *Oncogene* **21**:5182-5187.

Tsurumi T, Kobayashi A, Tamai K, Yamada H, Daikoku T, Yamashita Y, Nishiyama Y. (1996) Epstein-Barr virus single-stranded DNA-binding protein: purification, characterization, and action on DNA synthesis by the viral DNA polymerase. *Virology* **222**:352-364.

Tsurumi T, Fujita M, Kudoh A (2005). Latent and lytic Epstein-Barr virus replication strategies. *Rev Med Virol.* **15**:3-15.

Tunyaplin C, Shapiro MA, Calame KL. (2000). Characterization of the B lymphocyte induced maturation protein-1 (Blimp-1) gene, mRNA isoforms and basal promoter. *Nucleic Acids Res.* **28**:4846-4855.

Tunyaplin C, Shaffer AL, Angelin-Duclos CD, Yu X, Staudt LM, Calame KL. (2004) Direct repression of *prdm1* by Bcl-6 inhibits plasmacytic differentiation. *J. Immunol.* **173**:1158-1165.

Turner CA Jr, Mack DH, Davis MM. (1994) Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin secreting cells. *Cell* **77**:297-306.

Tusher VG, Tibshirani R, Chu G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A.* **98**:5116-5121.

Uchida J, Yasui T, Takaoka-Shichijo Y, Muraoka M, Kulwichit W, Raab-Traub N, Kikutani H. (1999) Mimicry of CD40 signals by Epstein-Barr virus LMP1 in B lymphocyte responses. *Science* **286**:300-303.

Uphoff CC, Denkmann SA, Steube KG, Drexler HG. (2010) Detection of EBV, HBV, HCV, HIV-1, HTLV-I and -II, and SMRV in human and other primate cell lines. *J Biomed Biotechnol.* 904767.

Ushmorov A, Ritz O, Hummel M, Leithauser F, Moller P, Stein H, Wirth T (2004). Epigenetic silencing of the immunoglobulin heavy-chain gene in classical Hodgkin lymphoma-derived cell lines contributes to the loss of immunoglobulin expression. *Blood* **104**:3326-3334.

Valentine R, Dawson CW, Hu C, Shah KM, Owen TJ, Date KL, Maia SP, Shao J, Arrand JR, Young LS, O'Neil JD. (2010) Epstein-Barr virus-encoded EBNA1 inhibits the canonical NF-kappaB pathway in carcinoma cells by inhibiting IKK phosphorylation. *Mol Cancer.* **9**:1.

Vallabhapurapu DS, Schimpl A, Berberich I. (2006) B Lymphocyte-induced maturation protein-1 (Blimp-1) is sufficient to trigger an unfolded protein response and XBP-1 processing in B cells. *Signal Transduction.* **1**:62-68.

Vasanwala FH, Kusam S, Toney LM, Dent AL. (2002) Repression of AP-1 function: a mechanism for the regulation of Blimp-1 expression and B lymphocyte differentiation by the B cell lymphoma-6 protooncogene. *J. Immunol.* **169**:1922-1929.

Vockerodt M, Morgan SL, Kuo M, Wei W, Chukwuma MB, Arrand JR, Kube D, Gordon J, Young LS, Woodman CB, Murray PG. (2008) The Epstein-Barr virus oncoprotein, latent membrane protein-1, reprograms germinal centre B cells towards a Hodgkin's Reed-Sternberg-like phenotype. *J Pathol.* **216**:83-92.

Vrzalikova K, Vockerodt M, Leonard S, Bell A, Wei W, Schrader A, Wright KL, Kube D, Rowe M, Woodman CB, Murray PG. (2011) Downregulation of BLIMP1-alpha by the EBV oncogene, LMP1, disrupts the plasma cell differentiation program and prevents viral replication in B cells: implications for the pathogenesis of EBV-associated B cell lymphomas. *Blood* PMID: 21411757 [Epub ahead of print]

Wang D, Liebowitz D, Kieff E. (1985) An EBV membrane protein expressed in immortalised lymphocytes transforms established rodent cells. *Cell* **43**: 831-840.

Wang D, Liebowitz D, Wang F, Gregory C, Rickinson A, Larson R, Springer T, Kieff E. (1988) Epstein-Barr virus latent infection membrane protein alters the human B-lymphocyte phenotype: deletion of the amino terminus abolishes activity. *J Virol.* **62**:4173-4184.

Wang F, Gregory C, Sample C, Rowe M, Liebowitz D, Murray R, Rickinson A, Kieff E. (1990) Epstein–Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. *J Virol.* **64**:2309-2318.

Wang S, Rowe M, Lundgren E. (1996) Expression of the Epstein Barr virus transforming protein LMP1 causes a rapid and transient stimulation of the Bcl-2 homologue Mcl-1 levels in B-cell lines. *Cancer Res.* **56**:4610-4613.

West JA, Viswanathan SR, Yabuuchi A, Cunniff K, Takeuchi A, Park IH, Sero JE, Zhu H, Perez-Atayde A, Frazier AL, Surani MA, Daley GQ. (2009) A role for Lin28 in primordial germ-cell development and germ-cell malignancy. *Nature.* **460**:909-913.

Williams H, McAulay K, Macsween KF, Gallacher NJ, Higgins CD, Harrison N, Swerdlow AJ, Crawford DH. (2005) The immune response to primary EBV infection: a role for natural killer cells. *Br J Haematol.* **129**:266-274.

Wilson JB, Bell JL, Levine AJ. (1996) Expression of Epstein–Barr virus nuclear antigen-1 induces B cell neoplasia in transgenic mice. *EMBO J.* **15**:3117-3126.

Wilson TM, Vaisman A, Martomo SA, Sullivan P, Lan L, Hanaoka F, Yasui A, Woodgate R, Gearhart PJ. (2005) MSH2-MSH6 stimulates DNA polymerase eta, suggesting a role for A:T mutations in antibody genes. *J Exp Med.* **201**:637-645.

Winberg G, Matskova L, Chen F, Plant P, Rotin D, Gish G, Ingham R, Ernberg I, Pawson T. (2000) Latent membrane protein 2A of Epstein-Barr virus binds WW domain E3 protein-ubiquitin ligases that ubiquitinate B-cell tyrosine kinases. *Mol Cell Biol.* **20**:8526-8535.

Winslow MM, Gallo EM, Neilson JR, Crabtree GR. (2006) The calcineurin phosphatase complex modulates immunogenic B cell responses. *Immunity* **24**:141-152.

Woisetschlaeger M, Jin XW, Yandava CN, Furmanski LA, Strominger JL, Speck SH. (1991) Role for the Epstein-Barr virus nuclear antigen 2 in viral promoter switching during initial stages of infection. *Proc Natl Acad Sci USA.* **88**:3942-3946.

Wolf J, Kapp U, Bohlen H, Kornacker M, Schoch C, Stahl B, Mucke S, von Kalle C, Fonatsch C, Schaefer HE, Hansmann ML, Diehl V. (1996) Peripheral blood mononuclear cells of a patient with advanced Hodgkin's lymphoma give rise to permanently growing Hodgkin-Reed Sternberg cells. *Blood.* **87**:3418-3428.

Wood VH, O'Neil JD, Wei W, Stewart SE, Dawson CW, Young LS. (2007) Epstein-Barr virus-encoded EBNA1 regulates cellular gene transcription and modulates the STAT1 and TGFbeta signaling pathways. *Oncogene* **26**:4135-4147.

Wu L, Nakano H, Wu Z. (2006) The C-terminal activating region 2 of the Epstein-Barr virus-encoded latent membrane protein 1 activates NF-kappaB through TRAF6 and TAK1. *J Biol Chem.* **281**:2162-2169.

Yamamoto R, Nishikori M, Kitawaki T, Sakai T, Hishizawa M, Tashima M, Kondo T, Ohmori K, Kurata M, Hayashi T, Uchiyama T. (2008) PD-1-PD-1 ligand interaction contributes to immunosuppressive microenvironment of Hodgkin lymphoma. *Blood* **111**:3220-3224.

Yates J, Warren LN, Sugden B. (1985) Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. *Nature* **313**:812-815.

Ye BH, Cattoretti G, Shen Q, Zhang J, Hawe N, de Waard R, Leung C, Nouri-Shirazi M, Orazi A, Chaganti RS, Rothman P, Stall AM, Pandolfi PP, Dalla-Favera R. (1997) The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. *Nat Genet.* **16**:161-170.

Yoshida T, Kimura N, Akiyoshi T, Ohshima K, Nagano M, Morioka E, Hisano S, Ohyashiki K, Kamada N, Tamura K. (1999) Jumping translocation of homogeneously staining region and tetraploidy with double minutes in acute myelomonocytic leukemia. *Cancer Genet Cytogenet.* **109**:40-44.

Young LS, Lau R, Rowe M, Niedobitek G, Packham G, Shanahan F, Rowe DT, Greenspan D, Greenspan JS, Rickinson AB. (1991) Differentiation-associated expression of the Epstein-Barr virus BZLF1 transactivator protein in oral hairy leukoplakia. *J Virol.* **65**:2868-2874.

Young LS, Dawson CW, Eliopoulos AG. (2000) The expression and function of Epstein-Barr virus encoded latent genes. *Mol Pathol.* **53**:238-247.

Young LS, Rickinson AB. (2004) Epstein-Barr virus: 40 years on. *Nat Rev Cancer.* **4**:757-768.

Yu J, Angelin-Duclos C, Greenwood J, Liao J, Calame K. (2000) Transcriptional repression by Blimp-1 (PRDI-BF1) involves recruitment of histone deacetylase. *Mol. Cell. Biol.* **20**:2592-2603.

Zelazowski P, Max EE, Kehry MR, Snapper CM. (1997) Regulation of Ku expression in normal murine B cells by stimuli that promote switch recombination. *J Immunol.* **159**:2559-2562.

Zeng Y, Zhong JM, Li LY, Wang PZ, Tang H, Ma YR, Zhu JS, Pan WJ, Liu YX, Wei ZN, et al. (1983) Follow-up studies on Epstein-Barr virus IgA/VCA antibody-positive persons in Zangwu County, China. *Intervirology* **20**:190-194.

Zhang YW, Xie HQ, Chen Y, Jiao B, Shen ZX, Chen SJ, Zhao WL. (2010) Loss of promoter methylation contributes to the expression of functionally impaired PRDM1β isoform in diffuse large B-cell lymphoma. *Int J Hematol.* **92**:439-444.

Zhao WL, Liu YY, Zhang QL, Wang L, Leboeuf C, Zhang YW, Ma J, Garcia JF, Song YP, Li JM, Shen ZX, Chen Z, Janin A, Chen SJ. (2008) PRDM1 is involved in chemoresistance of T-cell lymphoma and down-regulated by the proteasome inhibitor. *Blood* **111**:3867-2871.

Zheng B, Fiumara P, Li YV, Georgakis G, Snell V, Younes M, Vauthey JN, Carbone A, Younes A. (2003) MEK/ERK pathway is aberrantly active in Hodgkin disease: a signaling pathway shared by CD30, CD40, and RANK that regulates cell proliferation and survival. *Blood* **102**:1019-1027.

Zimber-Strobl U, Suentzenich KO, Laux G, Eick D, Cordier M, Calender A, Billaud M, Lenoir GM, Bornkamm GW. (1991) Epstein-Barr virus nuclear antigen 2 activates transcription of the terminal protein gene. *J Virol.* **65**:415-423.

blood

2011 117: 5907-5917
Prepublished online March 16, 2011;
doi:10.1182/blood-2010-09-307710

Down-regulation of BLIMP1α by the EBV oncogene, LMP-1, disrupts the plasma cell differentiation program and prevents viral replication in B cells: implications for the pathogenesis of EBV-associated B-cell lymphomas

Katerina Vrzalikova, Martina Vockerodt, Sarah Leonard, Andrew Bell, Wenbin Wei, Alexandra Schrader, Kenneth L. Wright, Dieter Kube, Martin Rowe, Ciaran B. Woodman and Paul G. Murray