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# **IMMUNOLOGICAL CONSEQUENCES OF EPSTEIN-BARR VIRUS REPLICATION**

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”Science... Never solves a problem without creating ten more.”  
George Bernard Shaw

“One of the advantages of being disorderly is that one is constantly making exciting  
discoveries”.  
Alan Alexander Milne

To my parents

# ABSTRACT

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus involved in the pathogenesis of a wide spectrum of malignant and non-malignant diseases. In healthy EBV carriers, the virus is believed to infect two major cellular targets - B lymphocytes and epithelial cells. While EBV latency is established predominantly, if not exclusively, in B-lymphocytes, virus replication can take place both in B cells and epithelial cells. Lytic replication ensures virus transmission to new carriers and replenishes the cellular reservoirs of virus persistence. The generally asymptomatic and harmless persistence of EBV relies on a tightly controlled immune response and distinct modes of virus/cell interactions observed at different stages of EBV life cycle. The aim of this thesis was to characterize the mutual influence of the host immune system and EBV at the replicative stage of virus infection.

We showed that EBV enters monocytes and inhibits their differentiation into dendritic cells (DCs) without the need of viral gene expression. The sensitivity of the cells to virus-induced apoptosis progressively decreases along the process of DC maturation and is strongly dependent on the cell type in which the virus replicated before infecting DC precursors, since epithelial-cells derived viruses exhibited a significantly stronger pro-apoptotic activity than their B cell-derived counterparts. The capacity of the virus to suppress DC development might help in delaying the establishment of EBV specific immunity before the pool of infected B cells reaches the size sufficient for long-term virus persistence.

During virus replication, both B cells and epithelial cells may escape recognition by cytotoxic CD8+ T cells through downregulation of MHC class I molecules. Our work demonstrated that MHC class I heavy-chain and  $\beta 2m$  mRNA and protein synthesis are inhibited during EBV replication. Several other characteristic changes observed in the MHC class I processing and presentation pathway during the lytic cycle were recapitulated by chemical inhibition of protein synthesis. These results were recently confirmed by others and the viral protein responsible for host-cell global protein synthesis shutdown was shown to be encoded by the BGLF5 open reading frame of the EBV genome.

Triggering of receptors of the tumor necrosis factor (TNF) superfamily participates both in determining the fate of B-lymphocytes during the process of their differentiation and in immunologic clearance of virus infected targets. Initiation of EBV lytic cycle counteracted sensitization to death induced by TNF-related apoptosis-inducing ligand (TRAIL) that resulted from B-cell receptor (BCR) triggering in Burkitt's lymphoma cells. Differential modulation of death-transmitting and decoy TRAIL receptors was associated with sensitization to TRAIL in response to BCR-triggering or protection from TRAIL by EBV lytic cycle. Interference with TRAIL-mediated checkpoints in B-cell differentiation may account for the involvement of EBV in autoimmune diseases. Decreased sensitivity to TRAIL may also protect EBV infected cells from recognition by CTL and NK-cells.

B-cell homeostasis is severely perturbed during malaria infection. We showed that the CIDR1 $\alpha$  domain of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a multiadhesive protein expressed during the erythrocytic phase of the parasite life cycle, binds to B cells and induces EBV replication. This might partly explain the increased EBV viral load during malaria infection and the increased risk of B cell immortalization in the ontogenesis of endemic Burkitt's lymphoma.

Results presented in this thesis strengthen the notion that EBV replication actively modulates the functioning of the immune system at different levels through complex interactions of viral products with several types of cells and contributes to immune suppression, autoimmunity and tumorigenesis through a number of mechanisms whose details require further characterization. Research lines defined by this work may lead to new approaches towards management of EBV associated diseases.

## LIST OF PUBLICATIONS

- I. **Guerreiro-Cacais AO**, Li L, Donati D, Bejarano MT, Morgan A, Masucci MG, Hutt-Fletcher L, Levitsky V.  
*Capacity of Epstein-Barr virus to infect monocytes and inhibit their development into dendritic cells is affected by the cell type supporting virus replication.*  
Journal of General Virology 2004; 85:2767-2778
  
- II. **Guerreiro-Cacais AO**, Uzunel M, Levitskaya J, Levitsky V.  
*Inhibition of heavy chain and beta2-microglobulin synthesis as a mechanism of major histocompatibility complex class I downregulation during Epstein-Barr virus replication.*  
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- III. **Guerreiro-Cacais AO**, Levitskaya J, Levitsky V.  
*A role of TRAIL in controlling signal integration in B-cells is revealed by the interference of Epstein-Barr virus with anti-Ig-induced sensitization to TRAIL.*  
Submitted manuscript
  
- IV. Chêne A, Donati D, **Guerreiro-Cacais AO**, Levitsky V, Chen Q, Falk KI, Orem J, Kironde F, Wahlgren M, Bejarano MT.  
*A molecular link between malaria and Epstein-Barr virus reactivation.*  
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## LIST OF ABBREVIATIONS

BAFF	B cell activating factor belonging to the TNF family
BCR	B cell receptor
BL	Burkitt's lymphoma
BLIMP-1	B-lymphocyte-induced maturation protein 1
CBP	CREB-binding protein
CIDR1 $\alpha$	Cysteine-rich interdomain region 1 $\alpha$
CMV	Cytomegalovirus
CR2	Complement receptor 2, CD21
CXCR4	Chemokine (CXC motif) receptor 4
DISC	Death-inducing signaling complex
EBNA	EBV nuclear antigen
EBERs	EBV-encoded small nonpolyadenylated RNAs
EBV	Epstein-Barr virus
FADD	Fas-associated death domain
FDC	Follicular dendritic cell
FLIP	Flice/caspase-8 inhibitory protein
$\gamma$ HV68	Murine gammaherpesvirus 68
GC	Germinal center
<i>Gld</i>	Generalized lymphoproliferative disease; FasL knockout mice
GM-CSF	Granulocyte macrophage colony-stimulating factor
HHV6	Human Herpesvirus 6
HHV7	Human Herpesvirus 7
HL	Hodgkin's lymphoma
HSV1	Herpes Simplex Virus 1
HSV2	Herpes Simplex Virus 2
ICAM-1	Intercellular adhesion molecule 1
IAP	Inhibitors of apoptosis
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Infectious mononucleosis
IRF	Interferon responsive factor
ITAM	Immunoreceptor tyrosine-based activation motif
KSHV	Kaposi's Sarcoma-associated herpesvirus
LC	Langerhans cell
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
<i>Lpr</i>	Lymphoproliferation; Fas-knockout mice
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NF- $\kappa$ B	Nuclear factor $\kappa$ B
NPC	Nasopharyngeal carcinoma

OHL	Oral Hairy Leukoplakia
PfEMP1	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
PK	Protein kinase
PLAD	Pre-ligand assembly domain
PML	Pro-myelocytic leukemia
PTLD	Post-transplant lymphoproliferative disease
RA	Rheumatoid arthritis
RIP	Receptor-interacting protein
SLE	Systemic lupus erythematosus
SMAC/Diablo	Second Mitochondria-derived Activator of Caspases/Direct IAP Binding Protein with Low PI
TBP	TATA-binding protein
TAP	Transporter associated with antigen processing
T-D	Thymus-dependent
T-I	Thymus-independent
TGF $\beta$	Transforming growth-factor $\beta$
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factors
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
UPR	Unfolded protein response
vhs	Virion host shutoff
VZV	Varicella-zoster virus
XPB1	X-box-binding protein 1
XLP	X-linked lymphoproliferative disease



# 1. INTRODUCTION

Infecting more than 90% of the world's population, Epstein-Barr virus (EBV) is often described as an evolutionarily very successful member of the herpes virus family. As all herpesviruses, EBV is able to persist in the host for life and in the majority of life-long carriers the infection seems to be asymptomatic. However, its ability to immortalize human B lymphocytes in culture makes EBV a strong candidate as a causing agent of disease. The link between EBV infection and cancer has been known for a long time, and increasing evidence has now also linked EBV to autoimmunity. The persistence and spread of EBV relies on both the establishment of latency and cycles of replication. Production of new virions is a tightly controlled process, which takes place in two major cell targets, epithelial cells and B lymphocytes, as they reach specific differentiation stages and anatomical sites. Although there is significant knowledge on how EBV exploits the biology of the B lymphocyte to establish latency and how this is controlled by the immune system, only recently the replicative cycle has been explored from the immunological perspective. Many questions arise from these studies: What are the requirements for the initiation of replication? Where does it occur? How does it modify phenotypically and functionally the cells that harbor this process and how are these cells perceived by the surrounding tissue and the immune system? Are virus-replicating cells as sensitive to immune control mechanisms as their uninfected counterparts? If the replicative cycle is completed, what is the impact of newly formed virus on the existing pool of latently infected cells and on the immune system of the host? Some of these questions become even more relevant in light of the participation of EBV in polymicrobial diseases as endemic Burkitt's lymphoma and in autoimmunity.

## 2. AIMS OF THE THESIS

The general goal of this thesis was to investigate the cross-talk between the immune system and cellular reservoirs supporting virus replication.

The specific aims were:

- to determine the impact of virions of different cellular origins, i.e. epithelial cells or B lymphocytes, on the inhibition of dendritic cell differentiation.
- to determine the mechanism of MHC class I is downregulated during EBV replication.
- to assess the sensitivity of B lymphocytes replicating EBV to death-receptor mediated apoptosis.
- to study the effect of malaria and malaria-induced polyclonal B-cell activation on EBV replication.

As a consequence of this work, we believe to have gained new insights into the role of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) in controlling the process of B-cell activation.

## 3. EPSTEIN-BARR VIRUS

### 3.1 OVERVIEW OF EBV BIOLOGY

Most viruses have been initially identified as causative agents of acute illnesses, but this was not the case for Epstein-Barr virus (EBV). Denis Burkitt, a British surgeon working in equatorial Africa in the late 1950's, originally described and isolated cells from an aggressive lymphoma that was to subsequently carry his name. The geographical and climatic distribution of this tumor as well as its increased presence in African children suggested that either environmental factors or infectious agents participated in its etiology. It was not until 1964 that Anthony Epstein and his colleagues, Yvonne Barr and Bert Achong, succeeded in culturing lymphoma cells from Burkitt's lymphoma patients and identified herpesvirus particles in these cells by electron microscopy [1]. Soon after, when a laboratory technician working with EBV developed infectious mononucleosis and seroconverted, the link between EBV and mononucleosis was established. It has since become clear that EBV infection is widespread in all human populations and that the virus persists in the majority of individuals as a lifelong asymptomatic infection of the B cell pool.

EBV is a large, double-stranded DNA virus belonging to the herpesvirus family. Primary EBV infection happens most often during childhood by transmission of the virus through saliva and is usually asymptomatic. When infection is delayed to adolescence, approximately 25% of cases can manifest as infectious mononucleosis (IM), an acute but self-limiting lymphoproliferative disease. A general characteristic of the herpesvirus family is the infection and replication in one cell target, followed by establishment of life-long latency in another cell type, with occasional reactivation of lytic replication that leads to virus spread and infection of new hosts. This classical theme is exploited to different extents by all members of the herpesvirus family, with each virus targeting specific cell types [1, 2].

Lytic replication of herpesvirus is divided in three distinct phases of gene expression: immediate-early (IE), with transcription of viral transactivators of replication which regulate expression of both cellular and other viral genes; early (E), which includes the expression of components of the viral DNA replication machinery; and late (L), when mostly structural proteins of the virus capsid, tegument and envelope are expressed. However, establishment of latency varies among herpesvirus members. Alpha- (HSV1, HSV2, VZV) and beta- (CMV, HHV6, HHV7) herpesviruses enter their target cells and immediately shut down all viral gene expression. On the other hand, gamma-herpesviruses (EBV and KSHV) contain a set of latency proteins essential for an initial step of strong target cell proliferation and amplification of the infected cell pool before silencing of gene expression [3].

A characteristic which sets EBV apart from other gamma-herpesvirus is self-sufficiency in inducing B cell transformation, whereas KSHV depends on the contribution from host factors such as T cell help. This is accomplished through expression of a set of nine EBV viral proteins, that include six nuclear antigens

(EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA3L1) and three membrane proteins (LMP1, LMP2A, and LMP2B), and two non-translated nuclear RNAs (EBERs). These genes are expressed in all *in vitro* infected and immortalized B cells (lymphoblastoid cell lines [LCLs]). Molecular genetic analysis has demonstrated that EBNA2, EBNA3A, EBNA3C, EBNA3L1, and LMP1 are critical for *in vitro* B-lymphocyte transformation while EBNA3B, LMP2A, and LMP2B are dispensable [2]. A model for the establishment of EBV latency in B lymphocytes has been proposed based on the pattern of expression of these viral genes observed in both EBV-positive tumors that stem from B cells in different stages of differentiation and *ex vivo* analyzed virus-infected normal B cell subsets (discussed further in this thesis) [4].

Based on frequencies of various EBV-associated malignancies one could surmise that the virus targets two major cell types: B lymphocytes, as evidenced by Burkitt's lymphoma (BL), post-transplant lymphoproliferative disease (PTLD) and Hodgkin's lymphoma (HL); and epithelial cells, infected in nasopharyngeal carcinoma (NPC), which is 100% EBV positive, and gastric carcinoma [2]. The virus can, nevertheless, infect other cell types such as cytotoxic lymphocytes, as exemplified by EBV-positive NK and T cell lymphomas, and interact and/or infect leukocytes, although these interactions remain to be studied further [5-7].

In spite of the fact that EBV infects more than 90% of humans without posing a major threat to health, it is one of the most potent transforming viruses *in vitro*. The balance between an asymptomatic carrier state and disease is nevertheless finely tuned. An evidence for this is X-linked lymphoproliferative disease, in which mutations in the SAP gene lead to a fatal acute primary EBV infection, even though carriers of this mutation do not show impaired immune responses against other pathogens [8]. Another evidence comes from immunosuppressed individuals, which are at higher risk of developing EBV-carrying lymphomas and show that the infection is largely kept in check by a functional immune response. Nevertheless, not every EBV-positive immunosuppressed individual develops tumors, and the tumors that arise are often oligoclonal [9].

An understanding of B lymphocytes, their activation, differentiation and elimination, is essential for comprehending the EBV life cycle.

## 3.2 B LYMPHOCYTES AS A RESERVOIR OF EBV

### 3.2.1 B cell responses to antigen

Depending on their biochemical characteristics, antigens induce two types of B cell responses which can be classified as thymus-dependent (T-D) or independent (T-I). TD responses are triggered by protein antigens that are processed and presented on MHC class II molecules to cognate T lymphocytes. T-I responses can be induced either by substances which trigger polyclonal B-cell activation by Toll-like receptor stimulation such as LPS, poly-IC and CpG (T-I type I), or by high molecular-weight antigens with repetitive epitopes, such as bacterial capsular polysaccharides, that simultaneously engage multiple B-cell receptors (BCRs) on specific B-cells (T-I type II) [10] [11]. B-cell responses require the participation of several functionally and phenotypically distinct subsets of B-cells.

In mice, mature B cells can be divided in four subsets: follicular, marginal zone, B-1a (CD5+) and B-1b (CD5-) [12]. Whereas both follicular and marginal zone B cells are generated from a common transitional B cells precursor in the periphery, the point in B cell development from which B-1 B cells arise is not known, but these cells have self-renewing capacity [13]. Follicular B cells recirculate between the blood and lymphoid follicles and give rise to T-D responses. Both marginal zone B cells, which reside close to the marginal sinus of the spleen, and B-1 B cells, which are present in peritoneal and pleural cavities but upon antigen activation migrate to the spleen to become antibody-secreting cells, generate T-I immune responses. B-1a B cells, which are characterized by CD5 expression, produce low affinity polyreactive IgM, termed “natural” antibodies, which provide the first line of defense against several pathogens but also weekly react against self-antigens [13]. While B-1a cells spontaneously produce antibodies against encapsulated pathogens, B-1b cells have been shown to be essential for adaptive responses to bacterial polysaccharides and for long-term protection [11].

Upon encounter with the antigen, activated B cells migrate to secondary lymphoid organs where they can follow two different fates depending on the affinity with which their BCR binds the antigen: B cells with high BCR affinity move into extrafollicular areas, proliferate and differentiate into short-lived antibody-secreting plasma cells that mostly lack somatic hypermutation, and B cells with lower affinity move into B cell follicles, proliferate and establish germinal centers [14]. Germinal centers (GCs) are organized structures histologically divided into light and dark zones. Light zones are composed of follicular dendritic cells (FDCs) that trap antigen complexes, GC T helper cells, tangible body macrophages that engulf dying cells, and activated B cells (centrocytes). The dark zone is composed mostly of highly dividing B cell blasts (centroblasts), a sparse network of stromal cells and some GC T cells and macrophages. The light zone is strategically located towards the source of antigen: in the spleen it is directed towards the marginal sinus where blood-borne antigens enter the tissue, in lymph nodes it is positioned close to the subcapsular sinus which receives afferent lymphatic drainage from the skin, mucosa and viscera, and on tonsils and Peyer's patches it is oriented towards the mucosal surface.

A long-standing model for the function of GC in T-D responses suggests that in the dark zone, antigen-activated B cell blasts (centroblasts), that lack surface immunoglobulin (Ig), rapidly divide and somatically hypermutate their antibody variable-region genes. Centroblasts then exit the cell cycle, re-express mutated Igs on the cell surface and migrate to the light zone as centrocytes. Since these cells express surface Igs with a range of affinities for the original antigen (and possibly, by random mutations, to any antigens, including self), they compete for binding to antigens exposed by FDCs. Centrocytes that successfully endocytosed and processed antigen, present epitopes to T helper lymphocytes with specificity to the same antigen. Help through CD40L and cytokines leads to Ig class switch and further differentiation of selected cells into high affinity antibody-secreting plasma cells and memory B cells [15]. The process of somatic hypermutation and selection leads to an increase in the average affinity of serum antibodies against target antigens over the course of an immune response and has been termed affinity maturation [16].

T-I type II antigens can stimulate extrafollicular foci of plasma cell production and short-lived GC which collapse soon after compartmentalization into dark and light zones, suggesting that signals from T cells are essential for maintaining the GC response. The B cells that arise from these reactions have very low levels of somatic hypermutation and Ig-class switch. Nevertheless, memory responses can be generated against polysaccharides in the absence of long lasting GCs [10, 17].

Recent studies have however demonstrated that the above described model of the GC reaction is not completely accurate. In reality, centrocytes and centroblasts are much more similar to each other than was previously believed. Cell cycle can be detected in both dark and light zones, although the dark zone seems to be enriched for rapidly dividing cells. Centroblasts, previously believed to lack surface Ig, actually do express them, albeit at low levels if compared to naïve B cells, but at comparable levels to centrocytes. Also, activated B cells seem to move in between both compartments at comparable rates, which suggests that centrocytes might return to the dark zone to complete additional rounds of mutation and selection [18]. The previous notion that centroblasts could be separated from centrocytes on the basis of higher CD77 expression has also been proven wrong [19]. Currently the only reliable marker, which distinguishes the two GC populations, is the chemokine receptor CXCR4, which is expressed more abundantly by B cells in the dark zone and whose downregulation seems to direct cells into the light zone. Recent studies also show that even though most B-cells do not cross the barrier between the mantle zone and the GC, memory B-cells can re-enter GCs and participate in renewed rounds of maturation [20].

Antibody-secreting plasma cells can develop from antigen-activated naïve marginal-zone B cells, follicular B cells, activated GC B cells and memory B cells. Which B cell subsets become terminally differentiated depends on the nature of the antigen, its dose and form and the location of the encounter. The first cells to differentiate into plasma cells are marginal-zone B cells which have, as B-1 B cells, antigen receptor repertoire skewed towards recognition of T-I type II antigens, but can also engage in TD responses. Due to their location at the border of the white pulp of the spleen, these cells respond well to blood-borne antigens. Circulating follicular B cells can also respond to

antigenic stimulation by differentiating into plasmablast and forming extrafollicular foci of plasma cells. Both these cells types secrete IgM without somatic hypermutation and are short-lived, but provide a rapid first-line defense against pathogens. Activated follicular B cells may also form GC, as described above. This response takes a longer time to develop, but leads to the generation of both memory B cells and plasma cells that secrete high affinity, isotype switched antibodies. Some of these plasma cells migrate to the bone marrow, where they reside for prolonged periods of time. Memory cells can rapidly differentiate into plasma cells upon secondary encounter with the antigen, or by bystander T cells stimulation in the presence of CpG-containing DNA which activates TLR9 without specific antigenic stimulation [21].

Two transcription factors, BLIMP-1 (B-lymphocyte-induced maturation protein 1) and XBP1 (X-box-binding protein 1), are crucial for plasma cell differentiation. These genes control all the processes related to the boost in Ig production and the induction of a physiological unfolded protein response (UPR) that enables the cell to cope with the increase in protein synthesis and the folding, post-translational modifications and export of these proteins to the extracellular environment [13].

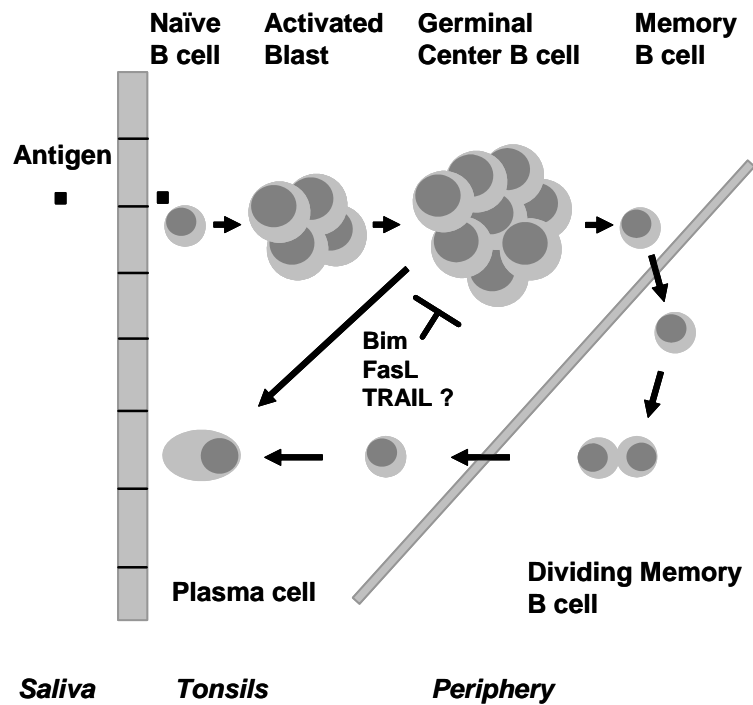


Figure 1. Follicular B cell response to antigen.

### 3.2.2 Apoptosis overview

The GC reaction is instrumental in generating memory B cells and plasma cells expressing antigen-specific high-affinity antibodies. Diversification of the B-cell repertoire in the GC through somatic hypermutation of the variable region of Ig heavy and light chain genes is a random process that not only leads to the generation of immunoglobulins with higher affinities but also produces B cell clones that either loose

the ability to recognize the original antigen or acquire increased affinities for self-structures, increasing the risk of autoimmunity [22]. Because only T cells with the appropriate antigenic specificity should be found in B cell follicles, only B cells that present relevant antigen-derived peptides on their MHC class II molecules receive T-cell mediated survival signals. B cells with no or irrelevant specificity are eliminated by apoptosis.

Apoptosis plays an important role in the development and maintenance of tissue homeostasis. Characteristics of apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation, ending with engulfment of apoptotic bodies by macrophages or neighboring cells, thereby preventing an inflammatory response. Apoptosis differs from necrosis, which is associated with loss of membrane integrity, cell swelling and disruption, resulting in damage to the surrounding tissue and inflammation. In mammals, a wide array of stimuli triggers two major apoptotic pathways: the extrinsic or death receptor mediated, and the intrinsic or mitochondrial pathway [23].

The extrinsic pathway is activated through death receptors, which include TNF-R1, Fas and TRAIL-R1 and TRAIL-R2, all belonging to the tumor necrosis factor (TNF) receptor superfamily. Members of the TNF ligand family are primarily produced as transmembrane proteins arranged in stable homotrimers. Death ligand stimulation results in oligomerization of the receptors and recruitment of the adaptor protein Fas-associated death domain (FADD) and pro-caspases 8 and 10, which together form the death-inducing signaling complex (DISC). Close proximity between these initiator caspases leads to autocatalytic cleavage and activation, and subsequent activation of the effector caspases 3, 6 and 7. Preassembly or self-association of death receptors through an extracellular pre-ligand assembly domain (PLAD), which differs from the ligand-binding domains, is critical for receptor-ligand interaction and downstream signaling. Another protein, cellular caspase-8 (FLICE)-inhibitory protein (cFLIP), with high homology to caspase-8 and 10, can also be recruited to the DISC. Four splice cFLIP variants have been identified so far at the level of protein expression: cFLIPL, cFLIPS, cFLIPR and p43FLIP. All isoforms are capable of dimerizing with caspase-8. Whereas the short (S) and Raji (R) isoforms are clearly anti-apoptotic due to a complete lack of the c-terminal catalytic domain, the outcome of dimerization between the long (L) isoform and caspase-8 might depend on levels of expression of these two proteins and can lead to either a limited caspase-8 or NF- $\kappa$ B activation. In different systems, the overexpression cFLIPL has been shown to either protect or induce apoptosis and, interestingly, the phenotype of cFLIP deficient T cells resembles that of FADD and caspase-8 deficient cells [24].

Whereas FasL binds to only one known receptor, TRAIL-induced apoptosis relies on the interplay between four known membrane-bound receptors. Whereas TRAIL-R1 and TRAIL-R2 are death-inducing and form DISC in a similar fashion to Fas, TRAIL-R3 and TRAIL-R4 work as decoys with either absent or truncated intracellular signaling domains, respectively. TRAIL-R3 is glycosylphosphatidylinositol (GPI)- anchored to the plasma membrane, resides in lipid rafts and titrates away TRAIL from death-inducing receptors [25]. TRAIL-R4 associates with TRAIL-R2 through a PLAD and inhibits apoptosis independently of ligand binding, a feature that sets TRAIL receptors



apart from other TNF family members that tend to only form homotrimers [26]. TRAIL-R4 also actively inhibits the formation of mixed TRAIL-R1 and R2 complexes [25]. Another difference between TRAIL and other TNF-family members is that upon ligand binding, death receptor internalization is not needed to initiate DISC formation and downstream caspase activation [27, 28]. Another level of complexity in TRAIL-receptor mediated killing is the ligand-induced clustering of the death-inducing receptors that relies on the presence of O-glycosilations on defined regions of their extracellular domains. Tumors cells deficient in the enzyme that promotes this glycosilation show reduced response to TRAIL [29].

TNF is a multifunctional pro-inflammatory cytokine. Two receptors are capable of binding TNF. TNF-R1 is ubiquitously expressed in most tissues and is the major mediator of TNF signaling, whereas TNF-R2 is mainly found in cell of the immune system and can only be fully activated by membrane bound TNF, but not by its soluble form. Whereas Fas and TRAIL receptors overwhelmingly induce apoptosis which masks their capacity to induce pro-survival signals through NF- $\kappa$ B, the cellular response to TNF often follows an opposite trend. Upon ligand binding, TNF-R1 induces formation of a complex, which includes TRADD, TRAFF2 and RIP (complex I) and leads to I $\kappa$ B kinase activation, I $\kappa$ B phosphorylation and its subsequent proteasomal degradation, releasing NF- $\kappa$ B dimmers, which migrate to the nucleus and induce transcription of pro-survival genes. Only in conditions where complex I fails to form does TNF-R1 bind FADD and induce caspase-8 activation (complex II) [23].

The intrinsic pathway, also referred to as the mitochondrial pathway, can be activated by stimuli that range from developmental cues, insufficient trophic support and intracellular damage. These signals converge to the members of the Bcl-2 family of pro- and anti-apoptotic proteins, whose ratio ultimately determines the fate of the cell. Bcl-2 family members can be divided in three groups, based on the number of Bcl-2 Homology (BH) domains. Anti-apoptotic members, like Bcl-2 and Bcl-X<sub>L</sub>, contain three to four BH domains, whereas the pro-apoptotic members are divided into either multi-BH domain (BH 1-3) proteins such as Bax and Bak, or BH3-only proteins such as Bim, Bad, Bid [30]. BH3-only proteins are regulated in response to pro-apoptotic signals either by an increase in gene expression or by post-translational mechanisms. Under normal conditions, Bax and Bak proteins are kept in check by binding to anti-apoptotic Bcl-2 family members. Apoptotic stimuli thus lead to neutralization of anti-apoptotic Bcl-2 family members by BH3 only proteins. This in turn licenses Bax/Bak to undergo conformational changes and to form pores on the outer mitochondrial membrane that leads to the release of soluble intramembrane proteins such as cytochrome c and disruption of mitochondrial functions. Cytochrome c binds to Apaf-1, forming a complex known as apoptosome, which in turn recruits and activates caspase-9, leading to activation of effector caspases. Another set of molecules released from the mitochondria (e.g. Smac/DIABLO) binds and sequesters inhibitors of apoptosis (IAPs), which under normal conditions inhibit the activation of caspase-3 and -9 [31].

Mammalian cells can be divided into two types according to their requirement for mitochondrial amplification of the apoptotic signal following death-receptor engagement. In type I cells, processed caspase-8 is sufficient to directly activate

downstream effector caspases. In type II cells, the signal following caspase-8 activation has to be transmitted to the mitochondria by caspase-8 mediated cleavage of Bid, which in turn leads to the release of cytochrome c by the mitochondria and formation of the apoptosome. Active caspase-9 activates caspase-3 which in turn cleaves caspase-8 completing the loop [23].

### **3.2.3 Apoptosis and the germinal center reaction**

The development of a T-D response subjects the B lymphocyte to at least three checkpoints. In the first checkpoint, antigen-specific activated B cells and T cells must meet in the B cell follicular border. Costimulation from T cells is needed to direct the B lymphocyte into the follicles for affinity maturation. After rounds of somatic hypermutation and proliferation, centroblast/centrocytes must be positively selected by binding specifically and with high affinity to antigen in the light zone of the GC (second checkpoint). The last checkpoint relies on the encounter with GC-residing helper T cells again specific for the cognate antigen [22]. The major players in this response are thus BCR-stimulation and T cell help in the form of CD40L, although other stimuli such as TLR engagement, BAFF and other cytokines are emerging as important modulators.

BCR triggering has been shown to induce apoptosis in the absence of concomitant T cell help. Several models on both B cell lines and primary B cells, that played with the strength of antigenic stimulation, show that the apoptotic signal delivered under these conditions correlates with the extent of BCR triggering, is independent of death receptors, FADD or caspase-8 but can be prevented by overexpression of Bcl-2 or Bcl-X<sub>L</sub> [32-34]. The proapoptotic Bcl-2 family member Bim has been implicated in this process [35]. Bim deficient mice produce more memory B cells that lack affinity matured antibodies and plasma cells secreting low affinity antibodies [36]. In one model, whereas both sIg-triggering or extensive sIg-crosslinking on BL cell lines in vitro induced apoptosis, only the latter induced alterations in Bim expression (degradation of BimX<sub>L</sub> but upregulation of BimL and BimS) that were compatible with induction of cell death. [37]. In another study, BCR triggering was shown to induce Bim upregulation and Bcl-2 downregulation, tipping the ratio towards apoptosis [35].

Several studies demonstrated the importance of Fas/FasL interactions in the elimination of autoreactive B cells by specific CD4<sup>+</sup> T cells in normal mice [38, 39]. Misguided T cell help in the form of CD40 stimulation induces expression of Fas on naïve and memory cells and renders them sensitive to Fas-induced apoptosis [40]. Even though CD40 stimulation also upregulates cFLIP expression, the balance between Fas upregulation and available pools of cFLIP still promotes cell death in response to FasL [41]. On the other hand, sIg-stimulation leads to protection from FasL-mediated apoptosis again by NF-κB dependent upregulation of cFLIP [42, 43]. Whether anti-apoptotic Bcl-2 family members can inhibit FasL induced apoptosis in B cells is a matter of dispute [44].

We found that in Burkitt's lymphoma cell lines, which exhibit the phenotype of germinal center centroblast/centrocytes, and in memory B cells, BCR stimulation leads to increased sensitivity to TRAIL-induced cell death, while conferring protection against FasL (**paper III**). In contrast, the reverse effect for both ligands is seen when cells are stimulated with CD40L alone, while concomitant triggering of BCR and CD40 leads to protection from both TRAIL and FasL. Although we have not extensively dissected the mechanisms of this sensitization, the increase in the death-inducing TRAIL-R2, the decrease in the decoy TRAIL-R4, and the decrease in the protective Bcl-2 family member Bcl-X<sub>L</sub> might be involved in the process.

A correlation between our in vitro system of BCR stimulation and *ex vivo* isolated peripheral blood B cells is the fact that memory B cells are more resistant to TRAIL than naïve B cells, and naïve cells are more resistant than circulating CD5+ B-1a cells. In a model in which sensitivity to TRAIL is determined by antigenic triggering and T cell help, memory cells, which have at some point received both signals, should be more resistant than naïve cells, whereas CD5+ B cells, which only received antigenic stimulation, should be more sensitive. Even though the difference between naïve and CD5+ B cells might be related to other innate characteristics of these two B cell subsets, it is interesting to note that under our experimental conditions, the effect of BCR triggering on TRAIL sensitization in memory B cells is long lasting and does not require concomitant sIg stimulation and TRAIL engagement, whereas the protective effect of CD40L is transient. The fact that memory B cells can upon re-encounter with the antigen migrate back into B cell follicles and undergo additional rounds of somatic hypermutation [20] underscores the possible significance of the sensitization to TRAIL as an additional checkpoint in this B cell compartment.

The roles of Bim and Fas in the control of immune responses had been highlighted previously. Although both Fas knockout and Bim knockout mice produce autoantibodies, the development of fatal SLE-like disease only happens in some mouse strains but not in others. Three recent studies addressed the effects of Fas and Bim deletion during both homeostasis and immune responses. While the shutdown of the acute T cell response against HSV1 depended only of Bim, downsizing of the T cell response to murine  $\gamma$ -herpesvirus depended on both factors [45]. These mice also developed severe SLE-like symptoms with the production of isotype-switched high affinity nuclear antibodies, expansion of activated B and T cell blasts and increased macrophage activation [46].

The importance of TRAIL in the immune system remains somewhat unclear. Although TRAIL-deficient mice show no gross phenotype [47], blocking or addition of TRAIL in specific models of infection, autoimmunity and immune surveillance against tumors modulates the response [48]. Recently TRAIL was implicated in controlling secondary expansions of CD8+ T cells that were primed in the absence of CD4+ T cell help [49]. Even though T cells are able to expand and acquire effector functions in the absence of CD4+ T cell help, upon re-challenge with antigen these "helpless" T cells are deleted by TRAIL. These results have been however contested by another study that claimed that TRAIL only delayed but not completely abolished secondary expansions of "helpless" CD8+ T cells [50]. TRAIL was also initially proposed to play a role in thymic negative selection, but this hypothesis was subsequently disproved [48].

Mouse knockout models have shown that TRAIL deficiency alone does not predispose for autoimmunity. However when autoimmunity was induced experimentally in these mice, the absence of TRAIL exacerbated the effects [51]. Similarly, while absence of TRAIL does not lead to spontaneous tumor development at early age (although it predisposes for lymphomas in older mice), when challenged with tumor models, these mice show poor tumor clearance and reduced survival [52]. However, when TRAIL-resistant tumor models were employed, treatment with exogenous TRAIL had tumor promoting effect, which again highlights the fact that TNF family members can exert both pro-survival and proapoptotic effects depending on the cell target [48].

TRAIL might be involved in controlling a specific subset of responses, which would also explain why its effect has been missed in the models studied so far. One evidence for this is that exogenous long-term administration of TRAIL-blocking antibodies into autoimmunity-prone FasL-deleted (*gld*) mice leads to an increase in serum auto-antibodies of the IgG1 subclass [53]. These mice preferentially produce auto-antibodies of IgG2a and IgG2b subclasses which are typically the antibody types produced under a Th1 response promoted by IFN $\gamma$ . IgG1 is produced preferentially in response to Th2 cytokines like IL-4. The fact that CD4<sup>+</sup> T cells with a Th2 phenotype have been shown to express TRAIL but not FasL and be more resistant to TRAIL than Th1 cells (which do express FasL but not TRAIL) [54], favors a scenario in which TRAIL might aid in restricting the development of somatically mutated centrocytes that either lost specificity to the target antigen or that developed undesired self-recognizing Igs in the context of Th2 responses. Interestingly, anti-TRAIL antibody administration had no impact on the splenomegaly, lymphadenopathy, T cell numbers and cytokine profiles characteristic of these mice, which again suggest that the impact of TRAIL in shaping immunity might be very specific.

### **3.2.4 The model of EBV persistence**

A model devised by David Thorley-Lawson proposes that EBV establishes latency by mirroring the differentiation steps taken by an antigenically triggered naive B cell during its progression through the GC reaction into the memory pool (reviewed in [4]). This proposition stems from similarities in the phenotype of EBV-infected B cell tumors with different types of latent gene expression and normal B lymphocytes at different stages of differentiation, from the known roles of latency proteins in the modulation of B cell activation, proliferation and survival and from the data obtained on the expression of these proteins in different subsets of B cells from infected individuals.

The model proposes that during primary infection EBV crosses the thin epithelium of the tonsils either directly or by a step involving infection of (and possibly replication in) the epithelial cell layer, and infects naïve B cells, which form the bulk of the underlying lymphoid bed. The virus switches on the full program of latency gene expression (growth program or latency III), involving all EBNAs and LMPs, which drives the naïve B cell into an activated proliferating state that resembles a B cell blast

generated in response to an antigen. The infected cells migrate to secondary follicles and change the transcription program to a more restricted form (default program or latency II), in which only EBNA1 and LMP1 and LMP2a are expressed. While for an uninfected B cell triggered by antigen the GC response depends on two major signals, BCR and CD40, in the case of EBV these signals are provided by LMP2a and LMP1, respectively (and see below). While LMP2a is sufficient to induce GC formation and somatic hypermutation [55], LMP1 drives isotype switching [56] and downregulation of BCL6 [57], a GC transcription factor that controls the exit of the memory cell from the GC. Therefore, a successive pattern of viral gene expression should exist, in which LMP2a is expressed before LMP1. Infected memory B cells leave the GC and shut down all viral gene expression (latency program), with the exception of the expression of EBNA1 during homeostatic cell division (EBNA1 only or latency I), and occasionally of LMP2a. This allows the virus to remain virtually invisible from immune recognition in the memory B cell compartment.

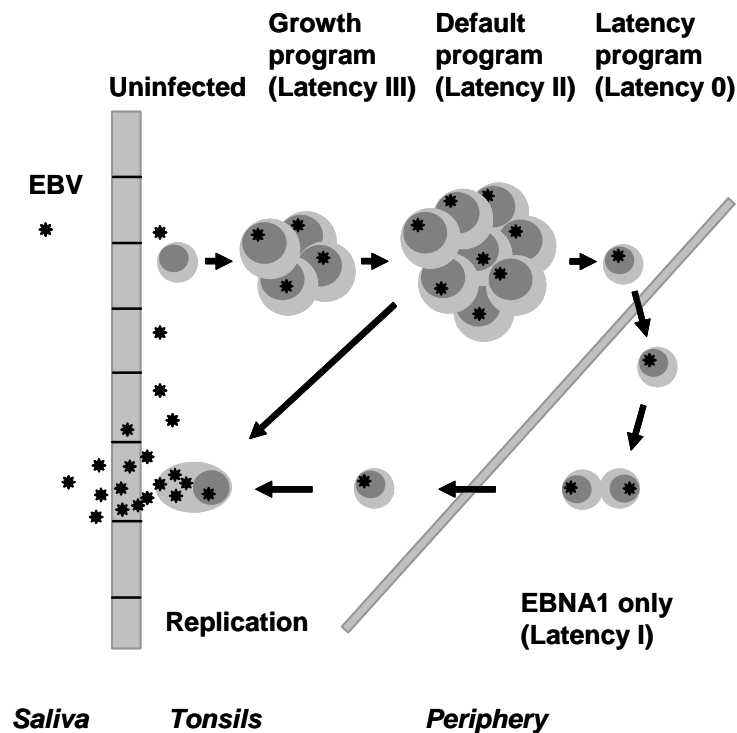


Figure 2. EBV exploits the normal steps of B cell differentiation..

There are a few apparent problems with this model. In vitro, EBV promiscuously infects any B cell and transforms it into a proliferating lymphoblast, showing no preference for naïve B cells. Another issue is that in IM, which represents the only accessible model of primary EBV infection, EBV most likely directly infects both memory and GC B cells because analysis of somatic hypermutation in proliferating EBV-infected memory and GC cells did not show diversification from a common infected naïve B cell precursor. Moreover, cells of the same clonal origin expressed different pattern of latent gene expression, and that did not correlate with the progression of these cells through the GC reaction [58]. A subsequent study again performed on B-cells from IM patients showed that EBV infected GC and memory

cells did not show signs of ongoing somatic hypermutation, whereas EBV negative cells in the same tonsils did, and that “aberrant” viral genes expression, with the presence of EBNA1 and LMP2a, but not LMP1 could be observed [59].

IM might be, however, quite different from asymptomatic primary infection and from the life-long carrier state. In IM the follicular structure of the tonsils is disrupted, and thus the GC environment which might be essential in triggering the shift of viral gene expression from the growth to the default program is lacking. Increased viremia could also lead to massive direct infection of GC and memory cells. Nonetheless, analysis of circulating infected B cells during the acute phase of IM shows that this population is almost entirely composed of memory B cells, up to 50% of which can be EBV-positive. However, these cells do not differ in phenotype and cell cycle distribution from infected peripheral blood B cell from healthy donors, nor do they support higher levels of lytic replication [60]. The same scenario is true for PTLN, in which infected cells that exit the lymph node are of the resting memory B cell phenotype. Cells that retain a lymphoblastoid phenotype are thus mostly restricted to lymph nodes and only on occasions when secondary mutations arise are they seen in the periphery [61].

A recent study has also revisited the issue of somatic hypermutation in post-GC memory B cells in IM patients, showing a higher rate of somatic hypermutation in EBV-positive memory B cells and their exclusion from the IgD<sup>+</sup> CD27<sup>+</sup> memory compartment, which seems to stem mostly from GC-independent immune responses to T-I antigens [62]. Interestingly, their pattern of somatic mutations shows hallmarks of true antigenic selection based on their frequency, type and location within the Ig gene, revealing that the memory phenotype does not only rely on surrogate signals provided by LMPs, but that LMPs only ensure that B cells with low antigenic affinity make it through the GC reaction [63].

### 3.3 EBV PROTEINS AND LATENCY

EBV-encoded latency proteins, along with promoting proliferation, also mediate strong anti-apoptotic activity. These two properties are essential in the establishment of latency since proliferating B cells are normally very susceptible to apoptosis, especially in the absence of an appropriate immunological stimulus. It also ensures the survival of infected cells long enough for them to enter the non-dividing memory B cells pool, which is intrinsically long-lived and a perfect reservoir for the virus. These same factors make however a major contribution to the malignant transformation of infected cells.

EBNA1 is expressed in all actively dividing EBV-infected cells and is responsible for binding the viral episome through its origin of replication (OriP) to the mitotic cellular DNA, assuring replication and transfer of virus genome to all daughter cells [64]. EBNA1 is also involved in the transcriptional control of other latency proteins, a function that is independent of episome maintenance [65]. However, B cell transformation can be achieved in the absence of EBNA1, albeit at much lower efficiency, through direct integration of the viral DNA into the cellular genome. LCLs generated in the absence of EBNA1 showed tumorigenicity comparable to LCLs established from wild-type virus, demonstrating that EBNA1 is not absolutely essential for transformation but dramatically increases its efficiency [66]. Transgenic mice constitutively expressing EBNA1 were shown to be prone to B-cell lymphoma development [67], but recent experiments by another group with animal of the same background failed to show such an effect [68]. Nevertheless, EBNA1 expression in BL cell lines also offers some protection from apoptosis [69].

The role of EBNA2 in transformation has been established by the fact that mutant EBV strains lacking this gene are unable to immortalize B cells in vitro. This has been largely attributed to the EBNA2-mediated transcriptional activation of LMP1 and LMP2a, and by its interaction with EBNA-LP, which together induce cell cycle progression during immortalization of infected resting B lymphocytes. LMP1 and LMP2a are however transcribed independently of EBNA2 in EBV associated tumors. EBNA3 family proteins act as repressors of EBNA2 and modulate its transactivational properties [2].

LMP1 is the main transforming protein of EBV [70]. LMP1 is an integral plasma membrane protein associated to lipid rafts with a pattern of signaling that mimics constitutive CD40 triggering in a ligand-independent manner, and is thus capable of activating both canonical (p65/p50) and non-canonical (p52/REL-B) NF- $\kappa$ B pathways, as well as MAP kinase and PI3 kinase pathways. Even though the upstream signaling might be slightly different between CD40 and LMP1, the sets of downstream genes activated by both pathways are largely overlapping. However, mice engineered to express the extracellular portion of CD40 with the LMP1 cytoplasmic signaling tail, which therefore needs CD40L triggering for activation, develop lymphadenopathy, splenomegaly, and production of autoantibodies, in spite of normal responses to

specific antigenic challenge [71]. LMP1 also induces the expression of cell-surface adhesion molecules and activation antigens, and upregulates anti-apoptotic proteins (e.g. Bcl-2, A20 and Mcl-1) in B cells. In epithelial cells, ectopic LMP1 expression leads to hyperproliferation, inhibition of differentiation, actin remodeling and invasiveness, along with protection from apoptosis [72].

LMP2a and its splice variant LMP2b are not essential for EBV-induced cell transformation in B lymphocytes, but aid in the transformation [73], migration and invasiveness potential of epithelial cells [74]. Signals from LMP2a mimic those of the BCR, which promote B lymphocyte survival and proliferation, and involve PI3 kinase and MAP kinase activation as well as immunoreceptor tyrosine-based activation motif (ITAM)-mediated recruitment of *src* and *syk* tyrosine kinases. It has been widely accepted that LMP2a blocks signaling through the BCR by excluding the BCR from lipid rafts where it would otherwise initiate signaling [72]. However, transgenic mouse models that concomitantly express LMP2a and transgenic BCRs show enhanced responses to specific antigens due to increased survival and expansion of activated cells [75]. LMP2a also does not interfere with B cell tolerance induced by exposure to strong stimuli in an *in vivo* model where B cells react to a self-antigen. On the other hand, LMP2a bypasses anergy induction in response to low levels of antigen [76]. If constitutively expressed during B cell ontogeny, LMP2a favors the expansion of the B-1 B cell compartment [77]. Even though it was known that LMP2a could rescue BCR-deficient B cells from apoptosis [78], recent studies have pointed to an essential role of LMP2a in promoting growth transformation and survival in germinal center B cells which lost BCRs due to crippling mutations [79, 80].

The small untranslated RNAs EBER-1 and -2 are accumulated at high levels during all forms of latency and regulate apoptosis through different mechanisms. EBER-1 interacts with the interferon-inducible protein kinase R (PKR), and inhibits its activation by double-stranded RNAs, protecting infected cells from IFN $\alpha$ -induced apoptosis [81]. EBER-2 has however a more prominent role in EBV-mediated growth transformation, as viruses lacking the coding sequence for this RNA were significantly less efficient in generating LCLs *in vitro*, and the cell lines generated proliferated at much lower rates, due to reduced autocrine IL-6 production [82]. These observations have been extended to epithelial cell lines, where EBERs induce the expression of growth factors that promote cell survival [83].

A challenge to the classical view of establishment of latency and its sole dependency on EBNA2-driven gene expression was the demonstration that two virus-encoded Bcl-2 homologues, BHRF1 and BALF1, which are normally expressed during lytic replication, are essential for transformation of new B cell targets [84]. These genes have redundant functions, are needed for the establishment of latency but are dispensable for further proliferation of latently infected cells, which matches their transient expression immediately after infection, preceding that of EBNA2. Even though BZLF1 is also transiently expressed as an immediate-early gene during primary infection [85], it is not involved in the induction of BHRF1 and BALF1 at this stage. This might be explained by the fact that the EBV genome is delivered unmethylated to the nucleus, which allows the access of a number of cellular transcription factors to the viral DNA. The



expression of EBNA2 might thereafter restrict the expression of these lytic genes and ensue the progression of latency gene expression.

The establishment of latency is therefore dependent on initial protection from apoptosis when resting B lymphocytes are infected, followed by intense proliferation of target cells once the full growth program is expressed. Whereas the rescue from apoptosis is clear, the need for rounds of proliferation of infected targets is supported by the fact that a murine  $\gamma$ HV68 engineered to block proliferation of the infected target cell failed in establishing latency, in spite of no impairment of initial replication [86].

### 3.4 REPLICATION AND THE ESTABLISHMENT OF PERSISTENCE

Lytic replication of EBV may constantly take place in healthy virus carriers, since shedding of virus particles is regularly revealed by their presence in saliva [87, 88]. Histological analysis of tonsil tissue revealed rare cells supporting virus replication which resembled, in phenotype, antibody-secreting plasma cells. Since EBV-infected memory B cells recirculate primarily between the peripheral blood and the tonsils [89], they were hypothesized to be the source of these plasma cells supporting virus replication. It is now established that differentiation of EBV-bearing memory cells into plasma cells triggers virus replication in tonsils of healthy carriers [90] and the induction of replication, in this context, depends on the intracellular plasma-cell environment rather than on the factors triggering plasma cell differentiation. It has since been shown that the plasma cell differentiation transcription factor XBP-1 directly induces transcription of the immediate-early BZLF1 gene [91]. Differentiation of B lymphocytes into antibody-secreting cells generates an unfolded protein response (UPR) to cope with ER stress generated by heightened demands in protein folding, glycosylation and export. Both the UPR and the master regulator of plasma cell differentiation Blimp-1, induce XBP-1 expression. We have observed that incubation of Akata BL cells with the ER N-linked glycosylation inhibitor tunicamycin, which induces ER stress, leads to a significant increase in virus reactivation (data not shown). However, XBP-1 expression alone is not sufficient for activation of virus replication that implies participation of additional factors. A candidate for this role is protein kinase D (PKD), a histone deacetylase inhibitor induced upon BCR triggering, which synergizes with XBP-1 in the activation of both BZLF1 and BRLF1 transcription [92].

Whereas plasma cell differentiation is a lengthy process, reactivation of virus replication seen in *in vitro* triggered BL cell lines follows a much shorter kinetics (see **paper II**). BCR-triggering and other stimuli that induce rapid BZLF1 transcription such as TGF $\beta$  concomitantly induce apoptosis in the cells that fail to enter the lytic cycle, indicating that this second mechanism of induction might operate when EBV-carrying memory B cells are subjected to stress, and allows the virus to escape by replication. Nevertheless, apoptosis induction is neither necessary nor sufficient for EBV reactivation [93].

For efficient induction of replication, BZLF1 needs to transactivate the other immediate early transcription factor, BRLF1. Expression of both proteins is needed for induction of all subsequent early and late viral genes. However, due to extensive methylation and silencing of the viral genome in latent infection, it was not clear how replication could be initiated under these circumstances. BZLF1 became the first transcription factor known to preferentially bind to, and activate, a methylated promoter (i.e. BRLF1) [94]. BZLF1 is also expressed as an immediate-early gene following primary infection of B cells and this expression is directly activated by cellular factors without the need of de novo protein synthesis [85], but it does not lead to lytic replication (see previous section).

After BZLF1 and BRLF1 activation, the subsequent cascade of gene expression continues with the early genes, some of which are involved in viral DNA replication,

and late genes, which are expressed after viral DNA synthesis and include components of the virion [95]. Replication also leads to extensive activation of cellular genes that participate directly in the process of virus production, protection from apoptosis and modulation of immune responses. Surprisingly, growth program (latency III) genes are expressed late during replication, reaching levels comparable to those observed in LCLs, and their expression is sensitive to inhibitors of viral DNA replication [96]. Whereas LMP1 was known to be expressed during lytic cycle, to aid in cell survival and to be essential for viral particle egress [97], the impact of latency III genes in this context is not known.

During the lytic cycle, the EBV genome is amplified more than 100-fold. Intermediates of viral DNA replication are found as concatemeric molecules, probably resulting from rolling-circle DNA replication, which are subsequently cleaved into unit-length genomes and packaged into virions in the nucleus [98]. EBV capsids bud through the inner nuclear membrane and thereby acquire a primary envelope which subsequently fuses with the outer nuclear membrane, resulting in nucleocapsid release into the cytoplasm [99]. Here, the majority of the tegument, a structure composed of a multitude of different proteins that links the capsid and the envelope, is added to nucleocapsids, which obtain their final envelope by budding into glycoprotein-containing Golgi-derived vesicles. Thus, a process of nucleocapsid envelopment, de-envelopment and re-envelopment must occur during EBV egress [100].

In spite of being considered a B lymphotropic virus, EBV is also capable of infecting epithelial cells. Whereas both replication and latency associated genes can be detected in single cells of oral hairy leukoplakia (OHL) of immunocompromised individuals [101], in nasopharyngeal carcinoma (NPC) the infection is usually characterized by the default program (latency II). Evidence of EBV infection and replication of the oropharyngeal epithelium of healthy carriers has been difficult to obtain [102] but methods with increased sensitivity have now detected EBV in *ex vivo* samples of nasopharyngeal mucosa [103, 104]. Nevertheless, the factors that control latency versus replication in epithelial cells remain elusive.

The discovery that EBV virions derived from B cells and epithelial cells differ in their capacity to infect different cell types, led to new insights on the mechanisms which regulate cellular tropism of EBV [105]. As other herpesvirus, EBV possesses a number of glycoproteins inserted into the virus envelope which play specific roles during attachment, fusion and penetration of the virion into the target cells. Gp350/220 is the most abundant envelope protein and mediates the attachment of the virion to B cells by binding complement-receptor 2 (CR2; CD21), resulting in capping of the receptor and endocytosis of the virus [106]. Glycoproteins gH and gL form a heterodimer in which gL plays a role in folding and transport of gH to the plasma membrane. The dimer, together with another glycoprotein, gB, is involved in the fusion process in both B cells and epithelial cells [107]. Gp42, which has homologs only among lymphocryptovirus, binds to MHC class II molecules, which act as coreceptors if present on the target cell [108] and can be incorporated into a trimeric complex with gH/gL [109]. Finally, the multispan virus membrane protein BMRF2 binds to integrins on the basolateral membranes of polarized epithelial cells [110].

The fact that gp42 can bind to MHC class II molecules also within the cell supporting replication and be targeted for degradation led to the discovery that virions can have different stoichiometry of glycoproteins in the envelope depending on the cell they bud from [105]. If the virus replicates in a B lymphocyte, the resulting virions have lower levels of gH/gL/gp42 complexes, but a higher amount of gH/gL dimmers. These virions are more efficient in infecting epithelial cells, which have a (unidentified) ligand for gH and for which the presence of gp42 has an inhibitory effect for fusion. In this case, BMRF2 aids in the binding [110] whereas gB is essential for fusion [107]. On the other hand, virions that bud from epithelial cells, which lack MHC class II, have much higher amounts of trimeric gH/gL/gp42 complexes which are essential, along with gp350-CR2 interactions, for efficient infection of B cells. Although epithelial cell-derived viruses are much more infectious for B cells than B cell-derived viruses, for epithelial cells the difference between an epithelial and a B cell derived virus still holds but is less striking [107].

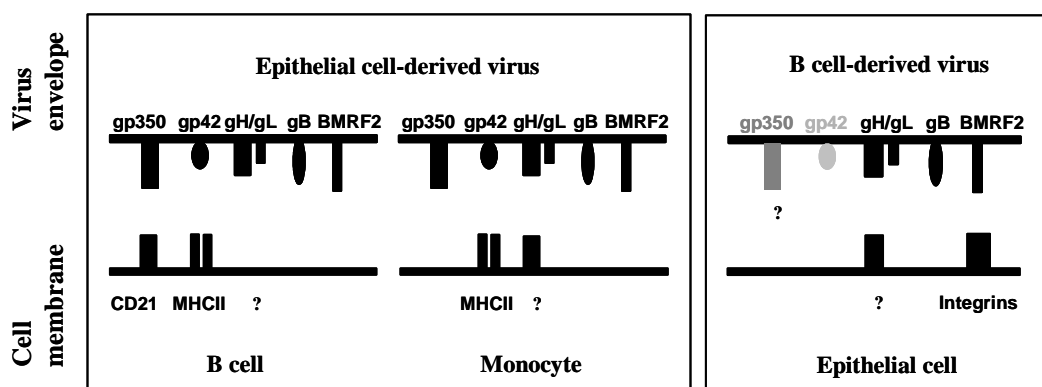


Figure 3. EBV virions acquire different proportions of envelope glycoproteins depending on the nature of the virus-producing cell, which influences their capacity to infect different cell targets

The dynamics of the crosstalk between the cell reservoirs of EBV latency or EBV replication is not well understood. Analysis of virions shed in saliva demonstrated a higher proportion of gp42 as compared to virions generated from LCLs of the same donors, and EBV from saliva was better at binding B cells than epithelial cells, suggesting an epithelial origin of the virus [111]. Integrins, the binding partners for BMRF2, are expressed at the basolateral membranes of epithelial cells and thus only enable cell-free virus to infect unidirectionally. Cell-cell contact is needed for virus infection from the apical membrane to occur [104]. The fact that the estimated amount of B lymphocytes replicating below the tonsillar epithelium at any given time are too low to support the nearly constant shedding seen during the healthy carrier state [88], together with the abovementioned evidences, would argue for an amplification step in virus production during shedding rather than during infection [9]. However, once the epithelium is infected, virions are released towards both apical and basal membranes.

B cells have been recently shown to behave as highly effective transfer vehicles of plasma membrane-bound virus to epithelial cells. The efficiency of the process

correlated with the levels of CD21; was epithelial cell specific, since neither endothelial cells nor fibroblasts could be infected; infection involved synapse formation but not cell fusion and happened within 10 minutes. Moreover, infected B cells carried additional virions on the cell surface for up to 2 days [112]. This study also shows that the presence of gp350, although important for B cell binding and infection, is deleterious for epithelial infection and that possibly the binding of gp350 to CD21 on the carrying B cell unmasks other envelope components for infection of epithelial cells.

Two subsequent studies extended this model to monocytes/macrophages and Langerhans cells (LCs). In one, EBV was shown to infect blood-borne and oral epithelium LCs, with a pattern of gene expression that ranged from latency in blood to replication in mucosa [7]. In the other, analysis of oral hairy leukoplakia (OHL) sections revealed that both macrophages and LCs were lytically infected by EBV and located both to submucosal and intraepithelial areas. *Ex vivo* infected monocytes, macrophages and LCs cells migrated to intraepithelial sites of oral explants, whereas control-infected B lymphocytes did not, and that these cells spread EBV to keratinocytes. This study also confirmed the transfer of virus from infected B lymphocytes to monocytes in vitro [6]. Since LCs migrate to all types of epithelial tissues with equal affinity, it could explain why EBV shedding is detectable not only from the oropharyngeal epithelium, as would be expected from the recirculation model of plasma cells, but also from genital and lactating mammary epithelia.

The infection of leukocytes by EBV is a long-standing observation. Both neutrophils and monocytes can be infected in vitro and the infection modulates cytokine and chemokine secretion, phagocytic capacity and apoptosis [5]. Whereas some of these effects are attributed to replication, others are solely dependent on viral adsorption. Previous work in our laboratory showed that EBV infection inhibited the development of dendritic cells from monocyte precursors cultured in vitro with differentiating factors (GM-CSF and IL-4), whereas no apoptotic effect could be observed in the absence of the cytokines. Additionally, no viral gene expression was detected in infected cells [113]. We extended our analysis to demonstrate that the death-inducing effect of the virus is dependent on the composition of the viral envelope (**paper I**). Virions derived from epithelial cells, and thus rich in gH/gL/gp42 trimers, are much more efficient in entering monocytes and inducing apoptosis than B cell-derived virus. Whereas monocytes, like epithelial cells, do not have a receptor for gp350 but express a ligand for gH, they do, as B lymphocytes, express MHC class II as a ligand for gp42. Interestingly, our analysis of envelope glycoprotein composition showed that virions derived from B lymphocytes contain decreased amounts of gp350. Whether gp350 binds intracellularly to CR2 and is degraded in a similar way as gp42/MHC class II complexes is not known.

EBV is able to bind to TLR2 on monocytes and induce secretion of the chemokine MCP-1 [114]. IL-6, GM-CSF and leukotriene production are also induced by EBV binding to monocytes without viral gene expression, through yet unknown ligands [5]. It is therefore possible that a crosstalk between pathways stimulated during adhesion of the virus (TLR2, MHC class II and a putative gH ligand), and external stimuli such as the cytokine environment (GM-CSF and IL-4), modulates the fate of these cells. If no additional triggering is given, infected monocytes respond by secreting cytokines that,

in the case of IL-6 and GM-CSF, boost the growth of EBV transformed B cells. However, if forced to differentiate into antigen-presenting dendritic cells, monocytes die by apoptosis and ensure that the immune response is delayed until the pool of infected B cells is large enough. The general importance of antigen presentation for priming of T cell responses in the context of EBV has been highlighted recently by the demonstration that humanized mice depleted of plasmacytoid dendritic cells showed increased mortality in response to EBV infection [115]. Therefore, EBV infection of monocytes may play a dual role in EBV life cycle promoting EBV spread in primary infection and transmission to new hosts as well as delaying antigen presenting function of cells differentiating into DCs in the early stages of the immune response.

## **3.5 CELLULAR IMMUNE RESPONSES AND IMMUNE SUBVERSION**

### **3.5.1 NK cell responses**

Cells of the innate immune system act in synergy to provide the first line of defense against pathogens. Although NK cells have an established role in controlling CMV and HSV infections, their contribution in the protection from EBV has been questioned by the fact that NK cell-deficient patients suffered primarily from other herpesvirus-related diseases and that the immune control of  $\gamma$ HV68, a mouse  $\gamma$ -herpes virus, did not seem to rely on NK cells [116]. Yet, NK cells can inhibit EBV-induced growth transformation of resting B cells in vitro through the secretion of IFN $\gamma$ , which is in turn dependent on dendritic cell (DC)-derived IL-12 [3] or plasmacytoid DC derived IFN $\alpha$  [115]. Stimulation of TLR3 (which binds dsRNA) and TLR9 (which binds to unmethylated CpG DNA) by EBV-derived factors is essential for the activation of myeloid DCs [117] or plasmacytoid DCs, respectively [115]. Interestingly, inhibition of growth transformation of B lymphocytes in vitro is most efficiently achieved with tonsil-derived NK cells, which are at the site of primary infection, as compared to their blood counterparts [117]. X-linked lymphoproliferative (XLP) disease, caused by a mutation which affects the cytotoxic function of both T cell and NK cells and leads to fatal primary EBV infection [118], together with a recently identified primary immunodeficiency that specifically targets the NK cell compartment and is associated with EBV-driven lymphoproliferation [119], underscores the fact that NK cells are important in limiting the establishment and/or expansion of the reservoir of latent EBV infection. NK cells are also capable of recognizing and killing EBV infected targets supporting lytic replication, which heavily downregulate MHC class I expression, but not cells in latency [120]. This sets EBV apart from CMV which escapes T cell recognition by MHC class I downregulation, but possesses additional mechanisms for protection against NK mediated lysis.

### **3.5.2 T cell recognition**

During IM up to 40% of the total circulating CD8<sup>+</sup> T cell pool can be devoted to one individual virus-derived epitope, with most reactivities against IE proteins, followed by E antigens and much fewer against L proteins. Reactivities against latent proteins are less pronounced, reaching up to 5% of total CD8<sup>+</sup> T cells, and target mostly EBNA 3A, 3B and 3C proteins. Epitope recognition varies both qualitatively and quantitatively between IM and post-IM/memory stages. Interestingly, in these individuals a significant fraction of the circulating CD8<sup>+</sup> T cell pool is still devoted to the control of the asymptomatic infection. CD4<sup>+</sup> T cell responses during IM are much less robust than CD8<sup>+</sup> T cell responses and are represented by a broader range of specific reactivities [121].

One of the first recognized mechanism of immune escape in EBV was the evasion of CD8<sup>+</sup> T cell responses by EBNA1, which contains an internal 250 amino acid glycine-alanine repeat which, if transferred to other proteins, protects them from proteasomal processing and suppresses their MHC class I presentation [122, 123]. EBNA1 was later

shown to be processed and presented on MHC class I if provided as an exogenous antigen, through a TAP-independent mechanism [124], and the relevance of this as an immune evasion strategy was further challenged by the detection of EBNA1-specific cytotoxic CD8<sup>+</sup> T cells in humans [3]. However, recognition of LCLs by EBNA1-specific CTLs is weak. Furthermore, recent experiments with rhesus lymphocryptovirus (rhLCV) infection, which recapitulates EBV infection in humans, show that although strong priming against EBNA1 takes place, these CTLs are unable to kill latently infected targets [125].

Analysis of CD8<sup>+</sup> T cell clones against representative epitopes derived from IE, E or L lytic cycle proteins, showed that their capacity to recognize cells supporting EBV replication decreased along with the progression through the lytic cycle. This implied that the antigen processing capacity of these cells became progressively impaired as the replication proceeded [126]. It was by then already known that cells supporting EBV replication had low levels of MHC class I and II [121], and this was subsequently suggested to rely at least in part on the inhibition of peptide transport into the ER by inhibition of the transporters associated with antigen processing (TAPs), which mediate the transfer of peptides from the cytoplasm into the lumen of the ER for subsequent loading onto MHC class I molecules [127]. We showed that even though the contribution of functional inactivation of the TAP heterodimer into the observed downregulation of MHC class I molecules cannot be excluded, shutdown of host cell protein synthesis plays a major role in the decrease of MHC class I molecules in EBV infected cells supporting virus replication (**paper II**).

Two E lytic cycle proteins have been identified now that subvert MHC class I-mediated immune recognition. BNLF2a is responsible for blocking peptide transport to the ER by inhibiting both peptide and ATP binding to TAPs [128]. There is no sequence homology between BNLF2a and the TAP-inhibitory proteins ICP47 of HSV or US6 from HCMV. Whereas ICP47 is also cytoplasmic and acts as a high affinity competitor for peptide binding, it does not affect ATP binding to TAPs. US6, on the other hand, affects ATP binding to TAPs through its ER luminal domain but does not interfere with peptide binding [129]. BNLF2a has a unique structure and mechanism of action, and homologues among other  $\gamma$ 1-herpesvirus that infect old-world primates.

BGLF5 on the other hand is a viral DNase/alkaline exonuclease whose expression leads to the shutoff of protein synthesis due to increased mRNA turnover, resembling in function the SOX protein from KSHV [130]. The capacity of both BGLF5 and SOX to block protein synthesis does not, however, depend on their exonuclease function [131]. Whereas gene products with alkaline exonuclease activity exist throughout the herpesvirus family, neither  $\alpha$ - nor  $\beta$ -herpesvirus have RNA-turnover functions associated with them. Whereas HSV-1 expresses the virion host shutoff (vhs) protein that degrades cellular mRNA, HCMV probably compensates for its absence by additional mechanisms of immune subversion that target MHC class I heavy chain to degradation (US2, US11) or block their egress from the ER (US3) [129].

A number of other factors could also modulate the expression of MHC class I during EBV replication, of which the IE lytic cycle promoter BZLF1 and the E protein BMLF1 might play prominent roles. BZLF1 has been implicated in regulating a



number of cellular pathways. First, pro-myelocytic leukemia (PML) bodies, present in the nucleus, have been implicated in controlling MHC class I transcription [132] and are a site for EBV replication [133]. BZLF1 interferes with several molecules that participate in PML nuclear body aggregates (PML protein, Sumo-1 and CBP), some of which are also involved in a multi-proteins complex termed “enhanceosome” which drives MHC class I heavy chain and  $\beta$ 2m transcription. Specially CBP (CREB-binding protein), which is present in limited amounts in the cell and is needed for optimal induction of transcription by BZLF1, might be “consumed” during EBV replication and withdrawn from participation in other processes. Second, BZLF1 also reduces the transcriptional activity of the NF- $\kappa$ B p65 subunit, which is activated downstream of antigen receptors [134], and could counteract BCR triggered upregulation of MHC class I, since the MHC class I promoter also has NF- $\kappa$ B responsive elements. Third, while during latency LMP1 promotes activation of IRF-7, which results in TAP2 upregulation and enhanced immunogenicity, during lytic replication BZLF1 interacts with IRF7. Whereas this interaction does not affect nuclear shuttling of IRF7, even in response to dsRNA, it inhibits expression of IRF7 target genes (IFN $\alpha$ 4, IFN $\beta$  and TAP2) and results in their reduced mRNA levels [135, 136]. Finally, BZLF1 reduces both protein and mRNA levels of the TATA-binding protein TBP, which is used by all RNA polymerases and is therefore central to the process of gene expression [137]. In one experimental model, however, BZLF1 transfection did not induce MHC class I downregulation, but only prevented its upregulation by LMP1 expression [121].

EBV BMLF1 (also referred to as SM, Mta and EB2) is a nuclear RNA-binding, early lytic cycle protein that is essential for lytic EBV replication, and has homology with HSV ICP27 and CMV UL69. BMLF1 regulates gene expression at the level of mRNA stability, processing and nuclear export and is essential for EBV DNA replication [138]. Most cellular genes as well as latent and immediate-early lytic cycle genes contain introns, whereas the majority of early and late lytic cycle genes do not. BMLF1 has a selective preference for intronless genes, and although it does not affect their rate of transcription, it increases their half-life and aids in shuttling them out of the nucleus, since intronless genes are generally poorly exported [139, 140]. At the same time, it inhibits expression of target genes containing constitutive splicing signals. BMLF1 has also been shown to bind to Sp110b protein, an interferon-inducible component of PML bodies, which is co-opted by BMLF1 to become a component of the cellular machinery that BMLF1 uses to facilitate EBV lytic gene expression [141]. It is possible that BMLF1 acts in concert with BGLF5 to preferentially target host genes to degradation, as it has been shown for the HSV-1 ICP27 and vhs [130]

EBV also encodes a viral homologue (BCRF1, vIL-10) with 83% of sequence identity to human IL-10 (hIL-10). Whereas hIL-10 has both immunosuppressive and immunostimulatory functions, the viral counterpart retains the ability to suppress inflammatory cytokine production and inhibit MHC class II expression on monocytes and macrophages and prevent T cells proliferation, but it does not costimulate thymocyte proliferation nor does it induce MHC class II expression on B cells [142]. Although IL-10 has dampening effects on antigen presentation, it is questionable whether vIL-10 would play a role in lytic cycle-induced MHC class I reduction since it is expressed as a L gene and MHC class I downregulation is an early event. The

influence of both hIL-10 and vIL-10 might be of importance *in vivo* by diminishing T cell-derived IFN $\gamma$  production and supporting B cell survival [143].

Due presumably to cross-priming, EBV-specific CD4<sup>+</sup> T cell responses contain more reactivities to L lytic cycle proteins than CD8<sup>+</sup> T cell responses and probably recognize target cells that have exogenously acquired virion proteins rather than cells that have been infected by the virus. Interestingly, CD4<sup>+</sup> T cell clones against structural proteins of the virion were capable of recognizing primary B cells immediately following virus infection, presumably by the presentation of processed antigens of the virion on MHC class II molecules, and decreased transforming efficiency of B cell *in vitro* by secreting IFN $\gamma$ . However, EBV can actively interfere with CD4<sup>+</sup> T cell recognition through gp42. gp42 interacts intracellularly with MHC class II molecules at all stages of their maturation and is produced both as a membrane-bound and soluble form during lytic replication. The soluble form, which can also be shed, is able to directly bind to MHC class II and protect cells from CD4<sup>+</sup> T cell recognition due to steric hindrance of MHC class II/TCR interaction [144, 145].

### 3.5.3 Target cell elimination

Following recognition by cells of the immune system, infected target cells must be eliminated. Elimination by effector cells can be achieved by either the release of cytotoxic granules containing perforin and granzymes, or by death receptor-mediated killing. While analyzing MHC class I synthesis and export during EBV replication (**paper II**), we observed an accumulation of calreticulin, an ER chaperone essential for MHC class I assembly in the ER, at the cell surface. Calreticulin has been shown to interact with perforin and inhibit perforin mediated killing. This protection seems to occur independently of direct interaction with perforin and a mechanism whereby calreticulin stabilizes the membrane and prevents polypore formation of perforin has been proposed [146].

Death-receptor mediated killing is involved in the control of immune responses and in the clearance of virus-infected and tumor cells. TRAIL has attracted special attention since it was demonstrated that it kills preferentially tumor cells while sparing most primary cells [147]. Moreover, TRAIL seems to play an important role in the killing of cells infected by dengue [148], hepatitis B [149], influenza [150] and HCMV [151]. Few studies have, however, addressed the role of EBV in modulating sensitivity to TRAIL. EBV-positive B cell lymphoma lines generated from patients with PTLN, and thus displaying the latency III program, are insensitive to TRAIL, in spite of high receptor expression. The resistance relies on receptor-proximal inhibitory mechanisms, since neither receptor capping nor caspase-8 activation could be observed in response to TRAIL [152]. Similarly, *in vitro* infected EBV-negative cell lines acquire resistance to TRAIL and FasL [153], which relies partially on the expression of LMP1, downstream activation of NF- $\kappa$ B and expression of cFLIP. In two reports, however, LMP1, alone or in the context of latency III, was shown to induce Fas expression, as a true mimic of CD40L, and to sensitize cells to FasL [154, 155].

In **paper III** we show that, during EBV replication, target cells become insensitive to TRAIL. This effect is probably mediated by a number of factors. EBV replication leads to downregulation of both TRAIL-R1 and TRAIL-R2 from the cell surface. Whereas TRAIL-R1 probably follows the same fate as MHC class I and is decreased due to inhibition of protein synthesis, TRAIL-R2 accumulates inside the cell, and is a likely contributor for concomitant decoy TRAIL-R4 retention, since these receptors are preassembled on the plasma membrane [26]. Since neither protein synthesis nor ER stress were able to mimic the effect of replication on the retention of the receptors, it is possible that virus-specific mechanisms operate at this level to protect cells from TRAIL-mediated apoptosis. Adenoviruses downregulate TRAIL-R2 by receptor internalization and degradation [28], whereas strategies for retention and/or degradation of other immunomodulatory receptors have been described for herpesviruses [156]. Induction of replication by BCR triggering leads to expression of lytic cycle proteins including LMP1, and thus mimics the signals received by centrocytes during clonal selection in the GC. We have shown that when both signals are given in combination they protect target cells from TRAIL and FasL-induced cell death, an effect mediated by the interplay of both cFLIP and mitochondrial proteins such as Bcl-2 [157]. Additionally, BHRF1, an E protein which shows partial sequence and functional homology to Bcl-2, can protect epithelial and B cell lines from Trail-, FasL- and TNF-induced apoptosis, downstream of Bid cleavage [158].

TNF $\alpha$  induces a wide range of genes that play a role in the immune and inflammatory response, including molecules of the MHC complex, adhesion molecules, chemokines and cytokines. In addition it plays a role in induction of apoptosis, acting as an antiviral cytokine directly cytotoxic to virus-infected cells. It had been shown previously that BZLF1 prevents TNF $\alpha$ -induced upregulation of ICAM-1 and TNF $\alpha$ -induced cell death, through the downregulation of TNF-R1 promoter activity, leading to downregulation of surface receptor expression (Morrison and Kenney, 2004). In **paper III** we could recapitulate, in our system, the effect of EBV replication on receptor expression, but we could not observe any additional protection conferred by receptor modulation, probably due to the reasons highlighted above.

Viral FLIPs (vFLIPs) were first identified in non-human  $\gamma$ -herpesviruses and in KSHV. vFLIPs all resemble the short form of human cFLIP, which only contains two sets of DED but no caspase-like domain [24]. We have identified by immunoblotting an extra band of lower molecular weight than caspase 8 but higher than caspase 9, that could be revealed by different anti-caspase antibodies, only in Akata cells supporting virus replication (data not shown). Though the size resembles that of a pro-caspase, we have not yet determined the nature of this cross-reactive protein.

EBV devotes more than 80% of its genome to lytic replication. While a large part of it is clearly necessary for DNA replication, assembly and export of virions, the function of many of these genes remains unknown. The rather recent establishment of lytic replication models in vitro, although maybe not very useful for the assessment of replication in the context of an immune response, will bring many more insights into the biology of the virus.

## 4. EBV AND BURKITT'S LYMPHOMA

Burkitt's lymphoma is a non-Hodgkin B cell lymphoma that develops either as a facial or abdominal tumor, and has one of the highest rates of cell proliferation (doubling time of 24-48h) of any human tumor [159]. There are three epidemiologically distinct variants of BL: endemic, sporadic and immunodeficiency-associated. The originally described endemic form of BL occurs in the equatorial regions of Africa and Papua New Guinea, where malaria is holoendemic. Endemic BL is 100% EBV-genome positive and mostly affects children. In parts of Africa, it accounts for up to 74% of all malignancies in childhood. Sporadic forms vary in their geographical distribution, incidence, association with EBV and mainly affect young adults and children. In areas with intermediate incidence, such as Brazil and North Africa, EBV is present in up to 85% of tumors, but only in 15% of sporadic cases seen in developed countries [2].

BL tumor cells usually express IgM and markers of GC centroblasts, such as CD10, CD77 and BCL6. Nevertheless, it is not clear whether the tumor stems from a GC or memory B cell. On one hand, BL cells have undergone somatic hypermutation and express the appropriate markers of GC origin. On the other hand, these tumors grow in sites other than lymphoid tissue and EBV, if present, exhibits a pattern of gene expression which resembles that of circulating latently infected memory B cells, with the sole expression of EBNA1 and the non-coding EBER RNAs (present in all forms of EBV latency) [9].

The hallmark of all BL tumors is the translocation between the *c-Myc* proto-oncogene and one of the Ig heavy or light chain loci [1]. *c-Myc* plays a critical role in regulating cell proliferation, differentiation and apoptosis. Although expression of *c-Myc* is normally tightly regulated, once translocation occurs, expression of the gene becomes constitutive and potently drives S phase cell cycle progression [69, 160]. Interestingly, the translocation breakpoints both in the *c-Myc* and Ig genes differ between endemic BLs and the sporadic or HIV-associated BLs [1].

Endemic BL is now well established as a polymicrobial disease. There is overwhelming evidence for the participation of both EBV and malaria in the disease, as well as the potential contribution of other mechanisms in the process that ultimately leads to *c-Myc* translocation (described below).

The initial evidence that implicated EBV in BL tumorigenesis was the discovery that EBV-positive BL tumors carry clonal EBV genomes in all tumor cells [161]. Spontaneous loss of EBV during in-vitro passage of BL cell lines leads to an increase in susceptibility to apoptosis [162], and both EBNA1 and EBERs have been implicated in protection against apoptosis and increase in tumorigenicity [69]. Even though it has been assumed that only EBNA1 and EBERs are expressed in BL cells, additional forms of gene expression were described recently, in that BL cells of the same tumor were shown to express either the latency I pattern, all EBNA1 without EBNA2 expression, or all six EBNA1s, in all cases without detectable expression of LMP proteins. These different forms of latency also conferred variable protection against different apoptotic

stimuli and showed that expression of all EBNA2 induced the highest level of protection [163]. These results support the notion that EBV acts as an inhibitor of apoptosis rather than as an inducer of growth-transformation in BL, and counteracts the pro-apoptotic effect of excessive *c-Myc* expression. However, this issue is not resolved since EBV infection has been shown to lead to genomic instability, with latency I being sufficient for this effect [164].

Comparison of VDJ rearrangements in tumors of endemic or sporadic origin also revealed that endemic (EBV positive) tumors have higher mutation rates than sporadic tumors, and signs of antigenic selection, which were absent in sporadic tumors. This study, together with the evidence that the pattern of *c-Myc* translocation differs between endemic and sporadic forms, suggests that EBV negative sporadic cases arise from an early centroblast cell, whereas EBV-positive tumors derive from a memory post-germinal center cell [160].

The alleged involvement of malaria infection in BL pathogenesis have relied on three major correlations. In patients with BL, the frequency of the sickle-cell trait, which offers protection against severe infection of *P. falciparum* malaria, is lower than would be expected based on a random distribution [165]. Also, the incidence of BL has dropped in areas with efficient mosquito eradication programs [166] or where widespread treatment of the population with anti-malarial drugs has been implemented for a window of time [167].

Chronic exposure to malaria has profound effects on the immune system leading to paradoxical polyclonal B cell activation, hypergammaglobulinemia, and autoantibody production, in combination with loss of T cell responses, deletion of memory B cells and a general state of immune suppression [168]. Of the four species that infect man, *P. falciparum* causes the most severe morbidity and is responsible for essentially all mortality associated with malaria. The high pathogenicity of *P. falciparum* is attributed to the ability of the parasite to infect erythrocytes of all developmental stages and its capacity of promoting adherence of infected red blood cell to vascular endothelium (sequestration) and to other uninfected cells. This process in turn relies on the expression of the variant adhesin *Plasmodium falciparum* membrane protein 1 (PfEMP1) during the erythrocytic phase [169].

PfEMP1 contains functionally conserved domains that are, nevertheless antigenically variable. Rapid appearance and mutually exclusive expression of different variants of PfEMP1 allow for the generation of heterogeneous adhesive phenotypes within a clone and promote the evasion from protective immune responses. PfEMP1 has been shown to bind to CD36, CD31, non-immune Igs, ICAM-1 and heparan sulphate. The extracellular cysteine-rich interdomain region 1 alpha (CIDR1 $\alpha$ ) of PfEMP1 has been recently shown to bind to sIgs and act as a T-I polyclonal B cell activator in a manner similar to protein A of *S. aureus* [170], which might explain the polyclonal B cell activation and hypergammaglobulinemia seen during malaria. This effect was shown preferentially on memory B cells and resembled sIg triggering by the pattern of gene expression induced [171].

We show that in addition to inducing B cell activation and survival, recombinant CIDR1 $\alpha$  also induces EBV replication in infected target cells (**paper IV**). This effect was seen in the model BL cell line Akata, tonsil B cells of healthy EBV-infected individuals and PBMCs of children with BL. The fact that CIDR1 $\alpha$  preferentially stimulates memory B cells, in which EBV establishes latency and that it can induce replication in these cells might represent a mechanism by which malaria infection leads to an increase in numbers of EBV virions that in turn are capable of infecting new target cells. The fact that memory B cells have been recently shown to return to GCs and undergo additional rounds of affinity maturation [20], coupled to the fact that, especially in the spleen, these cells are in close proximity with infected erythrocytes, could lead to the infection of a large pool of GC B cells in different stages of differentiation. This could also partly explain why alternative gene expression programs can be detected in BL cells, apart from the most common latency I [163]. Additionally, anti-VCA antibodies increase in the months or years preceding the onset of BL [172] and antibodies against BZLF1 rise during acute malaria infection [173] together with EBV viral loads in peripheral blood. Cell-free EBV DNA in plasma is also found in children in malaria endemic areas, and is cleared with antimalaria treatment [174].

Cytotoxic T-cell mediated immunosurveillance to both EBV latency and lytic antigens is reduced in children of holoendemic malaria areas [175], and might be due in part to increased levels of IL-10 which both inhibit T cell responses and stimulate B lymphocytes. Thus, increased circulating virus, infection of a bigger B cell pool, coupled by decreased recognition and elimination of infected cells, deregulated viral gene expression and disruption of lymphoid organ architecture during malaria [176] could contribute to an increase in the chance of a *c-Myc* translocation to be rescued. Again, the fact that BLs are clonal and that sporadic cases of BL, which are not associated with either EBV or malaria, happen at much lower frequencies speaks for the rare occurrence of the translocation.

Infected erythrocytes have also been shown to express the TLR9 ligand hemozoin [177]. TLR9 is expressed constitutively on memory B cells and can be induced in naïve B cells upon BCR triggering [178]. It remains to be addressed what effect hemozoin alone or in combination with CIDR1 $\alpha$  has on EBV infected B lymphocytes.

Other mechanisms have been proposed to aid in the increase of *c-Myc* translocation frequency in BL patients. First, acquisition of immunity to arboviruses (e.g. Yellow fever) coincides better with the age distribution of endemic BL than acquisition of immunity to either malaria or EBV, and arboviruses have been suggested to induce oncogenic alterations in concert with DNA viruses, such as EBV. Second, plants of the genus *Euphorbia*, which are present in areas that overlap with malaria and EBV and are used for medicinal purposes, produce tumor-promoting substances which resemble the phorbol ester TPA, a protein kinase C (PKC) activator. Outbreaks of arboviral infections have coincided with clustered manifestations of BL and thus raise the possibility that arbovirus and plant-derived carcinogens act as cofactors of BL development along with malaria and EBV [159].

## 5. EBV AND AUTOIMMUNITY

Involvement of EBV in the pathogenesis of autoimmune diseases has been proposed a long time ago. Growing evidence implicates EBV as a risk factor for rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and multiple sclerosis (MS). SLE and MS patients are almost 100% EBV positive as compared to 90% in the general population. Even more striking differences were observed in pediatric cohorts of MS, where 83% of patients are EBV seropositive as compared to 42% of seroprevalence in controls. EBV viral loads in peripheral blood are increased up to 40-fold in SLE patients and up to 10-fold in RA patients as compared to controls. Furthermore, infectious mononucleosis (IM) does not only increase the likelihood of developing hematologic malignancies such as HL, but also increases by two fold the risk of developing MS. Patients with the history of IM have increased anti-EBNA1 Ig titers more than 10 years before the onset of MS symptoms [179, 180].

The contribution of EBV to autoimmunity has been attributed to molecular mimicry between EBV antigens, such as EBNA1, and self-antigens [181]. Whereas this cannot be disregarded, another possibility is that EBV directly immortalizes autoreactive B lymphocytes. In this respect, LMP2a may play a prominent role due to its capacity to rescue B cells with crippling BCR mutations, to bypass anergy induction by self-antigen and to bypass pre-plasma cell tolerance checkpoints in transgenic mouse models of autoimmune disease. LMP2a transgenic mice recapitulate several features characteristic for patients with SLE such as increased numbers of circulating plasma cells, decreased amounts of the negative regulator of BCR signaling Lyn and increased B cell-survival cytokine BAFF expression. SLE patients also have increased numbers of LMP2a positive cells in the peripheral blood [180].

Central in the study of autoimmunity are the mechanisms by which autoreactive cells are deleted during the GC reaction checkpoint, which can generate B cells with self-reactive BCRs. As previously mentioned, both Bim and FasL have been implicated in the quality control during the GC reaction. Mice lacking Bim develop lymphadenopathy and splenomegaly, display increased amounts of circulating autoantibodies, and accumulate IgG-secreting plasma cells resulting in hypergammaglobulinemia [46]. Fas-mediated apoptosis is required for normal lymphocyte homeostasis and peripheral immune tolerance. In Fas- (lpr) and FasL- (gld) deficient mice and in patients with the same deletions, abnormal accumulation of lymphocytes often result in systemic autoimmunity [46].

The TRAIL pathway may be also involved in autoimmunity. Chronic blockade of TRAIL by antibody exacerbates collagen-induced arthritis and experimental autoimmune encephalomyelitis in mouse models of human rheumatoid arthritis and multiple sclerosis. TRAIL deficiency also enhances the susceptibility of mice to autoimmune arthritis and diabetes [23]. TRAIL, however, is mostly involved in preventing disease progression than in the initiation of autoimmunity, since TRAIL knockout mice have a largely normal phenotype [48]. Interestingly, LMP2a expression in B cells of transgenic animals did not induce any obvious signs of autoimmunity

either, and these animals had to be crossed with animals transgenic for a self-reactive BCR specific to observe a brake of tolerance [76]. This suggests that both TRAIL deficiency and LMP2a expression can independently confer a survival advantage to autoreactive B cells, that would otherwise be either deleted or anergized, respectively. If our model proposed in **paper III** is correct, B cells triggered by antigen in the absence of T cell help should be more susceptible to TRAIL-induced cell death. This hypothesis implies that cells receiving BCR-like signal through LMP2a should be more sensitive to TRAIL. Our unpublished experiments support this notion (data not shown). It is possible that the phenotype observed in transgenic mice in the presence of LMP2a or in the absence of TRAIL under autoimmune-predisposing conditions, is much milder than what would be seen in double-knockout animals. Along these lines of reasoning, a synergistic effect on the development of autoimmunity was observed for inactivation of Fas and Bim in double-knockout mice [46, 182].

B-1a B cells secrete “natural” IgM antibodies with wide a spectrum of reactivities. Due to their weak affinity to self-antigens these cells were first proposed to mediate autoimmunity, but were subsequently shown not to be the high affinity antibody-producing cells in autoimmune diseases. However, B-1a B cell numbers are increased in mouse models of autoimmunity, they are potent producers of IL-10, and bear excellent antigen-presenting capacity [183]. We have shown that human circulating CD5+ B cells, which should correspond to B-1a B cells, are extremely sensitive to death induced by TRAIL (**paper III**), and if proven to be implicated in autoimmunity, might constitute interesting targets for immunotherapies.

EBV-infected B cells from patients with SLE also have aberrant latent and lytic gene expression in the blood. Whereas LMP2a is occasionally detected in infected memory B cells in the periphery, without any concomitant expression of other viral genes, some SLE patients show expression of LMP1 and BZLF1. It is not known, however, whether these are the memory B cells secreting high affinity antibodies [184].

During primary EBV encounter, virus infection can lead to the immortalization of large numbers of B lymphocytes, some of which carry potentially self-reactive specificities. Otherwise, random self reactivities can be acquired during somatic hypermutation in the GC. Due to the concert action of LMP2a and LMP1 these cells might escape all the checkpoints controlled by Bim (due to BCR-only activation), FasL (due to CD40L-only triggering) or TRAIL (due to BCR-only activation). LMP2a would also ensure that these cells are not anergized by low levels of peripheral antigen, enhance the generation of plasma cells, increase the level of antigen presentation by both MHC class I and II, and costimulatory molecules that could aid in the activation of self-reactive T lymphocytes, the major players in certain autoimmune diseases such as MS [180]. Additionally, LMP2a increases the sensitivity of B cells to TLR ligands, and TLR9 has been implicated in the recognition of dsDNA during SLE [185]. It is not surprising, in this context, that IM could represent an overblown version of this scenario, with disruption of lymphoid architecture where many more B cells can be immortalized at random and where viral gene expression is disregulated. Autoimmunity, therefore most probably relies on a plethora of triggering factors, with specific HLA backgrounds, deficient clearance of apoptotic cells containing DNA,



antigenic mimicry and EBV infection working in concert to subvert immune response checkpoints.

## 6. CONCLUSION

Methods of molecular genetics and development of new models to study the virus lytic cycle have revealed new mechanisms of immune escape during EBV replication and underscored the importance of EBV interaction with unconventional (non-B cell/non-epithelial cell) cellular targets such as monocytes and dendritic cells. These data has to be incorporated in a model of EBV persistence that becomes significantly more complex than previously proposed but helps to explain certain paradoxes.

During primary infection, EBV is passed through saliva as cell-associated virus or as free virions, and might directly infect epithelial cells through their apical membranes, or somehow cross the epithelium and be picked up by naïve B lymphocytes, the most abundant B cell subset present at this site in the tonsils. The virus switches on the growth program in infected B cells and drives their migration to B cell follicles, where they divide and form GCs. During migration, B lymphocytes, which retain surface-bound virions during prolonged periods of time, transfer these viruses directly to epithelial cells or to phagocytes. Virions passed through saliva have a high tropism for MHC class II expressing cells (i.e. B cells and other APCs) and might also infect them directly. Depending on the phenotype of the recipient cells and the local cytokine environment, this transfer or direct infection can lead to different outcomes. If infected, monocytes and Langerhans cells might migrate into the epithelium, initiate replication and pass the virus onto neighbouring epithelial cells. Phagocytes might also bind EBV and secrete B cell growth-promoting cytokines (IL-6, GM-CSF) without any need of viral gene expression, or, if triggered to differentiate into dendritic cells, be deleted by apoptosis to avoid premature antigen presentation. Infection of immature dendritic cells might also induce unresponsiveness to other maturation signals, as observed for other  $\gamma$ -herpesviruses [186].

Both cell-bound and free virus can efficiently infect epithelial cells through the basolateral membranes. Once infected, epithelial cells are able to shed virus both into saliva and into the epithelium. This replication step seems to be essential for efficient establishment of latency as suggested by the analysis of  $\gamma$ HV68 infection in mice. At some point, however, local stimulation of antigen presenting cells leads to activation of NK cells, which limit the extent by which newly infected B cells initiate the growth program, and T cells, which initiate EBV-specific responses.

Crucial for successful establishment of latency is the transient expression of certain lytic cycle genes (BHRF1 and BALF1) immediately after the infection of B lymphocytes, which provide early rescue signals from apoptosis, until EBNA2 latency genes are activated and drive B cell expansion. The importance of proliferation for the establishment of latency is again supported by the  $\gamma$ HV68 model. Proliferation of EBV infected blasts and their seeding of GCs probably requires LMP2a and LMP1, which mimic stimuli provided by the BCR and CD40, respectively. Both signals rescue infected B lymphocytes at the multiple checkpoints that normally ensue that centroblasts with crippled or self-reactive BCR generated by somatic hypermutation, or B cells activated by bystander help, are deleted. Whether participation of true antigenic

triggering takes place, and whether TRAIL has any limiting influence at this point of the reaction is unknown. In any case, infected B cell exit the GC with the phenotype of an antigen-selected memory B cells, which expresses either no EBV genes or LMP2a alone and may express EBNA1 during homeostatic division.

Latency with limited or no antigen expression in the memory B cell compartment ensures life-long persistence of the virus. However, continuous shedding of virions through saliva and the fact that epithelial cells do not seem to, under normal circumstances, carry latent EBV, mean that the virus has to reactivate occasionally in other cellular reservoirs. Plasma cell differentiation in response to antigen in the tonsils provides sufficient signals for lytic cycle initiation. EBV carrying Langerhans cell precursors that migrate to all mucosal tissues might provide additional sources of virions found in saliva.

Replication in B lymphocytes is associated with a number of immune escape mechanisms that limit recognition by EBV-specific CD8<sup>+</sup> T cells (MHC class I downregulation by BGLF5 and BNLF2a, and gp42 blocking of MHC class II) and the susceptibility of targets to elimination (TRAIL, TNF and IFN $\gamma$  resistance) [187]. How replication in other cells is triggered and progresses is not known, but the fact that latency genes are expressed at late stages of the lytic cycle and strongly inhibit apoptosis might result in survival of the host cell after termination of the replicative cycle (see figure 4 for a summary).

EBV congregates mechanisms of induction of proliferation, inhibition of apoptosis and immune escape in the B lymphocyte, a cell type central to the establishment of immune responses. In light of this knowledge, it is very surprising that EBV infection is not associated with an even higher incidence of malignancies and autoimmunity. Further analysis of the mechanisms which ensure the relatively innocent persistence of EBV in the human population will help to understand the pathological consequences of EBV infection.

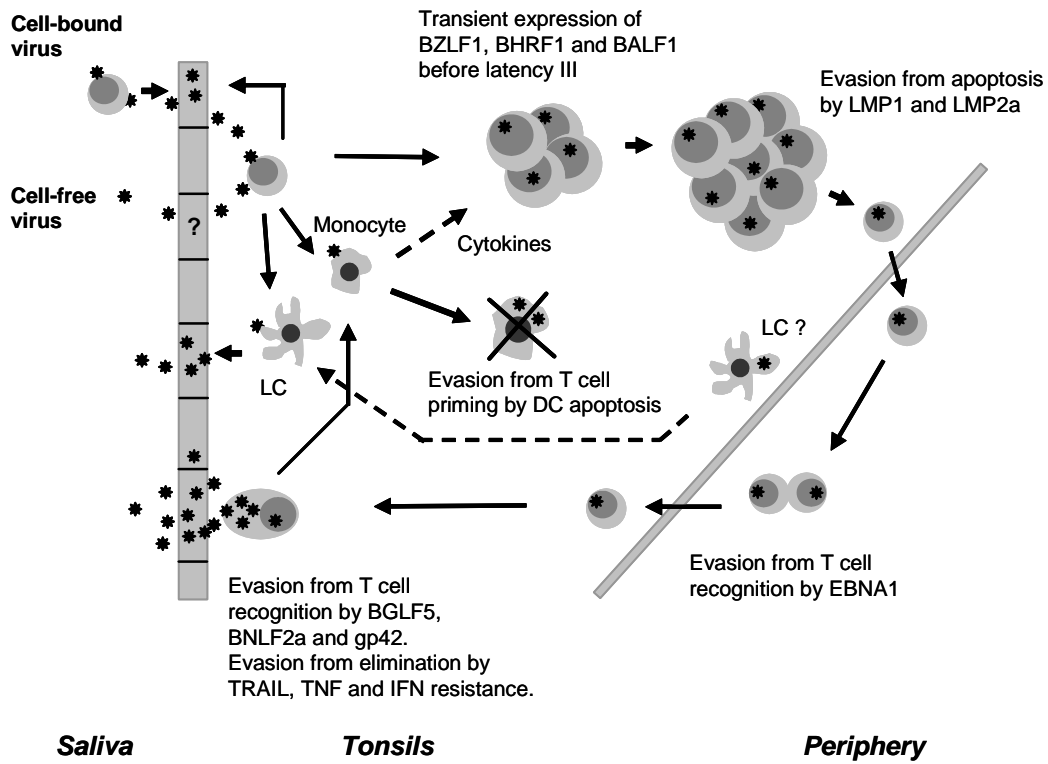


Figure 4. Some of the strategies employed by EBV to evade immune recognition.

## 7. ACKNOWLEDGEMENTS

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