Mechanisms of immune evasion in Epstein-Barr virus infection

Anna M. Gram

About the cover: To understand the phenotype displayed by a cell, we need to take a look behind the scenes and realize that it is a net sum of ongoing processes.

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Anna Magdalena Gram

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Promotoren:	Prof. dr. Rob C. Hoeben
	Prof. dr. Emmanuel J.H.J. Wiertz
Co-promotor:	Dr. Maaike E. Ressing

Promotiecommissie:	Prof. dr. Tom H.M. Ottenhoff
	Dr. Mirjam H.M. Heemskerk
	Prof. dr. Frank J.M. van Kuppeveld, Universiteit Utrecht
	Prof. dr. Martine J. Smit, Vrije Universiteit Amsterdam

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Chapter 1

General introduction

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Herpesviruses

Herpesviruses are enveloped viruses with a linear double-stranded DNA genome. Their genome sizes range from 125 to 230 kilobase pairs harboring at least 70 to 200 coding and up to 70 non-coding gene products [1]. These gene products give rise to viral proteins and several RNA species including mainly micro-RNAs and few non-coding RNAs. The virions of herpesviruses consist of an icosahedral capsid containing the densely packaged genome, the tegument surrounding the capsid, and the lipid envelope with several glycoproteins.

To date, up to 200 distinct herpesvirus species have been identified in mainly vertebrates [1], of which 100 have been classified by the International Committee on Taxonomy of Viruses (ICTV). As herpesviruses have generally a narrow host range, it is, therefore, likely that more than the 200 identified herpesviruses exist. The order of *Herpesvirales* consists of the families of *Herpesviridae*, *Alloherpesviridae*, and *Malacoherpesviridae* [2]. The hosts of *Herpesviridae* are mammals, birds and reptiles, while *Alloherpesviridae* and *Malacoherpesviridae* infect fish and amphibians, and bivalves, respectively. The family of *Herpesviridae* is divided into the three subfamilies α-, β-, and γ-*Herpesvirinae*. In humans, eight herpesvirus species have been identified. According to the guidelines of the ICTV, they are named human herpesvirus (HHV) 1 through 8, but their traditional names are widely used. The human herpesvirus species are α-herpesviruses Herpes simplex virus (HSV)-1, -2 (HHV-1 and -2), Varizella Zoster virus (VZV, or HHV-3), the β-herpesviruses human cytomegalovirus (HCMV, or HHV-5), HHV-6 and -7, as well as the two γ-herpesviruses Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8), and Epstein-Barr virus (EBV or HHV-4) [3]. Human herpesviruses are typically highly prevalent. Epstein-Barr virus, for instance, is carried by about 90% of the adult world population [4]. KSHV is an exception with only very low prevalence in most Western societies (around 3%), while its prevalence is higher in areas such as the Mediterranean basin or sub-Saharan Africa (15-25% and 50-60%, respectively) [5].

A hallmark of herpesvirus infection is the establishment of life-long latent infection. This is also reflected by the name of the virus family originating from the Greek word 'herpein' meaning 'to creep'. Latency is characterized by limited viral gene expression without production of viral progeny. In contrast to other viruses establishing latent or chronic infection, such as human immunodeficiency virus (HIV), papillomaviruses (HPV), or hepatitis C virus (HCV), persistence of herpesviruses usually does not result in life-threating diseases. Primary infection with herpesviruses or reactivation from latency cause symptoms as seen for HSV-1 (cold sore) and VZV (chickenpox and shingles, respectively), but can also be asymptomatic as in many cases of EBV infection. The absence of severe symptoms may be interpreted as a delicate balance of immune control of herpesviruses and good adaptation of the virus to the host. Due to millions of years of co-evolution between herpesviruses and humans, herpesviruses have acquired successful strategies to evade innate and adaptive immune recognition including innate immune receptors (e.g TLRs) and downstream signalling [6,7,8], the complement system [9], and antigen presentation [10,11,12] without causing major damage of the human host.

Life cycle of Epstein-Barr virus

EBV is the prototypic human γ-herpesvirus belonging to the genus of Lymphocryptoviruses. In general, it is transmitted via saliva. Typically, EBV infects and replicates in epithelial cells and B lymphocytes. The entry mechanism of EBV into these cells is dependent on different cell surface molecules [13]. Interestingly, virions produced in epithelial cells appear to have an increased tropism for B cells and vice versa [14]. In a new host, EBV infects epithelial cells in the oropharynx, lytically replicates in these cells and disseminates to naïve B cells. According to the current model of the EBV life cycle [15], EBV infects naïve B cells, which then start to proliferate in response to the growth-transformation program of the nine latency-associated EBV proteins. During differentiation of EBV-infected B cells into memory B cells, the long-term reservoir of EBV, expression of latent EBV proteins is continuously reduced. Finally, a quiescent stage is reached in which no EBV proteins are expressed (latency 0). Different stages of latency (latency I, II, and III) are characterized by expression of distinct sets of latent EBV proteins [16]. Besides latent proteins, EBV expresses the two non-coding EBV-encoded small RNAs (EBER) and about 40 miRNAs organized in two clusters, the BART and BHRF1 miRNAs, during the latent stages [17]. The functions of these non-coding RNAs are now beginning to be elucidated and growth-transforming, anti-apoptotic, and immune evasive functions have been suggested [18]. Interestingly, miRNAs have been detected in EBV virions, allowing their release in newly infected cells [19]. Moreover, EBV miRNAs can be transferred via exosomes from EBV-infected cells to uninfected recipient cells *in vitro*, and may in this way regulate uninfected cells and cell types not typically infected by EBV [20,21].

Reactivation from latency and lytic replication of EBV in memory B cells is associated with terminal differentiation into plasma cells. The molecular mechanism and circumstances of reactivation are, however, ill-defined. Lytic replication is highly organized and consists of three distinct phases. During the immediate-early phase, the viral transactivators are expressed, which drive expression of the early phase proteins that are required for viral DNA replication and expression of the structural proteins during the late phase. New virions are assembled and infectious virus is released, which might be further amplified in epithelial cells shedding virus into saliva. Lytic replication does not remain unnoticed by the immune system as many immunogenic or immune-stimulatory viral molecules are generated. Finally, the host's immune system controls and limits lytic replication of the virus.

Clinical manifestation of Epstein-Barr virus infection

EBV is the causative agent of infectious mononucleosis (IM), a disease characterized by sore throat, lymphoadenopathy, fever, and fatigue [13]. Onset of symptoms correlates with an exaggerated expansion of mononuclear cells, mainly EBV-specific $CD8^+$ T cells [13,22]. This characteristic expansion is also reflected by the name of the disease. IM is a self-limiting disease that mainly occurs in adolescents and adults upon primary EBV infection. A prospective study among EBV-naïve university students revealed that more than 75% of these students developed infectious mononucleosis upon primary EBV infection [23]. At young

age, EBV infection is asymptomatic in most cases, yet children might develop IM. In fact, IM has first been described by a pediatrician [24]. To date, it is unclear why IM is more common at adolescent or adult age. One hypothesis is that pre-existing immunity primes adults to respond stronger to EBV as they may have acquired more T cell clonotypes cross-reactive with EBV epitopes. However, only a minor fraction of $CD8^+$ T cells from EBV-positive individuals show reactivity to both influenza and EBV peptides [25]. Another explanation is offered by a study showing that lytically EBV-infected B cells are preferably recognized by a specific NK cell subset, which is decreased in frequency in adults compared to children [26].

EBV infection of primary B cells *in vitro* results in growth-transformation enabling prolonged culturing of these lymphoblastoid cell lines (LCL). Given the growth-transforming properties of EBV, it may not come as a surprise that various malignancies are associated with EBV, which are often of B lymphocyte or epithelial origin. Indeed, EBV was the first tumor virus described. EBV-associated malignancies were found to generate different sets of EBV latency proteins, thereby distinct latency stages were characterized. In 1964, EBV was first identified in Burkitt's lymphoma (latency I), a B cell lymphoma [27]. Nowadays, EBV infection is also recognized to be associated with Hodgkin's lymphoma, nasopharyngeal and gastric carcinoma (all latency II), lymphoproliferative disease in the immunocompromised (latency III) and other malignancies (reviewed in [28]).

Lessons from immune deficiencies

Inherited and acquired immune deficiencies as well as transplantation-related immunosuppression are pre-disposing conditions for life-threatening EBV-associated diseases [28,29,30]. These immunodeficiencies are characterized by defects in T and/or natural killer (NK) cell functions, thereby highlighting the importance of these cells in the control of EBV infection.

Primary immunodeficiencies

Most of the primary (or inherited) immunodeficiencies predisposing for EBV-associated diseases are also linked to other pathogenic infections including viral, bacterial, and fungal infections. As an exception, manifestation of the deficiencies X-linked lymphoproliferative disease (XLP) and X-linked inhibitor of apoptosis protein deficiency (XIAP) is mainly associated with primary EBV infection causing severe and often fatal IM [31,32]. Moreover, life-threatening conditions as haemophagocytic lymphohistiocytosis (HLH) or dysgammaglobulinaemia might develop [29]. In case of XLP, lymphomas can arise as well, but those have not been observed in XIAP.

Although XLP patients have normal NK and T cell numbers, these cells display a functional defect. In addition, XLP patients lack invariant NKT (iNKT) cells, a small subset of specialized T cells. Defects were attributed to mutations in the gene *SH2D1A* present on the X-chromosome. This gene encodes the signalling lymphocyte activation molecule (SLAM)-associated protein SAP [33,34]. The adaptor protein SAP allows signalling of several members

of SLAM receptors, which are required for interactions between B cells and NK or T cells [34]. SAP is required to transfer activating signals from SLAM receptors, while in its absence the signal is inhibitory. Since SAP is expressed in NK and T cells, these cells cannot be activated by (EBV-infected) B cells in XLP patients [35,36]. Moreover, SAP appears to be crucial for iNKT cell development explaining the absence of this cell type in XLP patients [37].

In females, one of the two X-chromosomes is randomly silenced in somatic cells. Interestingly, in females with heterozygous SAP expression, EBV-specific $CD8^+$ T cells are exclusively SAP-positive [38] suggesting that SAP-negative T cells cannot contribute to control of the B lymphotropic virus EBV. In non-EBV-specific T cells, SAP-deficiency and expression is equally distributed.

In conclusion, XLP patients are selectively prone to EBV-associated disease, since EBV-infected B cell cannot be properly controlled by NK, T, and iNKT cells.

XIAP patients display a similar phenotype upon infection with EBV, but the underlying molecular mechanism is different. These patients have decreased iNKT cell numbers, but normal T and NK cell numbers [32]. NK cells of XIAP patients appear to be functional, in contrast to those in XLP patients. In XIAP patients, the *BIRC4* gene is mutated resulting in a defective XIAP protein. In healthy individuals, XIAP is present in lymphocytes, myeloid cells, and NK cells [32]. XIAP prevents cell death by inhibiting several caspases promoting apoptosis. Therefore, T cells in XIAP patients show increased sensitivity towards cell death-inducing stimulation. How this excess of apoptosis in lymphocytes contributes to abnormal responses to EBV remains to be determined. As in XLP patients, heterozygous female carriers show a non-random X chromosome inactivation in leukocytes [32] supporting the importance of XIAP in cells controlling EBV infection.

Patients with primary immunodeficiencies are rare; therefore it may be challenging to picture the full phenotype and the consequences and contribution of the absent or dysfunctional molecule, which could also have an indirect, e.g. immunodevelopmental effect. Individuals with a secondary or acquired immunodeficiency are more abundant. Observed complications following EBV infection in these patients support the notion of T cell immunity being crucial in control of EBV.

Secondary immunodeficiencies

A typical secondary immunodeficiency is progression to acquired immunodeficiency syndrome (AIDS) following upon HIV infection. In HIV-positive individuals, Burkitt's lymphomas (BL) are more common than in the general population [28]. Another malignancy observed in AIDS patients is B cell lymphoproliferative disease during late AIDS progression. This malignancy may be associated with a decrease in T cell surveillance and is therefore, less often seen since introduction of highly active anti-retroviral therapy (HAART), which prevents progression to late stage AIDS.

Transplantation patients receiving an immunosuppressive therapy targeted to T cells to prevent graft rejection may also develop EBV-associated lymphoproliferative disease. It

has been demonstrated that this is due to diminished T cell control, as patients with post-transplant lymphoproliferative disease (PTLD) can be effectively treated by adoptive transfer of EBV-reactive T cells [39].

In summary, EBV is the etiological agent of immunopathological diseases, such as IM, and several malignancies. Primary immunodeficiencies selectively affecting T and/or NK cell function, but also induced immunosuppression, contribute to our understanding of EBV control.

Immune responses to EBV

Control of EBV infection is achieved by the interplay of innate and adaptive immune responses. Innate immune responses precede adaptive immune responses. The innate immune system comprises different kind of cells like NK cells, macrophages, dendritic cells (DCs) and granulocytes; and soluble components such as the complement system. The composition of innate immune stimulation determines the ensuing adaptive immunity [40]. Various cell types, but especially innate immune cells, recognize foreign pathogen-associated molecular patterns (PAMPs) by their pattern recognition receptors (PRRs). Generally, viral infections are sensed by PRRs, such as Toll-like receptors (TLRs), cytosolic DNA and RNA sensors [41,42,43,44]. Especially viral nucleic acids are recognized by these receptors, but also other molecules such as viral glycoproteins might be sensed by PRRs.

Innate immune recognition

In contrast to the well-established role of T cell immunity for EBV control, the contribution of innate immune sensors to recognition of EBV infection *in vivo* is less clear, which might be due to the long incubation period of around 40 days after primary EBV infection before first symptoms arise and that virus reactivation is asymptomatic. A recent study could detect a transient type I interferon (IFN) signature in some individuals 3 to 15 days prior to onset of IM symptoms, possibly indicative of innate immune activation [22].

Cells targeted by EBV express a variety of PRRs involved in initial sensing of viral infection. These receptors initiate signaling cascades that culminate in activation of the transcription factors interferon regulatory factors (IRF) 3 and 7 or NF-κB. Nuclear translocation of IRF3/7 induces production of type I interferons (IFN I) and NF-κB-induced transcription activates anti-apoptotic and inflammatory processes, thereby creating an environment hostile to viral replication [42]. Various PRR signaling pathways are activated during EBV infection.

Toll-like and RIG-I-like receptors

The Toll-like receptors (TLRs) TLR2, TLR3, and TLR9 as well as the RNA sensor RIG-I have been implicated in the detection of EBV infection [45]. TLRs are membrane-bound receptors present on the cell surface or in the endosomal compartment, while RIG-I resides in the cytoplasm.

TLR2 is present on the cell surface of monocytes. TLR2 typically senses lipopeptides, yet it has been suggested to sense EBV, possibly its glycoproteins present on the viral envelope [46]. The non-structural protein dUTPase of EBV has been shown to stimulate TLR2-mediated NF-κB activation and it has been suggested that this protein is present in exosomes released by infected cells [47,48]. RNA can be recognized by the ubiquitously expressed cytoplasmic RIG-I molecule and the endosomal TLR3 present in conventional DCs and other immune cells. TLR3 senses dsRNA, while RIG-I responds to ssRNA species. Yet, both sensors were shown to be stimulated by the single-stranded EBERs of EBV, which can form stem-loop structures [49,50,51]. The endosomal TLR9 present in B cells and plasmacytoid DCs (pDCs) responds to DNA. TLR9 has been shown to be stimulated by EBV DNA [52].

Nuclear and cytoplasmic DNA sensors

Several cytosolic DNA sensors have been identified in recent years [53,54]. These DNA sensors can induce type I IFN responses by the STING-TBK1-IRF3 pathway or inflammasome activation resulting in caspase-1-mediated IL-1β/IL-18 maturation and pyroptosis [41,53,55].

ALR inflammasomes

The DNA-sensing AIM-like receptors (ALR) absent in melanoma 2 (AIM2) and IFN-inducible protein 16 (IFI16) can form inflammasomes. Inflammasomes are associated with two different functions, namely cytokine processing and pyroptosis, which are both caspase-1 dependent. In brief, pyroptosis is characterized as caspase-1 mediated cell death and differs to some extent morphologically from apoptosis [56]. Recently, gasdermin D was found to be a substrate for caspase-1 (and other caspases including the human caspase-4, and -5, as well as murine caspase-11). The N-terminal part of cleaved gasdermin D promotes pyroptosis [57,58]. Thus far, pyroptosis has been observed in macrophages, DCs, and neurons. The relevance of pyroptosis to viral infection is at current unclear. The best studied effector mechanism of inflammasomes is processing of the cytokines pro-IL1β and pro-IL-18 into mature and bioactive cytokines by caspase-1 [59]. These are then exported by an ill-defined and unconventional secretion pathway.

IL-1β has a potent pyrogenic effect. It activates immune cells and promotes upregulation of adhesion molecules on endothelial cells, thereby helping activated immune cells, such as neutrophils, to migrate to the site of infection. Mature IL1β signals through the IL1 receptor (IL-1R). IL1R is constitutively expressed on many cell types. In view of its strong effects, IL-1β release is tightly controlled at multiple levels, such as transcription, mRNA stability, protein cleavage by inflammasomes, and secretion [60]. Furthermore, downstream signalling of the cytokine is regulated by IL-1Rα, a soluble antagonist of IL1R.

The importance of regulated IL-1β secretion is underscored by the severe clinical symptoms in patients with autoinflammatory disorders, which are characterized by uncontrolled IL1β release. Examples of these rare auto-inflammatory disorders include cryopyrin-associated periodic syndrome (CAPS), familial Mediterranean fever (FMF), and TNF receptor-associated

periodic syndrome (TRAPS), which are caused by mutations in NLRP3, pyrin (a regulator of ASC), and p55 TNF receptor, respectively [61]. Patients present symptoms ranging from fever, fatigue, and rash to joint, bone, and skin inflammation, serositis and deafness, and mental retardation in severe cases [62,63,64]. Patients can be treated effectively with either recombinant IL1Rα (anakinra), recombinant IL-1R-$Fc_{\gamma 1}$ fusion protein (rilonacept), or monoclonal antibodies neutralizing IL-1β (canakinumab) [61]. So far, knowledge is missing concerning susceptibility to infectious diseases in these patients.

IL-18 is the second cytokine being processed into its bioactive form by inflammasomes. In contrast to IL-1β, IL-18 has no pyrogenic activity; induction of *de novo* transcription of pro-IL-18 is not needed, as it is constitutively expressed in multiple cell types [65]. Similar to IL-1β, IL-18 signals through a heterodimeric IL-18 receptor (IL-18R) that contains a TIR domain activating NF-κB, p38, and JNK. Expression of IL-18R has been reported in T_H1 cells, some myeloid cells, as well as intestinal epithelial cells [59,60]. IL-18, in conjunction with IL-12, is a potent inducer of the type II IFN response via activation of natural killer cells [66].

The sensor molecule AIM2 was identified by several groups and it was shown to form an ASC-dependent inflammasome [67,68,69,70]. AIM2 contains a HIN200 domain and a PYD domain, which interacts with ASC and thereby, recruits pro-caspase-1. The ligand of AIM2, dsDNA, has been identified and direct binding of DNA to the HIN200 domain was demonstrated [68].

Infections by the *Orthopoxvirus* vaccinia virus and by the *beta-herpesvirus* murine cytomegalovirus (mCMV) were found to be sensed by AIM2 in murine macrophages [68,71]. The contribution of the AIM2 inflammasome in vaccinia virus infections *in vivo* still needs to be assessed. In macrophages infected with mCMV *in vitro* AIM2 mediated both IL-1β processing and the expression of IFN-β, the latter occurring independently of inflammasome activation [71]. *In vivo* studies with mCMV infections revealed that serum IL-18 levels as well as splenic $IFN\gamma^+$ natural killer cell numbers were reduced, while viral load in the spleen was increased in AIM2- or ASC-deficient mice compared to wild-type mice [71].

Interestingly, the AIM2 inflammasome did not recognize the *alpha-herpesvirus* HSV-1, although this virus is known to trigger IL-1β release [72]. This suggests the existence of alternative, yet unidentified, inflammasome initiators.

Like AIM2, IFI16 belongs to the ALR family, but its cellular localization is different: whereas AIM2 acts strictly cytosolically, IFI16 is mainly localized in the nucleus due to its nuclear localization sequence (NLS). IFI16 can relocalize to the cytoplasm in an acetylation-dependent manner [73]. IFI16 was suggested to act as a nuclear dsDNA sensor in Kaposi's sarcoma-associated herpesvirus (KSHV)-infected endothelial cells and in EBV-infected B-LCLs [74,75]. Subsequently, IFI16 assembles a functional inflammasome in the nucleus resulting in caspase-1 activation and processing of IL-1β in the cytoplasm, although IFI16-dependent IL1β secretion was not studied. Formation of the nuclear inflammasomes was assessed by co-immunoprecipitation and colocalization studies of different inflammasome components such as ASC and caspase-1. Caspase-1 activation and cytosolic IL-1β processing

were decreased upon IFI16 knockdown during KSHV infection. Assembly of the IFI16 inflammasome in the nucleus is remarkable as all other known inflammasomes are activated and assembled in the cytoplasm. Activation of IFI16 and subsequent inflammasome formation might be restricted to DNA viruses, which replicate in the nucleus. Previous studies could not demonstrate the formation of an IFI16 inflammasome in 293T cells or THP-1 macrophages [67,68], thus formation of an IFI16 inflammasome might be restricted to certain cell types.

Type I IFN response

IFI16 is the only DNA sensor reported to form inflammasomes and induce IFN I responses. Initially IFI16 was shown to sense HSV-1, thereby leading to IFN-β induction via STING [76]. IFI16 is requires for DNA-induced type I IFN production via the STING-TBK1-IRF3 axis in differentiated THP1 cells [77]. However, it is unclear how IFI16 contributes to STING activation.

Most recently, the cytosolic DNA sensor cGAS has been identified and it seems that this sensor plays a major role in the induction of type I IFN responses during infection with DNA viruses [78], although several additional proteins were suggested to act as cytoplasmic DNA sensors [79]. Upon dsDNA binding, cGAS produces the second messenger molecule cyclic GMP-AMP (cGAMP) that binds to the adaptor protein STING resulting in its activation [80]. cGAMP can also be transferred to neighboring cells via gap junctions and even viral particles have been found to carry cGAMP, thereby possibly stimulating STING without the need of DNA sensing [81,82,83]. STING resides in the ER membrane, but translocates to the perinuclear region upon activation. Subsequently, TBK1 and IRF3 are recruited and phosphorylated. Consequently, dimerized pIRF3 is imported to the nucleus where type I IFN transcription is initiated.

It is well known that incoming HSV-1 triggers this DNA sensing pathway in monocytes [78,80]. Also KSHV has been found to stimulate type I IFN responses in endothelial cells via this pathway [84]. Moreover, the first viral interference strategies of the cytosolic DNA sensing pathway have been identified suggesting that immune recognition via this pathway is relevant in vivo [84,85,86]. To date, it is not known whether EBV is sensed by the cGAS-STING pathway. It is even unclear whether human B cells are equipped, except for IFI16, with the respective molecules.

In conclusion, several EBV-derived molecules trigger distinct PRRs resulting in activation of various pathways. These different innate immune signals aid in shaping the ensuing adaptive immune response via induction of pro-inflammatory cytokines and type I IFNs.

Antigen presentation

Activation of the adaptive immune system largely depends on recognition of pathogen-specific antigens. B cells secrete antibodies that help clearing extracellular virions, while pathogen-specific T cells help to eliminate virus-infected cells. To promote cellular anti-viral immunity, antigen presentation is required. Generally, viral peptide antigens are presented by two kinds of antigen-presenting molecules, major histocompatibility complex (MHC) class I (or HLA I in humans) and MHC class II (or HLA II in humans) to cytotoxic $CD8^+$ or helper $CD4^+$ T cells, respectively. Primary EBV infection induces strong, virus-specific T cell responses targeting both lytic and latent EBV-derived epitopes [30]. Analysis of EBV-specific $CD8^+$ T cells revealed that T cells with reactivity towards peptides derived from immediate-early and early EBV proteins were more frequent than those directed against late EBV peptides [87]. Immunodominance of immediate-early and early peptides has been attributed to immune evasion of HLA I-mediated antigen presentation during the early phase of lytic replication, thereby inhibiting presentation of late antigens. There is no evidence of immunodominance of certain HLA II-presented peptides reflected by an even distribution of peptide reactivity of all lytic phase antigens in $CD4^+$ T cells. In contrast to $CD8^+$ T cell responses, $CD4^+$ T cells appear to be rather directed against latent peptides.

Peptide antigen presentation

In general, all nucleated cells express HLA I on their cell surface and are therefore, equipped to present peptide epitopes derived from viral antigens to cytotoxic T lymphocytes (CTL). Presented epitopes are derived from endogenously synthesized proteins including viral proteins. Proteins are degraded by the proteasome into smaller peptide fragments that are transported by the transporter associated with antigen presentation (TAP) from the cytosol into the ER lumen. There antigenic peptides can be further processed before loaded into the groove of HLA I molecules, a heterodimer consisting of the HLA I heavy chain and β_2-microglobulin (β_2m). Peptide-loaded HLA I molecules are transported from the ER to the Golgi and finally to the cell surface by the secretory pathway.

Surface expression of HLA II is far more restricted in comparison to HLA I. HLA II is primarily present on specialized antigen-presenting cells (APCs) such as DCs, monocytes, macrophages, and B cells. Peptides presented by HLA II are derived from exogenous proteins and pathogens taken up by APCs. Peptide processing and loading occurs in the so-called MHC II compartment (MIIC). HLA II is a heterodimer consisting of an α- and a β-chain. In the ER, newly synthesized HLA II associates with the invariant chain and is then transported to the late endocytic MIIC. The invariant chain is processed by cathepsins, which are proteases present in this compartment. Eventually CLIP, the part of the invariant chain residing in the binding groove of HLA II, is exchanged for the antigenic peptide. Peptide-loaded HLA II molecules are transported to the cell surface.

In humans, three genes code for the classical HLA I molecules HLA-A, -B, and –C; and another three for the HLA II molecules HLA-DR, -DQ, and –DP [88]. Moreover, these

genes exhibit allelic polymorphism; thereby each individual possesses an almost unique combination of HLA genes. The non-classical HLA I molecules HLA-E, -F, and –G as well as the HLA II molecules HLA-DM and –DO are monomorphic. Similarly, MHC-like molecules are non-polymorphic [89]. There are different families of MHC-like molecules, one of them being the CD1 family presenting lipid instead of peptide antigen to NKT cells (Fig.1 on page 25).

Lipid antigen presentation

The human CD1 family consists of the five members CD1a-e. CD1 isoforms differ by intracellular trafficking pathways and antigen-binding specificities, thereby fulfilling distinct functions. CD1a-c and CD1d present lipid antigens to T cells, including natural killer T (NKT) cells and γδ T cells [90,91,92], whereas CD1e has been suggested to assist in lipid loading of the antigen-presenting CD1 molecules [93]. In mice, only two forms of CD1d (CD1d1 and CD1d2) are present. Most effector functions are CD1d1-dependent, also supported by the inability of CD1d2 to support NKT cell selection [94]. CD1d is reported to be expressed by various antigen-presenting cells including B cells, DCs, monocytes, and macrophages [95]. Moreover, certain epithelial cells and hepatocytes have this surface marker [96].

Invariant NKT cells and their role in anti-viral immunity

NKT cells recognize lipids presented by CD1d molecules and are divided into two subsets; invariant (type I) NKT (iNKT) cells and diverse (type II) NKT cells [97,98]. iNKT cells are of particular interest as their response bridges the innate and adaptive immune system by rapid secretion of vast amounts of polarizing cytokines.

iNKT cells form an important subpopulation of CD1d-restricted T cells. The name iNKT cell originates from concurrent cellular expression of receptors that hallmark NK cells and a (semi-) invariant T cell receptor (TCR), which consists of an invariant α-chain (Vα24-Jα18 in humans, Vα14-Jα18 in mice) paired with one of a limited set of β chains (Vβ11 in humans, Vβ2, Vβ7, or Vβ8 in mice). In mice, iNKT cells constitute a significant fraction of T cells, while in humans their representation is rather low, suggestive of a different role for the immune response in both species.

iNKT cells are activated in multiple ways. Firstly, TCR engagement of CD1d complexes presenting pathogen-derived lipid antigens can lead to iNKT cell activation. Secondly, innate signals and cytokines such as IL-18 can activate iNKT cells independent of TCR signaling. The receptors NKG2D [99] and TIM-1 [100] can activate iNKT cells both independently and as co-stimulatory signals in concert with TCR triggering. Lastly, CD1d-mediated presentation of altered self-lipids in combination with cytokine signals can effectively activate iNKT cells. The mechanism of activation and the local polarizing cytokine environment dictate the subsequent iNKT cell response.

Upon activation, iNKT cells rapidly produce large amounts of cytokines, including IFN-γ that has direct anti-viral effects and boosts NK cell activation. In addition, iNKT cells can

mediate cytolysis, as a consequence of granzyme B, perforin, TRAIL, and FasL expression. Thus, iNKT cells could directly eliminate pathogens and tumor cells, although the *in vivo* importance of iNKT cell-induced cytotoxicity in general remains to be assessed. It is, however, clear that iNKT cells can mediate direct immune defense in the course of microbial infection, as was shown in *Salmonella typhimurium* and *Mycobacterium tuberculosis* infection models in mice [101,102,103]. Through preferential secretion of T_H1 or T_H2 cytokines, iNKT cell skew $CD4^+$ T cell responses and determine the quality of ensuing adaptive immunity. Interestingly, iNKT cells were suggested to limit EBV-mediated B cell transformation *in vitro* [104]. In recent years, evidence is accumulating that iNKT cells contribute significantly to anti-viral defense [96].

Susceptibility of iNKT cell-deficient humans so far appears restricted to herpesviral infections, especially EBV infection. In contrast, mice lacking expression of CD1d and/or iNKT cells were found to be vulnerable to a variety of viruses, including HSV-1 [105,106], HSV-2 [107], respiratory syncytial virus (RSV) [108], and influenza virus [109]. Furthermore, activation of iNKT cells by treatment with α-GalCer protected mice or decreased susceptibility in infection models studying diverse viruses, including murine cytomegalovirus (MCMV) [110], HSV-2 [111], RSV [108], influenza virus [109,112], HBV [113], and diabetogenic encephalomyocarditis virus (EMCV-D) [114]. This suggests that iNKT cells have a protective role in viral infections. However, pathogenic effects of iNKT cells have also been reported in dengue virus-infected mice [115]. In humans, iNKT cells might contribute to pathogenesis in DENV infection as activation of these cells was associated with disease severity during the febrile phase [116].

CD1d antigen presentation

CD1d molecules are composed of a heavy chain and β_2-microglobulin (β_2m). This structural homology with classical MHC class I molecules is reminiscent of the function shared by CD1d and MHC class I proteins, i.e. presenting antigens [117]. However, the diverse nature of antigens presented by either molecule is reflected in their antigen-binding grooves: whereas the grooves of highly polymorphic MHC class I are well-suited for binding defined peptides, lipid tails fit snugly into the hydrophobic pockets of CD1d, exposing the more polar moieties for TCR recognition.

Before lipids can be inserted into the antigen-binding groove of CD1d, they must first be extracted from the hydrophobic lipid bilayer into aqueous solution, a process that is facilitated by lipid transfer proteins. Distinct lipid transfer proteins vary in their modes of action and lipid-binding specificities [118]. Thus, lipid transfer proteins may facilitate preferential binding of certain lipid species by CD1d, thereby conferring a level of antigen selectivity.

In addition, the route of CD1d trafficking influences the lipid repertoire presented by CD1d molecules. After association of CD1d heavy chains with β_2m in the endoplasmic reticulum (ER), CD1d molecules travel via the Golgi compartment to the plasma membrane. The majority of CD1d leaves the ER in association with β_2m, yet this association is not an

absolute requirement for ER exit. In fact, surface expressed murine CD1d heavy chains are still capable of eliciting an NKT cell response in the absence of β_2m [119,120]. However, human cells expressing predominantly free CD1d heavy chains displayed a significantly reduced ability to activate iNKT cells, suggesting that CD1d/β_2m complexes are the functional unit of lipid antigen presentation in humans [121]. Furthermore, association of CD1d heavy chains with β_2m is required for resistance to lysosomal degradation [122]. In this way, β_2m might influence the lipid repertoire presented by CD1d molecules.

Similar to MHC class II, CD1d molecules survey endocytic compartments for the presence of antigens. Endosomal targeting signals in the cytoplasmic tail of the CD1d heavy chain regulate its trafficking. A threonine-based sequence targets the lipid-presenting molecules to the plasma membrane. Removal of this signal from the CD1d tail or mimicking phosphorylation of the threonine residue redirects CD1d molecules to endolysosomal compartments [121]. A tyrosine-based sorting motif (YXXZ; Y is tyrosine, X a random amino acid, and Z a bulky hydrophobic amino acid) is required for internalization of surface CD1d complexes [123]. In humans, this motif is recognized by adaptor protein (AP)-2, directing CD1d to early endosomes [124], while association of murine CD1d with AP-3 allows to gain access to late endosomes and lysosomes [125]. In humans, the cytoplasmic tail of CD1d lacks the consensus sequence required for association with AP-3 [126]. As a result, human CD1d mostly surveys early endocytic compartments. Still, a fraction of CD1d molecules (both human and mouse) gains access to the endolysosomal system via an alternative trafficking pathway, relying on binding of CD1d with invariant chains or MHC class II/invariant chain complexes. A dileucine motif in the cytoplasmic tail of the invariant chain directs associated CD1d and/or MHC class II molecules to the endolysosomal system, including the MHC class II-loading compartment (MIIC) [127,128,129]. Due to the restricted expression of MHC class II molecules, this alternative CD1d trafficking pathway is mostly constrained to professional APCs. Finally, the threonine-based targeting signal mediates re-expression of CD1d at the cell surface, where the CD1d molecules present their lipid cargo for surveillance by iNKT cells.

Lipid antigens presented by CD1d molecules

CD1d molecules present both pathogen-derived lipids and endogenous lipids. The first CD1d-restricted lipid antigen found to activate iNKT cells was α-galactosylceramide (α-GalCer), a glycosphingolipid compound derived from marine sponges [130]. Originally, this lipid was identified in a screen for compounds with anti-tumor activity [131]. For a long time, α-GalCer remained the only known CD1d-restricted antigen and it is still widely used in functional assays. The last decade has provided insights into the nature of other, biologically relevant, lipid antigens capable of activating iNKT cells.

A large diversity of self-lipid species bound to human CD1d has been identified by elution studies. Among those were glycerophospholipids having a variety of polar head groups and containing either one, two, or four radyl chains. Furthermore, several sphingomyelins and glycosylated sphingolipids were also eluted from CD1d molecules [132].

CD1d can present lipids from pathogenic bacteria including *Sphingomonas* [133,134,135], *Borrelia burgdorferi* [136], and *Streptococcus pneumonia* [137]. The common feature of those lipids is the α-linked glycan headgroup, a feature shared with α-GalCer. For a long time, it was believed that mammalian cells cannot produce α-glycosylceramides, which were thought to represent a specific antigenic determinant of CD1d-presented, pathogen-derived lipids. However, it was recently shown that murine cells constitutively produce small amounts of α-glycosylceramides, although it remains to be determined how these lipids are synthesized in mammalian cells [138]. Another study provided evidence that a minor constituent of the endogenous glycosylceramide fraction was stimulatory for iNKT cells [139].

The identity of physiologically relevant, stimulatory lipid antigens presented by CD1d in the context of viral infection remains, at this time, incompletely understood. As opposed to microbial CD1d lipid antigens, virus-specific lipids do not exist. Therefore, the iNKT cell stimulatory lipids presented by CD1d during viral infection must be of host cell origin. This poses an intrinsic risk of undesired self-reactivity. To avoid this, self-lipids presented by CD1d should only be stimulatory towards iNKT cells during conditions of cellular stress, such as infection or carcinogenesis. Interestingly, the cellular lipid profile was found to be altered during hepatitis B virus (HBV) infection, leading to increased activation of NKT cells. Whereas diverse NKT cells were stimulated by HBV-induced lysophosphatidylethanolamine, different lipid(s) were responsible for the activation of iNKT cells, although their nature was not identified [140].

Alterations in CD1d lipid presentation induced by viral infection appear linked to activation of pattern-recognition receptors, such as Toll-like receptors (TLRs). TLR engagement could effectuate changes in CD1d antigen presentation in various ways such as increased synthesis or reduced degradation of antigenic self-lipids as well as different trafficking of CD1d or increased cell surface expression of this molecule. Stimulation of myeloid DCs with TLR ligands resulted in enhanced iNKT cell activation, which was dependent on both CD1d expression and cytokine secretion of IL-12 or type I IFN [141,142,143]. Stimulation of various TLRs, among which were the virus-sensing TLR3, 7, and 9, altered the expression of transcripts favoring β-glucopyranosylceramide synthesis. Yet the identity of the stimulatory lipids remains elusive at this point.

Reduced degradation of antigenic self-lipids by the lysosome-resident enzyme α-galactosidase A (α-Gal-A) has been suggested to induce iNKT cell activity [144]. Moreover, TLR stimulation caused a temporary decrease in α-Gal-A activity. Thus, the TLR-dependent inhibition of α-Gal-A activity would provide a mechanistic link between TLR-mediated pathogen recognition and the generation, and subsequent presentation, of antigenic self-lipids by CD1d [144].

The trafficking route of CD1d molecules affects lipid presentation. A subset (5-10%) of CD1d molecules associates with MHC class II complexes [129]. In TLR-stimulated mature DCs, MHC class II molecules are dramatically relocalized from intracellular endosomal compartments to the cell surface. As a consequence, the MHC class II-associated pool of

CD1d molecules would not encounter certain lipid species along the endolysosomal route. In line with this, the presentation of exogenous lipid antigens by CD1d molecules is reduced for mature DCs [145]. In further support, mice that lack the MHC class II-associated invariant chain exhibit defects in the localization of MHC class II molecules to the endolysosomal route [146]. In these same invariant chain-deficient mice, CD1d-mediated lipid presentation of endosome-derived model antigen α-GalGalCer is also much reduced [103].

CD1d mRNA levels were found to be increased upon infection of DCs with herpes simplex virus type 1 (HSV-1) or human cytomegalovirus (HCMV). Stimulation of TLR7 also elevated CD1d mRNA expression levels. The increased CD1d mRNA levels were accompanied by enrichment of CD1d proteins at the cell surface and enhanced activation and proliferation of iNKT cells [147]. On monocytes, CD1d was upregulated upon dengue virus infection, which might contribute to iNKT cell activation [116].

In conclusion, TLR-mediated recognition of viral infection leads to altered lipid presentation by CD1d molecules, thereby affecting the activation of iNKT cells. However, it has been suggested recently that robust iNKT cell activation in response to bacterial or viral pathogens might also be achieved by TLR stimulation in the absence of antigenic stimulation [148].

Viral evasion of CD1d-restricted antigen presentation

Considering the importance of iNKT cells in anti-viral defense, it may not come as a surprise that viruses have acquired strategies to modulate detection by iNKT cells. Viruses, and herpesviruses in particular, are well known to evade cytotoxic T lymphocyte (CTL) detection by reducing MHC class I surface display [149,150,151]. More recently, the first observations on virus-induced CD1d downregulation and iNKT cell evasion were reported.

Human immunodeficiency virus (HIV) has been shown to escape iNKT cell recognition (reviewed in [152]). A marked depletion of iNKT cells is observed after HIV infection, most likely resulting from cytolytic infection combined with activation-induced cell death. HIV further escapes iNKT cell recognition by downregulating CD1d surface display. The three viral proteins Vpu, Nef, and gp120 were shown to be involved in this process. Incubation of cells with recombinant HIV gp120 protein resulted in downregulation of CD1d surface levels [153], although the mechanism of action remains to be elucidated. HIV Nef accelerates the internalization of CD1d from the plasma membrane, retaining these lipid-presenting molecules in the *trans*-Golgi network [154]. Nef-induced downregulation acts via the tyrosine-based targeting motif located in the cytoplasmic tail of CD1d [154,155]. Finally, HIV Vpu retains CD1d molecules in early endosomes, thereby impairing recycling of CD1d from endocytic compartments to the cell surface [156].

Infection with either vaccinia virus (VV) or vesicular stomatitis virus (VSV) resulted in reduced activation of iNKT cells, although CD1d surface levels remained unchanged. The two viruses modulated MAPK signaling and subsequent intracellular CD1d trafficking, thereby presumably altering the lipid repertoire presented by CD1d for iNKT cell recognition [157].

The VV-encoded proteins B1R and H5R were found to be involved in evasion from CD1d-restricted iNKT cells [158]. Yet, B1R is a viral kinase that phosphorylates H5R, a transcription factor involved in late viral protein expression, and thus the effects of B1R and H5R on iNKT cell evasion may be indirect.

Human papillomavirus (HPV) type 16 inhibits MHC class I- and class II-restricted peptide presentation through expression of the small hydrophobic E5 protein [159,160,161]. In addition, HPV interferes with CD1d-resticted lipid presentation. Expression of HPV E5 protein caused a reduction in both cell surface and total CD1d protein levels. HPV E5 interacts with calnexin and prevents exit of CD1d molecules from the ER. Although mechanistic details are lacking, upon cellular expression of HPV E5, CD1d molecules end up in the cytosol, where they are degraded by the proteasome [162].

Products of herpesviruses HSV-1, KSHV, and EBV have been shown to interfere with trafficking of CD1d. In KSHV-mediated downregulation of surface CD1d, the viral proteins K3 and K5 have been implicated [163], which also are known to target HLA I for lysosomal degradation [164,165]. Although CD1d molecules enter the endocytic pathway as a consequence of K5-mediated ubiquitination of their cytoplasmic tails, the total cellular levels of CD1d remain virtually unchanged [163] suggesting that CD1d molecules appeared resistant to lysosomal degradation.

HSV-1 downregulates CD1d surface display by inhibiting recycling of CD1d molecules from endosomal compartments to the cell surface, leading to redistribution of CD1d to the limiting membranes of lysosomes. This HSV-1 induced downregulation is independent of the cytoplasmic tail of CD1d [166]. A subsequent study found that phosphorylation of the kinesin KIF3A by the serine-threonine kinase US3 of HSV-1 inhibited exocytosis from late endosomes/lysosomes to the cell surface of CD1d molecules [167].

In addition, the glycoprotein gB and US3 appear to cooperatively hamper CD1d-mediated antigen presentation to iNKT cells. gB was essential, but not sufficient, for CD1d downregulation during viral infection. Efficient CD1d downregulation required co-expression of an active US3 enzyme that modulates gB trafficking. CD1d trafficking is altered by the concerted action of gB and US3, redirecting CD1d to the *trans*-Golgi network [168].

Different B cell subsets like naïve, memory, and especially marginal zone-like B cells are CD1d-positive [169]. Ex vivo, cultured B cells show significantly decreased levels of CD1d molecules within several days. EBV infection of resting B cells even further decreased expression of CD1d in comparison to uninfected cells suggesting that EBV interferes with CD1d expression [104]. Transcription of CD1d molecules is regulated by retinoic acid receptor signalling in B cells [169]. Stimulation with retinoic acid rescued expression CD1d on cultured B cells as well as on EBV-infected B-LCLs. The observed increase of CD1d molecules resulted in enhanced iNKT cell stimulation in the presence of αGalCer. Interestingly, B-LCLs treated with retinoic acid, but not uninfected B cells, could stimulate iNKT cells even in the absence of αGalCer, pointing towards an endogenous ligand that is induced/upregulated by EBV infection of B cells [104]. The identity of this ligand remains elusive so far.

Immune evasion by Epstein-Barr virus

Many viral gene products of EBV are dedicated to functions that modulate anti-viral responses of the host. Investigation of immune evasion mechanisms acquired by EBV and other herpesviruses might provide useful insights into viral-specific immune defense and the immune system in general.

Evasion during the lytic cycle

About 80 proteins are expressed by EBV during its lytic cycle. Many of them interfere with molecules involved in immune responses of the innate and adaptive immune system. Some of them act highly specifically on certain host proteins, whereas others employ a mechanism of action with a broad target range. One of these broadly acting proteins is BGLF5, the EBV DNase (alkaline exonuclease) that is produced with early kinetics during the productive phase of infection [170] and is involved in a process termed shutoff.

Shutoff by BGLF5

Like other γ-herpesviruses, EBV inhibits cellular protein synthesis in productively infected cells through global mRNA destabilization. This is mediated by BGLF5, the protein first identified as a DNase [171], later its involvement in shutoff has been recognized [172]. BGLF5's additional RNase function utilizes the same catalytic site as its DNase activity, yet the substrate-binding site appears only partly shared by DNA and RNA substrates [173]. The promiscuous RNA degradation induced by EBV BGLF5 can affect immunologically relevant proteins. These include TLR2 and TLR9 that are capable of sensing EBV infection [46,52,174], but also the antigen-presenting molecules HLA I and HLA II [170,172] that present virus-derived peptides to $CD8^+$ and $CD4^+$ T cells, respectively. When *BGLF5* was expressed in isolation, $CD8^+$ T cell activation was decreased by 90%. During the lytic cycle, silencing of BGLF5 translation by 75% using shRNAs reduced, but not completely blocked, HLA I surface expression [174], indicating that other EBV gene products contribute to HLA I downregulation. It has been suggested that BGLF5 displays some selectivity, for example, TLR4 is not targeted by BGLF5 [46,174].

Evasion of antigen presentation

EBV compromises activation of both $CD8^+$ and $CD4^+$ T cells by interfering at various stages of the HLA class I and class II antigen presentation pathways, in particular during the productive phase of infection (**Figure 1**). During the early lytic cycle, BNLF2a and BILF1 inhibit HLA I-mediated antigen presentation on the cell surface, while gp42/gH/gL directly act during the late productive phase on HLA II.

BNLF2a is a gene product unique to lymphocryptoviruses of Old World primates. Expression of *BNLF2a* in isolation or in the context of EBV infection results in reduced $CD8^+$ T cell recognition. BNLF2a appears to deplete peptides from the ER through inhibition of

peptide import by the transporter associated with antigen presentation (TAP) [175,176]. *In vitro* infection with BNLF2a-deleted recombinant EBV restores T cell recognition of peptides expressed by these cells early after viral reactivation [177]. The mechanism of action of BNLF2a is exceptional among viral TAP inhibitors known to date. BNLF2a corrupts the binding of both peptides and ATP to the TAP complex, thereby blocking its transporter function and, ultimately, surface display of peptide/HLA complexes.

BILF1 encodes a constitutively active G-protein-coupled receptor (GPCR). The GPCR-signaling function is not required for downregulation of HLA I [178]. The underlying mechanism involves reduced transport of HLA I from the *trans*-Golgi network, as well as an increased turnover from the cell surface and, subsequently, enhanced degradation via lysosomal proteases [178]. This molecular mechanism is distinct from the ones identified for other viruses that induce degradation of HLA I. The cytoplasmic C-termini of both BILF1 and its targets are critical for HLA I downregulation. Most HLA I haplotypes are downregulated by BILF1, yet HLA-C alleles appear resistant [179]; the latter could deviate NK cells.

The relative contribution of BGLF5, BNLF2, and BILF1 to HLA I downregulation differs during the IE, E, and L phases of the EBV lytic cycle [180]. Knockdown of BNLF2a in donor LCLs primarily results in reduced activation of $CD8^+$ T cells specific for IE and E antigens, while BILF1 knockdown increases recognition of E and especially L antigens. Contrary to observations in overexpression studies, reducing BGLF5 expression displays limited effects on antigen recognition in any of the phases [180]. Timing of expression partially explains these differences, although some synergy between BNLF2a and BILF1 is also observed in reducing late antigen recognition.

Gp42 has initially been described as an entry receptor for EBV, binding to HLA class II molecules present on B cells. Additionally, gp42 acts as an immune evasion molecule. Its association with HLA class II / peptide complexes blocks T cell receptor (TCR) - class II interactions and precludes activation of $CD4^+$ T cells [181]. Whereas downregulation of HLA II occurs during productive EBV infection, this effect is not observed upon expression of gp42 in isolation [182], indicating that EBV employs additional HLA II evasion strategies. The viral interaction partners of gp42, gH and gL, cooperate to increase HLA II evasion (unpublished observation). In line with this, T cell activation was further diminished by additional inclusion of gH and gL in the gp42-HLA II complexes. Mechanistically, the major effect of gH/gL appeared to be stabilization and increased expression of gp42 (unpublished observation).

In addition to these ORFs that directly impair HLA II recognition, other EBV gene products indirectly interfere with $CD4^+$ T cell immunity. The immediate-early protein BZLF1 has been reported to impair IFNγ-signaling, thereby inhibiting CIITA promoter activity and, as a result, decreasing HLA II surface levels [183]. More recently, BZLF1 has been shown to impair HLA II presentation post-transcriptionally by interfering with the function of the invariant chain [184]. In this study, expression of BZLF1 in isolation resulted in approximately 50% reduction in $CD4^+$ T cell recognition. EBV also encodes a viral IL-10

homologue (BCRF1) that may act, similarly to host IL-10, as an anti-inflammatory cytokine able to inhibit and modulate CD4⁺ T cell priming and effector functions [185]. Moreover, BCRF1 has been shown to inhibit co-stimulatory molecules on human monocytes, which potentially results in inefficient priming and expansion of CD4⁺ T cells [186].

In conclusion, EBV has evolved multiple layers of immune evasion that interfere with the recognition of infected cells by CD8⁺ and CD4⁺ T cells. This wide range of evasion mechanisms explains how EBV can replicate and establish a life-long infection of its host, despite the existence of strong CD8⁺ and CD4⁺ T cell immunity against a broad repertoire

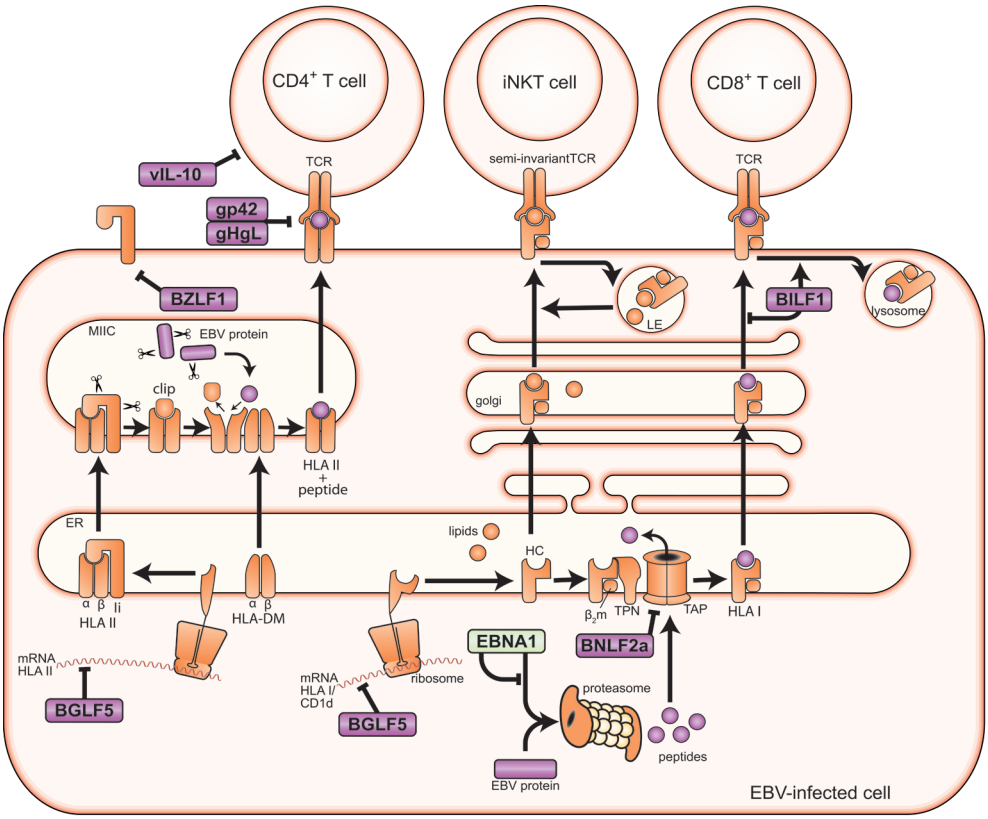

Figure 1 Antigen presentation pathways targeted by EBV. Multiple EBV-derived peptides are presented in the context of HLA I and II molecules to be scrutinized by specific T cells. Endogenous lipids are presented by CD1d to iNKT cells. Presentation to CD4⁺ T cells occurs by HLA II⁺ antigen-presenting cells, where EBV proteins are degraded in MHC class II-loading compartments (MIIC) and the resulting peptides are loaded onto HLA II molecules. For antigen presentation to CD8⁺ T cells, cytosolic EBV proteins, or fragments thereof, are degraded by the proteasome into peptides that are transported across the ER membrane by the transporter associated with antigen presentation (TAP) and are subsequently loaded onto newly synthesized HLA I molecules; mature HLA I/ peptide complexes travel through the Golgi compartments to the cell surface. Latent (green) and lytic (purple) EBV proteins interfere at various steps with activation of the cellular immune response.

of EBV antigens. Notably, the discovery of multiple EBV lytic cycle genes that co-operate to interfere with HLA class I and II antigen processing underscores the need for EBV to evade $CD8^+$ and $CD4^+$ T cell responses during replication, a time at which a large number of potential viral targets are expressed [187]. Together, these immune evasion strategies ensure a window for undetected replication of EBV. Additionally, these evasion mechanisms facilitate the establishment and maintenance of a life-long infection of the host.

Evasion of innate immunity

EBV interferes with innate immune responses at different levels. Modulation of the pro-inflammatory NF-κB and the IFN-inducing IRF pathways, which have a major role in induction of anti-viral innate immunity, has been reported as well as direct or indirect inhibition of cytokines and IFNs or modulation of their downstream effects [7]. One major modulator of innate immunity in lytic EBV infection is the immediate-early EBV transactivator BZLF1, which interferes with the above mentioned pathways.

BZLF1 interacts with the transcription factor IRF7 and inhibits its transcriptional activity on the IFNα4 and IFNβ promoters to prevent induction of an antiviral environment [188]. Productive EBV infection is associated with a reduction in NF-κB-dependent gene expression [189]. Viral BZLF1 and cellular NF-κB reciprocally inhibit each other's expression and, as a consequence, higher levels of NF-κB in the absence of BZLF1 favor EBV latency, whereas increased expression of BZLF1 upon lytic cycle induction overwhelms the limiting amount of NF-κB [190,191,192]. While NF-κB is still translocated to the nucleus, its transcriptional activity is suppressed by BZLF1, preventing induction of anti-viral immune effector mechanisms [190]. Moreover, EBV BZLF1 counteracts innate effector molecules in several ways. It downregulates the receptors for TNFα and IFNγ to reduce cellular responsiveness to these cytokines [183,193,194]. In addition, BZLF1 induces the suppressor of cytokine signaling SOCS3, which inhibits JAK/STAT signaling and thereby favors a state of type I IFN-irresponsiveness [195]. Additionally, SOCS3 reduces IFNα production by monocytes. Moreover, BZLF1 causes expression of the immunosuppressive cytokine TGFβ [196] and disrupts the formation of PML-bodies [197], which can have antiviral activity [198].

Besides BZLF1, the EBV proteins BRLF1, LF2, and BGLF4 prevent transcription of IRF proteins or interfere with their activity, thereby resulting in reduced production of type I IFNs [199]. Recently, Dunmire *et al.* reported that a clear systemic IFN response is observed during acute EBV infection, but this response lacks some key components compared to observations for other viruses [200]. This may illustrate the successful actions of the immune evasion mechanisms employed by EBV to repress secretion of interferon responsive genes.

TLR signaling pathways leading to NF-κB activation are tightly controlled by post-translational modifications, such as phosphorylation and ubiquitination [201,202]. EBV encodes the lytic proteins BGLF4 and BPLF1 that interfere with these modifications [203]. Being a component of the EBV tegument, BPLF1 could act both in productively as well as in newly infected cells [204,205].

EBV counteracts the pleiotropic host cytokine colony-stimulating factor 1 (CSF-1), which stimulates macrophage differentiation and IFNα secretion. To this end, EBV encodes a soluble form of the CSF-1 receptor, BARF1, that neutralizes the effects of host CSF-1 *in vitro*, leading to reduced IFNα secretion by EBV-infected mononuclear cells [206,207]. Mutating the BARF1 homologue in a related rhesus macaque lymphocryptovirus decreases viral load during primary infection and leads to a lower persistence setpoint *in vivo* [208].

The early-expressed EBV-encoded dUTPase (encoded by the BLLF3 gene) also modulates cytokine-induced responses. In a mouse model, dUTPase compromises lymphocyte responses, e.g. secretion of IFNγ [209]. In human cells, EBV dUTPase has seemingly opposing effects: it induces NF-κB activation in a TLR2/MyD88-dependent way [47,48,210], it inhibits lymphocyte proliferation, and it induces production of both proinflammatory cytokines as well as IL-10 [185,211]. Following this strategy, EBV appears to exploit the advantageous effects of NF-κB activation, while limiting ensuing anti-viral T cell responses.

Evasion during latent infection

Latent infection is characterized by limited EBV protein expression. In latency III cells, EBNA1-6 and LMP1 and 2 are expressed. During latency II, expression is restricted to EBNA1 and LMP1 and 2. Latency I involves expression of EBNA1 only, and latency 0 exists without any EBV protein generation. Some latency-associated proteins have immune modulatory functions, thereby facilitating immune evasion.

EBNA1

EBNA1, present during all latency stages, contains a long glycine-alanine repeat that inhibits translation as well as proteasomal degradation of EBNA1 through interference with processing by the 19S proteasomal subunit [212,213,214,215,216]. This strategy ensures sufficient EBNA1 levels to maintain the viral genome [217], while decreasing protein turnover to minimize viral antigen presentation to $CD8^+$ T cells. Initially, EBNA1-specific $CD8^+$ T cell responses were indeed not observed *in vitro* [213]. However, later studies did report EBNA1-specific T cell responses initiated by endogenously presented EBNA1-derived antigens [218,219,220]. Potential sources of these antigens include defective ribosomal products that lack the glycine-alanine repeat, or cross-presented exogenous antigens released by EBV-infected cells.

Other immune evasive actions of EBNA1 include inhibition of the canonical NF-κB pathway by interfering with phosphorylation of the IKK complex signaling intermediate [221] and modulation of the STAT1 and TGFβ signaling pathways [222].

EBNA2

EBNA2 applies a double-edged strategy by inducing low-level IFNβ production that leads to interferon-stimulated gene (ISG) production in BL cell lines [223], whereas anti-proliferative effects are neutralized by EBNA2-mediated inhibition of selected ISGs [224,225] and enhanced transcriptional activity of STAT3 [226] following IFNα production. STAT3 modulates IFN-induced immune responses through STAT1 and suppresses production of inflammatory mediators [227]. In addition, EBNA2 upregulates the IL-18 receptor on BL cells [228]. IL-18 plays a role in regulating innate and adaptive immune responses and is elevated in certain EBV-associated malignancies [229].

LMP1

LMP1 promotes B cell growth and survival by mimicking constitutive CD40 signaling to activate NF-κB, JNK, MAPK, JAK/STAT and PI3K signaling pathways [230]. These pathways affect many immunological processes and allow LMP1 to steer the host immune response (reviewed in [231]). LMP1-mediated NF-κB activation in EBV-immortalized B cells results in type I IFN production that stimulates STAT1 expression in autocrine and paracrine fashion [232,233,234]. STAT2 activity is inhibited by LMP1 [235]. LMP1-mediated upregulation of IRF7 benefits EBV by promoting cell growth, while at the same time an inhibitory IRF7 splice variant is induced to repress the adverse effects of type I IFN-production [236,237,238,239,240]. Furthermore, LMP1-mediated induction of JAK/STAT signaling pathways may be advantageous to EBV as the antiviral activities of ISGs prevent superinfection and facilitate establishment of latency [241,242]. Finally, LMP-1 mediated NF-κB activation reduces TLR9 surface expression [243] and supplies growth benefits to infected cells [244,245].

LMP2a and LMP2b

LMP2a inhibits NF-κB activity, IL-6 production, and subsequent JAK/STAT signaling pathways in carcinoma cell lines [246]. In contrast LMP2a induces NF-κB activation in B cells and uses the subsequently increased levels of anti-apoptotic Bcl-2 to protect infected cells from apoptosis in a transgenic mouse model [247]. Furthermore, LMP2a and LMP2b accelerate turnover of IFN receptors, resulting in decreased responsiveness of epithelial cells to IFNα and IFNγ [248].

EBV miRNAs

Apart from growth-transforming and anti-apoptotic functions, EBV miRNAs target several host genes involved in anti-viral immunity.

Among the first EBV miRNA targets identified was CXCL-11, a T cell attracting chemokine downregulated by EBV miRNAs BHRF1-3 [249]. Stress-induced NK cell ligands have been specifically investigated as potential viral miRNA targets. Initially, MICB was identified as a target of the HCMV encoded miR-UL112, and later it became apparent that also miRNAs

encoded by KSHV and EBV downregulate MICB expression. Inhibiting EBV miRNA BART2-5p results in increased NK cell killing *in vitro* [250]. Inflammasomes are induced by various cytoplasmic and nuclear sensors (e.g. NLRP3 and IFI16) and lead to production of the inflammatory cytokines IL-1β and IL-18 [251]. Although EBV has so far only been observed to activate inflammasomes through IFI16 [75], EBV miRNA BART15 downregulates the alternative inflammasome-activating sensor NLRP3 [252]. Co-culture of monocytic recipient cells with EBV$^+$ B cells secreting BART15-containing exosomes results in decreased IL-1β production. Additionally, EBV miRNAs regulate the IFNγ-STAT1 pathway in EBV$^+$ NK cells by downregulating IFNγ transcriptional regulator T-bet (BART20-5p), IFNγ (BART20-5p), and STAT1 (BART8) [253,254]. Inhibition of BART6-3p in a BL cell line caused upregulation of the IL-6 receptor chains (p80 and gp130) at the mRNA and protein level, indicating that BART6-3p may affect IL-6 signaling [255].

Outline of this thesis

Viruses, especially those causing persistent infections such as EBV, have acquired strategies to counteract the host's immune defense. During latency, viral protein production is limited or absent, thereby being virtually invisible to the immune system. Evasion strategies of EBV active during primary infection or upon reactivation are considered beneficial for establishment of latent infection or prolonged replication, respectively. Viral molecules interfering with antigen presentation by HLA I and HLA II have been identified previously. The aim of this thesis was to identify novel molecules directing immune evasion and investigate the respective underlying molecular mechanisms, thereby contributing to our understanding of EBV and the human immune system.

Chapter 2 describes an shRNA-based approach to silence translation of the shutoff protein BGLF5 in lytically EBV-infected B cells in order to unmask novel evasion targets of EBV. The non-classical lipid-presenting molecule CD1d was identified as a new target of BGLF5. Furthermore, the data suggested that likely additional mechanisms exist in EBV targeting CD1d. Identification of one of these molecules, EBV gp150, is described in **Chapter 3**. EBV gp150 appears to shield antigen-presenting molecules and other surface molecules on B cells by means of its abundant glycans. Thereby, EBV gp150 impairs T cell recognition of infected cells. In **Chapter 4**, allele specificity of the previously identified HLA I evasion molecule EBV BILF1 was investigated. Thereby, the molecular requirements in HLA I as well as in BILF1 that are needed for immune evasion were identified. Recent advances in the field of DNA sensing led to the question whether B cells might sense foreign DNA by the cGAS-STING pathway. **Chapter 5** describes that human B cells, the main target cells of EBV persistence, have the DNA sensors IFI16 and cGAS, but the majority of B cells tested lack STING protein. Irrespective of the presence of STING, human B cells were unresponsive to cytoplasmic DNA exposure. In **Chapter 6** the results of this thesis are summarized, and implications and future directions of this research are discussed.

1. Pellett PE, Roizman B, editors (2007) The Family Herpesviridae: A Brief Introduction.
2. Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, *et al.* (2009) The order Herpesvirales. Arch Virol 154: 171-177.
3. http://www.ictvonline.org/virusTaxonomy.asp.
4. Rickinson AB, Kieff E, editors (2007) Epstein-Barr virus.
5. Ganem D, editor (2007) Kaposi's sarcoma-associated Herpesvirus.
6. Amsler L, Verweij MC, DeFilippis VR (2013) The tiers and dimensions of evasion of the type I interferon response by human cytomegalovirus. J Mol Biol 425: 4857-4871.
7. Ning S (2011) Innate immune modulation in EBV infection. Herpesviridae 2: 1.
8. Paludan SR, Bowie AG, Horan KA, Fitzgerald KA (2011) Recognition of herpesviruses by the innate immune system. Nat Rev Immunol 11: 143-154.
9. Ojha H, Panwar HS, Gorham RD, Jr., Morikis D, Sahu A (2014) Viral regulators of complement activation: structure, function and evolution. Mol Immunol 61: 89-99.
10. van de Weijer ML, Luteijn RD, Wiertz EJ (2015) Viral immune evasion: Lessons in MHC class I antigen presentation. Semin Immunol 27: 125-137.
11. Griffin BD, Verweij MC, Wiertz EJ (2010) Herpesviruses and immunity: the art of evasion. Vet Microbiol 143: 89-100.
12. Zuo J, Rowe M (2012) Herpesviruses placating the unwilling host: manipulation of the MHC class II antigen presentation pathway. Viruses 4: 1335-1353.
13. Connolly SA, Jackson JO, Jardetzky TS, Longnecker R (2011) Fusing structure and function: a structural view of the herpesvirus entry machinery. Nat Rev Microbiol 9: 369-381.
14. Buisson M, Geoui T, Flot D, Tarbouriech N, Ressing ME, *et al.* (2009) A bridge crosses the active-site canyon of the Epstein-Barr virus nuclease with DNase and RNase activities. J Mol Biol 391: 717-728.
15. Thorley-Lawson DA, Hawkins JB, Tracy SI, Shapiro M (2013) The pathogenesis of Epstein-Barr virus persistent infection. Curr Opin Virol 3: 227-232.
16. Kempkes B, Robertson ES (2015) Epstein-Barr virus latency: current and future perspectives. Curr Opin Virol 14: 138-144.
17. Kang MS, Kieff E (2015) Epstein-Barr virus latent genes. Exp Mol Med 47: e131.
18. Skalsky RL, Cullen BR (2015) EBV Noncoding RNAs. Curr Top Microbiol Immunol 391: 181-217.
19. Jochum S, Ruiss R, Moosmann A, Hammerschmidt W, Zeidler R (2012) RNAs in Epstein-Barr virions control early steps of infection. Proc Natl Acad Sci U S A 109: E1396-1404.
20. Meckes DG, Jr., Shair KH, Marquitz AR, Kung CP, Edwards RH, *et al.* (2010) Human tumor virus utilizes exosomes for intercellular communication. Proc Natl Acad Sci U S A 107: 20370-20375.
21. Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, *et al.* (2010) Functional delivery of viral miRNAs via exosomes. Proc Natl Acad Sci U S A 107: 6328-6333.
22. Dunmire SK, Grimm JM, Schmeling DO, Balfour HH, Jr., Hogquist KA (2015) The Incubation Period of Primary Epstein-Barr Virus Infection: Viral Dynamics and Immunologic Events. PLoS Pathog 11: e1005286.
23. Balfour HH, Jr., Odumade OA, Schmeling DO, Mullan BD, Ed JA, *et al.* (2013) Behavioral, virologic, and immunologic factors associated with acquisition and severity of primary Epstein-Barr virus infection in university students. J Infect Dis 207: 80-88.
24. Balfour HH, Jr., Dunmire SK, Hogquist KA (2015) Infectious mononucleosis. Clin Transl Immunology 4: e33.
25. Clute SC, Watkin LB, Cornberg M, Naumov YN, Sullivan JL, *et al.* (2005) Cross-reactive influenza virus-specific CD8+ T cells contribute to lymphoproliferation in Epstein-Barr virus-associated infectious mononucleosis. J Clin Invest 115: 3602-3612.
26. Azzi T, Lunemann A, Murer A, Ueda S, Beziat V, *et al.* (2014) Role for early-differentiated natural killer cells in infectious mononucleosis. Blood 124: 2533-2543.

27. Epstein MA, Achong BG, Barr YM (1964) Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma. Lancet 1: 702-703.
28. Rickinson AB (2014) Co-infections, inflammation and oncogenesis: future directions for EBV research. Semin Cancer Biol 26: 99-115.
29. Parvaneh N, Filipovich AH, Borkhardt A (2013) Primary immunodeficiencies predisposed to Epstein-Barr virus-driven haematological diseases. Br J Haematol 162: 573-586.
30. Taylor GS, Long HM, Brooks JM, Rickinson AB, Hislop AD (2015) The immunology of Epstein-Barr virus-induced disease. Annu Rev Immunol 33: 787-821.
31. Purtilo DT, Cassel CK, Yang JP, Harper R (1975) X-linked recessive progressive combined variable immunodeficiency (Duncan's disease). Lancet 1: 935-940.
32. Rigaud S, Fondaneche MC, Lambert N, Pasquier B, Mateo V, *et al.* (2006) XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. Nature 444: 110-114.
33. Coffey AJ, Brooksbank RA, Brandau O, Oohashi T, Howell GR, *et al.* (1998) Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. Nat Genet 20: 129-135.
34. Sayos J, Wu C, Morra M, Wang N, Zhang X, *et al.* (1998) The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. Nature 395: 462-469.
35. Dong Z, Cruz-Munoz ME, Zhong MC, Chen R, Latour S, *et al.* (2009) Essential function for SAP family adaptors in the surveillance of hematopoietic cells by natural killer cells. Nat Immunol 10: 973-980.
36. Dupre L, Andolfi G, Tangye SG, Clementi R, Locatelli F, *et al.* (2005) SAP controls the cytolytic activity of CD8+ T cells against EBV-infected cells. Blood 105: 4383-4389.
37. Pasquier B, Yin L, Fondaneche MC, Relouzat F, Bloch-Queyrat C, *et al.* (2005) Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. J Exp Med 201: 695-701.
38. Palendira U, Low C, Chan A, Hislop AD, Ho E, *et al.* (2011) Molecular pathogenesis of EBV susceptibility in XLP as revealed by analysis of female carriers with heterozygous expression of SAP. PLoS Biol 9: e1001187.
39. Rooney CM, Smith CA, Ng CY, Loftin S, Li C, *et al.* (1995) Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. Lancet 345: 9-13.
40. Iwasaki A, Medzhitov R (2015) Control of adaptive immunity by the innate immune system. Nat Immunol 16: 343-353.
41. Wu J, Chen ZJ (2014) Innate immune sensing and signaling of cytosolic nucleic acids. Annu Rev Immunol 32: 461-488.
42. Takeuchi O, Akira S (2009) Innate immunity to virus infection. Immunol Rev 227: 75-86.
43. Thompson MR, Kaminski JJ, Kurt-Jones EA, Fitzgerald KA (2011) Pattern recognition receptors and the innate immune response to viral infection. Viruses 3: 920-940.
44. Kawai T, Akira S (2011) Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 34: 637-650.
45. Chijioke O, Azzi T, Nadal D, Munz C (2013) Innate immune responses against Epstein Barr virus infection. J Leukoc Biol 94: 1185-1190.
46. Gaudreault E, Fiola Sp, Olivier M, Gosselin J (2007) Epstein-Barr Virus Induces MCP-1 Secretion by Human Monocytes via TLR2. Journal of Virology 81: 8016-8024.
47. Ariza ME, Glaser R, Kaumaya PTP, Jones C, Williams MV (2009) The EBV-Encoded dUTPase Activates NF-kB through the TLR2 and MyD88-Dependent Signaling Pathway. The Journal of Immunology 182: 851-859.
48. Ariza ME, Rivailler P, Glaser R, Chen M, Williams MV (2013) Epstein-Barr Virus Encoded dUTPase Containing Exosomes Modulate Innate and Adaptive Immune Responses in Human Dendritic Cells and Peripheral Blood Mononuclear Cells. PLoS ONE 8: e69827.

49. Iwakiri D, Zhou L, Samanta M, Matsumoto M, Ebihara T, *et al.* (2009) Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from Toll-like receptor 3. J Exp Med 206: 2091-2099.
50. Samanta M, Iwakiri D, Kanda T, Imaizumi T, Takada K (2006) EB virus-encoded RNAs are recognized by RIG-I and activate signaling to induce type I IFN. EMBO J 25: 4207-4214.
51. Ablasser A, Bauernfeind F, Hartmann G, Latz E, Fitzgerald KA, *et al.* (2009) RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. Nat Immunol 10: 1065-1072.
52. van Gent M, Griffin BD, Berkhoff EG, van Leeuwen D, Boer IGJ, *et al.* (2011) EBV Lytic-Phase Protein BGLF5 Contributes to TLR9 Downregulation during Productive Infection. The Journal of Immunology 186: 1694-1702.
53. Paludan SR (2015) Activation and regulation of DNA-driven immune responses. Microbiol Mol Biol Rev 79: 225-241.
54. Orzalli MH, Knipe DM (2014) Cellular sensing of viral DNA and viral evasion mechanisms. Annu Rev Microbiol 68: 477-492.
55. Gram AM, Frenkel J, Ressing ME (2012) Inflammasomes and viruses: cellular defence versus viral offence. J Gen Virol 93: 2063-2075.
56. Lamkanfi M (2011) Emerging inflammasome effector mechanisms. Nat Rev Immunol 11: 213-220.
57. Kayagaki N, Stowe IB, Lee BL, O'Rourke K, Anderson K, *et al.* (2015) Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. Nature 526: 666-671.
58. Shi J, Zhao Y, Wang K, Shi X, Wang Y, *et al.* (2015) Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. Nature 526: 660-665.
59. Dinarello CA (2009) Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol 27: 519-550.
60. Sims JE, Smith DE (2010) The IL-1 family: regulators of immunity. Nature Reviews Immunology 10: 89-102.
61. Dinarello CA (2011) A clinical perspective of IL-1beta as the gatekeeper of inflammation. Eur J Immunol 41: 1203-1217.
62. Goldbach-Mansky R, Dailey NJ, Canna SW, Gelabert A, Jones J, *et al.* (2006) Neonatal-onset multisystem inflammatory disease responsive to interleukin-1beta inhibition. N Engl J Med 355: 581-592.
63. Leslie KS, Lachmann HJ, Bruning E, McGrath JA, Bybee A, *et al.* (2006) Phenotype, genotype, and sustained response to anakinra in 22 patients with autoinflammatory disease associated with CIAS-1/NALP3 mutations. Arch Dermatol 142: 1591-1597.
64. Ross JB, Finlayson LA, Klotz PJ, Langley RG, Gaudet R, *et al.* (2008) Use of anakinra (Kineret) in the treatment of familial cold autoinflammatory syndrome with a 16-month follow-up. J Cutan Med Surg 12: 8-16.
65. Puren AJ, Fantuzzi G, Dinarello CA (1999) Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1beta are differentially regulated in human blood mononuclear cells and mouse spleen cells. Proc Natl Acad Sci U S A 96: 2256-2261.
66. Chaix J, Tessmer MS, Hoebe K, Fuseri N, Ryffel B, *et al.* (2008) Cutting edge: Priming of NK cells by IL-18. J Immunol 181: 1627-1631.
67. Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES (2009) AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature 458: 509-513.
68. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, *et al.* (2009) AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 458: 514-518.
69. Roberts TL, Idris A, Dunn JA, Kelly GM, Burnton CM, *et al.* (2009) HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. Science 323: 1057-1060.

70. Burckstummer T, Baumann C, Bluml S, Dixit E, Durnberger G, *et al.* (2009) An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. Nat Immunol 10: 266-272.
71. Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, *et al.* (2010) The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nat Immunol 11: 395-402.
72. Muruve DA, Petrilli V, Zaiss AK, White LR, Clark SA, *et al.* (2008) The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. Nature 452: 103-107.
73. Li T, Diner BA, Chen J, Cristea IM (2012) Acetylation modulates cellular distribution and DNA sensing ability of interferon-inducible protein IFI16. Proc Natl Acad Sci U S A 109: 10558-10563.
74. Kerur N, Veettil MV, Sharma-Walia N, Bottero V, Sadagopan S, *et al.* (2011) IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi Sarcoma-associated herpesvirus infection. Cell Host Microbe 9: 363-375.
75. Ansari MA, Singh VV, Dutta S, Veettil MV, Dutta D, *et al.* (2013) Constitutive Interferon-Inducible Protein 16-Inflammasome Activation during Epstein-Barr Virus Latency I, II, and III in B and Epithelial Cells. Journal of Virology 87: 8606-8623.
76. Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, *et al.* (2010) IFI16 is an innate immune sensor for intracellular DNA. Nat Immunol 11: 997-1004.
77. Hansen K, Prabakaran T, Laustsen A, Jorgensen SE, Rahbaek SH, *et al.* (2014) Listeria monocytogenes induces IFNbeta expression through an IFI16-, cGAS- and STING-dependent pathway. EMBO J 33: 1654-1666.
78. Sun L, Wu J, Du F, Chen X, Chen ZJ (2013) Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science 339: 786-791.
79. Unterholzner L (2013) The interferon response to intracellular DNA: why so many receptors? Immunobiology 218: 1312-1321.
80. Wu J, Sun L, Chen X, Du F, Shi H, *et al.* (2013) Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. Science 339: 826-830.
81. Ablasser A, Schmid-Burgk JL, Hemmerling I, Horvath GL, Schmidt T, *et al.* (2013) Cell intrinsic immunity spreads to bystander cells via the intercellular transfer of cGAMP. Nature 503: 530-534.
82. Gentili M, Kowal J, Tkach M, Satoh T, Lahaye X, *et al.* (2015) Transmission of innate immune signaling by packaging of cGAMP in viral particles. Science 349: 1232-1236.
83. Bridgeman A, Maelfait J, Davenne T, Partridge T, Peng Y, *et al.* (2015) Viruses transfer the antiviral second messenger cGAMP between cells. Science 349: 1228-1232.
84. Ma Z, Jacobs SR, West JA, Stopford C, Zhang Z, *et al.* (2015) Modulation of the cGAS-STING DNA sensing pathway by gammaherpesviruses. Proc Natl Acad Sci U S A 112: E4306-4315.
85. Wu JJ, Li W, Shao Y, Avey D, Fu B, *et al.* (2015) Inhibition of cGAS DNA Sensing by a Herpesvirus Virion Protein. Cell Host Microbe 18: 333-344.
86. Lau L, Gray EE, Brunette RL, Stetson DB (2015) DNA tumor virus oncogenes antagonize the cGAS-STING DNA-sensing pathway. Science 350: 568-571.
87. Abbott RJ, Quinn LL, Leese AM, Scholes HM, Pachnio A, *et al.* (2013) CD8+ T cell responses to lytic EBV infection: late antigen specificities as subdominant components of the total response. J Immunol 191: 5398-5409.
88. Blum JS, Wearsch PA, Cresswell P (2013) Pathways of antigen processing. Annu Rev Immunol 31: 443-473.
89. Adams EJ, Luoma AM (2013) The adaptable major histocompatibility complex (MHC) fold: structure and function of nonclassical and MHC class I-like molecules. Annu Rev Immunol 31: 529-561.
90. Leslie DS, Vincent MS, Spada FM, Das H, Sugita M, *et al.* (2002) CD1-mediated gamma/delta T cell maturation of dendritic cells. J Exp Med 196: 1575-1584.
91. Spada FM, Grant EP, Peters PJ, Sugita M, Melian A, *et al.* (2000) Self-recognition of CD1 by gamma/

delta T cells: implications for innate immunity. J Exp Med 191: 937-948.
92. Godfrey DI, Stankovic S, Baxter AG (2010) Raising the NKT cell family. Nat Immunol 11: 197-206.
93. De Libero G, Mori L (2006) Mechanisms of lipid-antigen generation and presentation to T cells. Trends Immunol 27: 485-492.
94. Chen YH, Wang B, Chun T, Zhao L, Cardell S, *et al.* (1999) Expression of CD1d2 on thymocytes is not sufficient for the development of NK T cells in CD1d1-deficient mice. J Immunol 162: 4560-4566.
95. Exley M, Garcia J, Wilson SB, Spada F, Gerdes D, *et al.* (2000) CD1d structure and regulation on human thymocytes, peripheral blood T cells, B cells and monocytes. Immunology 100: 37-47.
96. Opasawatchai A, Matangkasombut P (2015) iNKT Cells and Their Potential Lipid Ligands during Viral Infection. Front Immunol 6: 378.
97. Rhost S, Sedimbi S, Kadri N, Cardell SL (2012) Immunomodulatory type II natural killer T (NKT) lymphocytes in health and disease. ScandJImmunol.
98. Godfrey DI, Stankovic S, Baxter AG (2010) Raising the NKT cell family. NatImmunol 11: 197-206.
99. Kuylenstierna C, Bjorkstrom NK, Andersson SK, Sahlstrom P, Bosnjak L, *et al.* (2011) NKG2D performs two functions in invariant NKT cells: direct TCR-independent activation of NK-like cytolysis and co-stimulation of activation by CD1d. EurJImmunol 41: 1913-1923.
100. Lee HH, Meyer EH, Goya S, Pichavant M, Kim HY, *et al.* (2010) Apoptotic cells activate NKT cells through T cell Ig-like mucin-like-1 resulting in airway hyperreactivity. JImmunol 185: 5225-5235.
101. Brigl M, Bry L, Kent SC, Gumperz JE, Brenner MB (2003) Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. NatImmunol 4: 1230-1237.
102. Behar SM, Dascher CC, Grusby MJ, Wang CR, Brenner MB (1999) Susceptibility of mice deficient in CD1D or TAP1 to infection with Mycobacterium tuberculosis. JExpMed 189: 1973-1980.
103. Sille FC, Martin C, Jayaraman P, Rothchild A, Fortune S, *et al.* (2011) Requirement for invariant chain in macrophages for Mycobacterium tuberculosis replication and CD1d antigen presentation. InfectImmun 79: 3053-3063.
104. Chung BK, Tsai K, Allan LL, Zheng DJ, Nie JC, *et al.* (2013) Innate immune control of EBV-infected B cells by invariant natural killer T cells. Blood 122: 2600-2608.
105. Grubor-Bauk B, Arthur JL, Mayrhofer G (2008) Importance of NKT cells in resistance to herpes simplex virus, fate of virus-infected neurons, and level of latency in mice. JVirol 82: 11073-11083.
106. Grubor-Bauk B, Simmons A, Mayrhofer G, Speck PG (2003) Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR. JImmunol 170: 1430-1434.
107. Ashkar AA, Rosenthal KL (2003) Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection. JVirol 77: 10168-10171.
108. Johnson TR, Hong S, Van KL, Koezuka Y, Graham BS (2002) NK T cells contribute to expansion of CD8(+) T cells and amplification of antiviral immune responses to respiratory syncytial virus. JVirol 76: 4294-4303.
109. Ishikawa H, Tanaka K, Kutsukake E, Fukui T, Sasaki H, *et al.* (2010) IFN-gamma production downstream of NKT cell activation in mice infected with influenza virus enhances the cytolytic activities of both NK cells and viral antigen-specific CD8+ T cells. Virology 407: 325-332.
110. van Dommelen SL, Tabarias HA, Smyth MJ, gli-Esposti MA (2003) Activation of natural killer (NK) T cells during murine cytomegalovirus infection enhances the antiviral response mediated by NK cells. JVirol 77: 1877-1884.
111. Iversen MB, Jensen SK, Hansen AL, Winther H, Issazadeh-Navikas S, *et al.* (2015) NKT cell activation by local alpha-galactosylceramide administration decreases susceptibility to HSV-2 infection. Immunobiology 220: 762-768.
112. Ho LP, Denney L, Luhn K, Teoh D, Clelland C, *et al.* (2008) Activation of invariant NKT cells enhances the innate immune response and improves the disease course in influenza A virus

infection. EurJImmunol 38: 1913-1922.
113. Kakimi K, Guidotti LG, Koezuka Y, Chisari FV (2000) Natural killer T cell activation inhibits hepatitis B virus replication in vivo. JExpMed 192: 921-930.
114. Exley MA, Bigley NJ, Cheng O, Tahir SM, Smiley ST, *et al.* (2001) CD1d-reactive T-cell activation leads to amelioration of disease caused by diabetogenic encephalomyocarditis virus. JLeukocBiol 69: 713-718.
115. Renneson J, Guabiraba R, Maillet I, Marques RE, Ivanov S, *et al.* (2011) A detrimental role for invariant natural killer T cells in the pathogenesis of experimental dengue virus infection. Am J Pathol 179: 1872-1883.
116. Matangkasombut P, Chan-In W, Opasawaschai A, Pongchaikul P, Tangthawornchaikul N, *et al.* (2014) Invariant NKT cell response to dengue virus infection in human. PLoS Negl Trop Dis 8: e2955.
117. Boes M, Stoppelenburg AJ, Sille FC (2009) Endosomal processing for antigen presentation mediated by CD1 and Class I major histocompatibility complex: roads to display or destruction. Immunology 127: 163-170.
118. Yuan W, Qi X, Tsang P, Kang SJ, Illarionov PA, *et al.* (2007) Saposin B is the dominant saposin that facilitates lipid binding to human CD1d molecules. ProcNatlAcadSciUSA 104: 5551-5556.
119. Brossay L, Jullien D, Cardell S, Sydora BC, Burdin N, *et al.* (1997) Mouse CD1 is mainly expressed on hemopoietic-derived cells. JImmunol 159: 1216-1224.
120. Amano M, Baumgarth N, Dick MD, Brossay L, Kronenberg M, *et al.* (1998) CD1 expression defines subsets of follicular and marginal zone B cells in the spleen: beta 2-microglobulin-dependent and independent forms. JImmunol 161: 1710-1717.
121. Liu J, Shaji D, Cho S, Du W, Gervay-Hague J, *et al.* (2010) A threonine-based targeting signal in the human CD1d cytoplasmic tail controls its functional expression. JImmunol 184: 4973-4981.
122. Zhu Y, Zhang W, Veerapen N, Besra G, Cresswell P (2010) Calreticulin controls the rate of assembly of CD1d molecules in the endoplasmic reticulum. JBiolChem 285: 38283-38292.
123. Sille FC, Boxem M, Sprengers D, Veerapen N, Besra G, *et al.* (2009) Distinct requirements for CD1d intracellular transport for development of V(alpha)14 iNKT cells. JImmunol 183: 1780-1788.
124. Lawton AP, Prigozy TI, Brossay L, Pei B, Khurana A, *et al.* (2005) The mouse CD1d cytoplasmic tail mediates CD1d trafficking and antigen presentation by adaptor protein 3-dependent and -independent mechanisms. JImmunol 174: 3179-3186.
125. Cernadas M, Sugita M, van der WN, Cao X, Gumperz JE, *et al.* (2003) Lysosomal localization of murine CD1d mediated by AP-3 is necessary for NK T cell development. JImmunol 171: 4149-4155.
126. Sugita M, Cernadas M, Brenner MB (2004) New insights into pathways for CD1-mediated antigen presentation. CurrOpinImmunol 16: 90-95.
127. Sille FC, Martin C, Jayaraman P, Rothchild A, Besra GS, *et al.* (2011) Critical role for invariant chain in CD1d-mediated selection and maturation of Valpha14-invariant NKT cells. ImmunolLett 139: 33-41.
128. Jayawardena-Wolf J, Benlagha K, Chiu YH, Mehr R, Bendelac A (2001) CD1d endosomal trafficking is independently regulated by an intrinsic CD1d-encoded tyrosine motif and by the invariant chain. Immunity 15: 897-908.
129. Kang SJ, Cresswell P (2002) Regulation of intracellular trafficking of human CD1d by association with MHC class II molecules. EMBO J 21: 1650-1660.
130. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, *et al.* (1997) CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science 278: 1626-1629.
131. Kobayashi E, Motoki K, Uchida T, Fukushima H, Koezuka Y (1995) KRN7000, a novel immunomodulator, and its antitumor activities. OncolRes 7: 529-534.
132. Cox D, Fox L, Tian R, Bardet W, Skaley M, *et al.* (2009) Determination of cellular lipids bound to

human CD1d molecules. PLoSOne 4: e5325.
133. Kinjo Y, Wu D, Kim G, Xing GW, Poles MA, *et al.* (2005) Recognition of bacterial glycosphingolipids by natural killer T cells. Nature 434: 520-525.
134. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C, III, *et al.* (2005) Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature 434: 525-529.
135. Sriram V, Du W, Gervay-Hague J, Brutkiewicz RR (2005) Cell wall glycosphingolipids of Sphingomonas paucimobilis are CD1d-specific ligands for NKT cells. EurJImmunol 35: 1692-1701.
136. Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, *et al.* (2006) Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. NatImmunol 7: 978-986.
137. Kinjo Y, Illarionov P, Vela JL, Pei B, Girardi E, *et al.* (2011) Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. NatImmunol 12: 966-974.
138. Kain L, Webb B, Anderson BL, Deng S, Holt M, *et al.* (2014) The identification of the endogenous ligands of natural killer T cells reveals the presence of mammalian alpha-linked glycosylceramides. Immunity 41: 543-554.
139. Brennan PJ, Tatituri RV, Heiss C, Watts GF, Hsu FF, *et al.* (2014) Activation of iNKT cells by a distinct constituent of the endogenous glucosylceramide fraction. Proc Natl Acad Sci U S A 111: 13433-13438.
140. Zeissig S, Murata K, Sweet L, Publicover J, Hu Z, *et al.* (2012) Hepatitis B virus-induced lipid alterations contribute to natural killer T cell-dependent protective immunity. NatMed.
141. Paget C, Mallevaey T, Speak AO, Torres D, Fontaine J, *et al.* (2007) Activation of invariant NKT cells by toll-like receptor 9-stimulated dendritic cells requires type I interferon and charged glycosphingolipids. Immunity 27: 597-609.
142. Salio M, Speak AO, Shepherd D, Polzella P, Illarionov PA, *et al.* (2007) Modulation of human natural killer T cell ligands on TLR-mediated antigen-presenting cell activation. ProcNatlAcadSciUSA 104: 20490-20495.
143. Zhu K, Yang J, Luo K, Yang C, Zhang N, *et al.* (2015) TLR3 signaling in macrophages is indispensable for the protective immunity of invariant natural killer T cells against enterovirus 71 infection. PLoS Pathog 11: e1004613.
144. Darmoise A, Teneberg S, Bouzonville L, Brady RO, Beck M, *et al.* (2010) Lysosomal alpha-galactosidase controls the generation of self lipid antigens for natural killer T cells. Immunity 33: 216-228.
145. Chen X, Wang X, Keaton JM, Reddington F, Illarionov PA, *et al.* (2007) Distinct endosomal trafficking requirements for presentation of autoantigens and exogenous lipids by human CD1d molecules. JImmunol 178: 6181-6190.
146. Boes M, van der WN, Peperzak V, Kim YM, Peters PJ, *et al.* (2005) In vivo control of endosomal architecture by class II-associated invariant chain and cathepsin S. EurJImmunol 35: 2552-2562.
147. Raftery MJ, Winau F, Giese T, Kaufmann SH, Schaible UE, *et al.* (2008) Viral danger signals control CD1d de novo synthesis and NKT cell activation. EurJImmunol 38: 668-679.
148. Holzapfel KL, Tyznik AJ, Kronenberg M, Hogquist KA (2014) Antigen-dependent versus -independent activation of invariant NKT cells during infection. J Immunol 192: 5490-5498.
149. Hansen TH, Bouvier M (2009) MHC class I antigen presentation: learning from viral evasion strategies. NatRevImmunol 9: 503-513.
150. Horst D, Verweij MC, Davison AJ, Ressing ME, Wiertz EJ (2011) Viral evasion of T cell immunity: ancient mechanisms offering new applications. CurrOpinImmunol 23: 96-103.
151. Horst D, Ressing ME, Wiertz EJ (2011) Exploiting human herpesvirus immune evasion for therapeutic gain: potential and pitfalls. ImmunolCell Biol 89: 359-366.
152. Li D, Xu XN (2008) NKT cells in HIV-1 infection. Cell Res 18: 817-822.
153. Hage CA, Kohli LL, Cho S, Brutkiewicz RR, Twigg HL, III, *et al.* (2005) Human immunodeficiency virus gp120 downregulates CD1d cell surface expression. ImmunolLett 98: 131-135.

154. Chen N, McCarthy C, Drakesmith H, Li D, Cerundolo V, *et al.* (2006) HIV-1 down-regulates the expression of CD1d via Nef. EurJImmunol 36: 278-286.
155. Cho S, Knox KS, Kohli LM, He JJ, Exley MA, *et al.* (2005) Impaired cell surface expression of human CD1d by the formation of an HIV-1 Nef/CD1d complex. Virology 337: 242-252.
156. Moll M, Andersson SK, Smed-Sorensen A, Sandberg JK (2010) Inhibition of lipid antigen presentation in dendritic cells by HIV-1 Vpu interference with CD1d recycling from endosomal compartments. Blood 116: 1876-1884.
157. Renukaradhya GJ, Webb TJ, Khan MA, Lin YL, Du W, *et al.* (2005) Virus-induced inhibition of CD1d1-mediated antigen presentation: reciprocal regulation by p38 and ERK. JImmunol 175: 4301-4308.
158. Webb TJ, Litavecz RA, Khan MA, Du W, Gervay-Hague J, *et al.* (2006) Inhibition of CD1d1-mediated antigen presentation by the vaccinia virus B1R and H5R molecules. EurJImmunol 36: 2595-2600.
159. Ashrafi GH, Haghshenas MR, Marchetti B, O'Brien PM, Campo MS (2005) E5 protein of human papillomavirus type 16 selectively downregulates surface HLA class I. IntJCancer 113: 276-283.
160. Ashrafi GH, Haghshenas M, Marchetti B, Campo MS (2006) E5 protein of human papillomavirus 16 downregulates HLA class I and interacts with the heavy chain via its first hydrophobic domain. IntJCancer 119: 2105-2112.
161. Zhang B, Li P, Wang E, Brahmi Z, Dunn KW, *et al.* (2003) The E5 protein of human papillomavirus type 16 perturbs MHC class II antigen maturation in human foreskin keratinocytes treated with interferon-gamma. Virology 310: 100-108.
162. Miura S, Kawana K, Schust DJ, Fujii T, Yokoyama T, *et al.* (2010) CD1d, a sentinel molecule bridging innate and adaptive immunity, is downregulated by the human papillomavirus (HPV) E5 protein: a possible mechanism for immune evasion by HPV. JVirol 84: 11614-11623.
163. Sanchez DJ, Gumperz JE, Ganem D (2005) Regulation of CD1d expression and function by a herpesvirus infection. JClinInvest 115: 1369-1378.
164. Coscoy L, Ganem D (2000) Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. ProcNatlAcadSciUSA 97: 8051-8056.
165. Ishido S, Wang C, Lee BS, Cohen GB, Jung JU (2000) Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. JVirol 74: 5300-5309.
166. Yuan W, Dasgupta A, Cresswell P (2006) Herpes simplex virus evades natural killer T cell recognition by suppressing CD1d recycling. NatImmunol 7: 835-842.
167. Xiong R, Rao P, Kim S, Li M, Wen X, *et al.* (2015) Herpes Simplex Virus 1 US3 Phosphorylates Cellular KIF3A To Downregulate CD1d Expression. J Virol 89: 6646-6655.
168. Rao P, Pham HT, Kulkarni A, Yang Y, Liu X, *et al.* (2011) Herpes simplex virus 1 glycoprotein B and US3 collaborate to inhibit CD1d antigen presentation and NKT cell function. JVirol 85: 8093-8104.
169. Allan LL, Stax AM, Zheng DJ, Chung BK, Kozak FK, *et al.* (2011) CD1d and CD1c expression in human B cells is regulated by activation and retinoic acid receptor signaling. J Immunol 186: 5261-5272.
170. Zuo J, Thomas W, van Leeuwen D, Middeldorp JM, Wiertz EJHJ, *et al.* (2008) The DNase of Gammaherpesviruses Impairs Recognition by Virus-Specific CD8+ T Cells through an Additional Host Shutoff Function. Journal of Virology 82: 2385-2393.
171. Baylis SA, Purifoy DJ, Littler E (1989) The characterization of the EBV alkaline deoxyribonuclease cloned and expressed in E. coli. Nucleic Acids Res 17: 7609-7622.
172. Rowe M, Glaunsinger B, van LD, Zuo J, Sweetman D, *et al.* (2007) Host shutoff during productive Epstein-Barr virus infection is mediated by BGLF5 and may contribute to immune evasion. Proc Natl Acad Sci U S A 104: 3366-3371.

173. Horst D, Burmeister WP, Boer IGJ, van Leeuwen D, Buisson M, *et al.* (2012) The Bridge in the Epstein-Barr Virus Alkaline Exonuclease Protein BGLF5 Contributes to Shutoff Activity during Productive Infection. Journal of Virology 86: 9175-9187.
174. van Gent M, Gram AM, Boer IG, Geerdink RJ, Lindenbergh MF, *et al.* (2015) Silencing the shutoff protein of Epstein-Barr virus in productively infected B cells points to (innate) targets for immune evasion. J Gen Virol 96: 858-865.
175. Hislop AD, Ressing ME, van Leeuwen D, Pudney VA, Horst Dl, *et al.* (2007) A CD8+ T cell immune evasion protein specific to Epstein-Barr virus and its close relatives in Old World primates. The Journal of Experimental Medicine 204: 1863-1873.
176. Horst D, van Leeuwen D, Croft NP, Garstka MA, Hislop AD, *et al.* (2009) Specific Targeting of the EBV Lytic Phase Protein BNLF2a to the Transporter Associated with Antigen Processing Results in Impairment of HLA Class I-Restricted Antigen Presentation. The Journal of Immunology 182: 2313-2324.
177. Croft NP, Shannon-Lowe C, Bell AI, Horst Dl, Kremmer E, *et al.* (2009) Stage-Specific Inhibition of MHC Class I Presentation by the Epstein-Barr Virus BNLF2a Protein during Virus Lytic Cycle. PLoS Pathog 5: e1000490.
178. Zuo J, Currin A, Griffin BD, Shannon-Lowe C, Thomas WA, *et al.* (2009) The Epstein-Barr Virus G-Protein-Coupled Receptor Contributes to Immune Evasion by Targeting MHC Class I Molecules for Degradation. PLoS Pathog 5: e1000255.
179. Griffin BD, Gram AM, Mulder A, van Leeuwen D, Claas FHJ, *et al.* (2013) EBV BILF1 Evolved To Downregulate Cell Surface Display of a Wide Range of HLA Class I Molecules through Their Cytoplasmic Tail. The Journal of Immunology 190: 1672-1684.
180. Quinn LL, Zuo J, Abbott RJM, Shannon-Lowe C, Tierney RJ, *et al.* (2014) Cooperation between Epstein-Barr Virus Immune Evasion Proteins Spreads Protection from CD8+ T Cell Recognition across All Three Phases of the Lytic Cycle. PLoS Pathog 10: e1004322.
181. Ressing ME, D. vL, Verreck FA, Gomez R, Heemskerk B, *et al.* (2003) Interference with T cell receptor-HLA-DR interactions by Epstein-Barr virus gp42 results in reduced T helper cell recognition. Proc Natl Acad Sci U S A 100: 11583-11588.
182. Ressing ME, D. vL, Verreck FA, Keating S, Gomez R, *et al.* (2005) Epstein-Barr virus gp42 is posttranslationally modified to produce soluble gp42 that mediates HLA class II immune evasion. J Virol 79: 841-852.
183. Morrison TE, Mauser A, Wong A, Ting JP-Y, Kenney SC (2001) Inhibition of IFN-y Signaling by an Epstein-Barr Virus Immediate-Early Protein. Immunity 15: 787-799.
184. Zuo J, Thomas WA, Haigh TA, Fitzsimmons L, Long HM, *et al.* (2011) Epstein-Barr Virus Evades CD4+ T Cell Responses in Lytic Cycle through BZLF1-mediated Downregulation of CD74 and the Cooperation of vBcl-2. PLoS Pathog 7: e1002455.
185. Brooks DG, Trifilo MJ, Edelmann KH, Teyton L, McGavern DB, *et al.* (2006) Interleukin-10 determines viral clearance or persistence in vivo. Nat Med 12: 1301-1309.
186. Salek-Ardakani S, Arrand JR, Mackett M (2002) Epstein–Barr Virus Encoded Interleukin-10 Inhibits HLA-Class I, ICAM-1, and B7 Expression on Human Monocytes: Implications for Immune Evasion by EBV. Virology 304: 342-351.
187. Ressing ME, Horst D, Griffin BD, Tellam J, Zuo J, *et al.* (2008) Epstein-Barr virus evasion of CD8(+) and CD4(+) T cell immunity via concerted actions of multiple gene products. Semin Cancer Biol 18: 397-408.
188. Hahn AM, Huye LE, Ning S, Webster-Cyriaque J, Pagano JS (2005) Interferon Regulatory Factor 7 Is Negatively Regulated by the Epstein-Barr Virus Immediate-Early Gene, BZLF-1. Journal of Virology 79: 10040-10052.
189. Keating Sa, Prince S, Jones M, Rowe M (2002) The Lytic Cycle of Epstein-Barr Virus Is Associated with Decreased Expression of Cell Surface Major Histocompatibility Complex Class I and Class II

Molecules. Journal of Virology 76: 8179-8188.
190. Morrison TE, Kenney SC (2004) BZLF1, an Epstein–Barr virus immediate-early protein, induces p65 nuclear translocation while inhibiting p65 transcriptional function. Virology 328: 219-232.
191. Dreyfus DH, Nagasawa M, Pratt JC, Kelleher CA, Gelfand EW (1999) Inactivation of NF-kB by EBV BZLF-1-Encoded ZEBRA Protein in Human T Cells. The Journal of Immunology 163: 6261-6268.
192. Gutsch DE, Holley-Guthrie EA, Zhang Q, Stein B, Blanar MA, *et al.* (1994) The bZIP transactivator of Epstein-Barr virus, BZLF1, functionally and physically interacts with the p65 subunit of NF-kappa B. Molecular and Cellular Biology 14: 1939-1948.
193. Morrison TE, Mauser A, Klingelhutz A, Kenney SC (2004) Epstein-Barr Virus Immediate-Early Protein BZLF1 Inhibits Tumor Necrosis Factor Alpha-Induced Signaling and Apoptosis by Downregulating Tumor Necrosis Factor Receptor 1. Journal of Virology 78: 544-549.
194. Bristol JA, Robinson AR, Barlow EA, Kenney SC (2010) The Epstein-Barr Virus BZLF1 Protein Inhibits Tumor Necrosis Factor Receptor 1 Expression through Effects on Cellular C/EBP Proteins. Journal of Virology 84: 12362-12374.
195. Michaud F, Coulombe Fo, Gaudreault E, Paquet-Bouchard C, Rola-Pleszczynski M, *et al.* (2010) Epstein-Barr Virus Interferes with the Amplification of IFNa Secretion by Activating Suppressor of Cytokine Signaling 3 in Primary Human Monocytes. PLoS ONE 5: e11908.
196. Cayrol C, Flemington EK (1995) Identification of cellular target genes of the Epstein-Barr virus transactivator Zta: activation of transforming growth factor beta igh3 (TGF-beta igh3) and TGF-beta 1. Journal of Virology 69: 4206-4212.
197. Adamson AL, Kenney S (2001) Epstein-Barr Virus Immediate-Early Protein BZLF1 Is SUMO-1 Modified and Disrupts Promyelocytic Leukemia Bodies. Journal of Virology 75: 2388-2399.
198. Saffert RT, Kalejta RF (2008) Promyelocytic leukemia-nuclear body proteins: herpesvirus enemies, accomplices, or both? Future Virology: Future Medicine. pp. 265-277.
199. Bentz GL, Liu R, Hahn AM, Shackelford J, Pagano JS (2010) Epstein–Barr virus BRLF1 inhibits transcription of IRF3 and IRF7 and suppresses induction of interferon-ß. Virology 402: 121-128.
200. Dunmire SK, Odumade OA, Porter JL, Reyes-Genere J, Schmeling DO, *et al.* (2014) Primary EBV Infection Induces an Expression Profile Distinct from Other Viruses but Similar to Hemophagocytic Syndromes. PLoS ONE 9: e85422.
201. Wertz IE, Dixit VM (2010) Signaling to NF-kB: Regulation by Ubiquitination. Cold Spring Harbor Perspectives in Biology 2: 1-19.
202. Deribe YL, Pawson T, Dikic I (2010) Post-translational modifications in signal integration. Nat Struct Mol Biol 17: 666-672.
203. Chang LS, Wang JT, Doong SL, Lee CP, Chang CW, *et al.* (2012) Epstein-Barr Virus BGLF4 Kinase Downregulates NF-kB Transactivation through Phosphorylation of Coactivator UXT. Journal of Virology 86: 12176-12186.
204. van Gent M, Braem SGE, de Jong A, Delagic N, Peeters JGC, *et al.* (2014) Epstein-Barr Virus Large Tegument Protein BPLF1 Contributes to Innate Immune Evasion through Interference with Toll-Like Receptor Signaling. PLoS Pathog 10: e1003960.
205. Johannsen E, Luftig M, Chase MR, Weicksel S, Cahir-McFarland E, *et al.* (2004) Proteins of purified Epstein-Barr virus. Proceedings of the National Academy of Sciences of the United States of America 101: 16286-16291.
206. Cohen JI, Lekstrom K (1999) Epstein-Barr Virus BARF1 Protein Is Dispensable for B-Cell Transformation and Inhibits Alpha Interferon Secretion from Mononuclear Cells. Journal of Virology 73: 7627-7632.
207. Strockbine LD, Cohen JI, Farrah T, Lyman SD, Wagener F, *et al.* (1998) The Epstein-Barr Virus BARF1 Gene Encodes a Novel, Soluble Colony-Stimulating Factor-1 Receptor. Journal of Virology 72: 4015-4021.

208. Ohashi M, Fogg MH, Orlova N, Quink C, Wang F (2012) An Epstein-Barr Virus Encoded Inhibitor of Colony Stimulating Factor-1 Signaling Is an Important Determinant for Acute and Persistent EBV Infection. PLoS Pathog 8: e1003095.
209. Padgett DA, Hotchkiss AK, Pyter LM, Nelson RJ, Yang E, *et al.* (2004) Epstein–Barr virus-encoded dUTPase modulates immune function and induces sickness behavior in mice. Journal of Medical Virology 74: 442-448.
210. Waldman WJ, Williams J, Lemeshow S, Binkley P, Guttridge D, *et al.* (2008) Epstein-Barr virus-encoded dUTPase enhances proinflammatory cytokine production by macrophages in contact with endothelial cells: Evidence for depression-induced atherosclerotic risk. Brain, Behavior, and Immunity 22: 215-223.
211. Glaser R, Litsky ML, Padgett DA, Baiocchi RA, Yang EV, *et al.* (2006) EBV-encoded dUTPase induces immune dysregulation: Implications for the pathophysiology of EBV-associated disease. Virology 346: 205-218.
212. Yin Y, Manoury B, Fahraeus R (2003) Self-Inhibition of Synthesis and Antigen Presentation by Epstein-Barr Virus-Encoded EBNA1. Science 5: 1371-1374.
213. Blake N, Lee S, Redchenko I, Thomas W, Steven N, *et al.* (1997) Human CD8+ T Cell Responses to EBV EBNA1: HLA Class I Presentation of the (Gly-Ala)–Containing Protein Requires Exogenous Processing. Immunity 7: 791-802.
214. Levitskaya J, Coram M, Levitsky V, Imreh S, Steigerwald-Mullen PM, *et al.* (1995) Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. Nature 375: 685-688.
215. Apcher S, Komarova A, Daskalogianni C, Yin Y, Malbert-Colas L, *et al.* (2009) mRNA Translation Regulation by the Gly-Ala Repeat of Epstein-Barr Virus Nuclear Antigen 1. Journal of Virology 83: 1289-1298.
216. Levitskaya J, Sharipo A, Leonchiks A, Ciechanover A, Masucci MG (1997) Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. Proc Natl Acad Sci U S A 94: 12616-12621.
217. Hochberg D, Middeldorp JM, Catalina M, Sullivan JL, Luzuriaga K, *et al.* (2004) Demonstration of the Burkitt's lymphoma Epstein-Barr virus phenotype in dividing latently infected memory cells in vivo. Proceedings of the National Academy of Sciences 101: 239-244.
218. Lee SP, Brooks JM, Al-Jarrah H, Thomas WA, Haigh TA, *et al.* (2004) CD8 T Cell Recognition of Endogenously Expressed Epstein-Barr Virus Nuclear Antigen 1. The Journal of Experimental Medicine 199: 1409-1420.
219. Tellam J, Connolly G, Green KJ, Miles JJ, Moss DJ, *et al.* (2004) Endogenous Presentation of CD8+ T Cell Epitopes from Epstein-Barr Virus-encoded Nuclear Antigen 1. The Journal of Experimental Medicine 199: 1421-1431.
220. Voo KS, Fu T, Wang HY, Tellam J, Heslop HE, *et al.* (2004) Evidence for the Presentation of Major Histocompatibility Complex Class IGÇôrestricted Epstein-Barr Virus Nuclear Antigen 1 Peptides to CD8+ T Lymphocytes. The Journal of Experimental Medicine 199: 459-470.
221. Valentine R, Dawson C, Hu C, Shah K, Owen T, *et al.* (2010) Epstein-Barr virus-encoded EBNA1 inhibits the canonical NF-kappaB pathway in carcinoma cells by inhibiting IKK phosphorylation. Molecular Cancer 9: 1.
222. Wood VHJ, O'Neil JD, Wei W, Stewart SE, Dawson CW, *et al.* (2007) Epstein-Barr virus-encoded EBNA1 regulates cellular gene transcription and modulates the STAT1 and TGF[beta] signaling pathways. Oncogene 26: 4135-4147.
223. Kanda K, Kempkes B, Bornkamm GW, Gabain Av, Decker T (1999) The Epstein-Barr Virus Nuclear Antigen 2 (EBNA2), a Protein Required for B Lymphocyte Immortalization, Induces the Synthesis of Type I Interferon in Burkitts Lymphoma Cell Lines. Biological Chemistry. pp. 213.
224. Aman P, von Gabain A (1990) An Epstein-Barr virus immortalization associated gene segment

interferes specifically with the IFN-induced anti-proliferative response in human B-lymphoid cell lines. The EMBO Journal 9: 147-152.
225. Kanda K, Decker T, Aman P, Wahlstr+Âm M, von Gabain A, *et al.* (1992) The EBNA2-related resistance towards alpha interferon (IFN-alpha) in Burkitt's lymphoma cells effects induction of IFN-induced genes but not the activation of transcription factor ISGF-3. Molecular and Cellular Biology 12: 4930-4936.
226. Muromoto R, Ikeda O, Okabe K, Togi S, Kamitani S, *et al.* (2009) Epstein–Barr virus-derived EBNA2 regulates STAT3 activation. Biochemical and Biophysical Research Communications 378: 439-443.
227. Ho HH, Ivashkiv LB (2006) Role of STAT3 in Type I Interferon Responses: Negative Regulation of STAT1-dependent Inflammatory Gene Activation. Journal of Biological Chemistry 281: 14111-14118.
228. Pagès F, Galon J, Karaschuk G, Dudziak D, Camus M, *et al.* (2004) Epstein-Barr virus nuclear antigen 2 induces interleukin-18 receptor expression in B cells. Blood 105: 1632-1639.
229. van de Veerdonk FL, Wever PC, Hermans MHA, Fijnheer R, Joosten LAB, *et al.* (2012) IL-18 Serum Concentration Is Markedly Elevated in Acute EBV Infection and Can Serve as a Marker for Disease Severity. Journal of Infectious Diseases 206: 197-201.
230. Kieser A (2007) Signal transduction by the Epstein-Barr virus oncogene latent membrane protein 1 (LMP1). Signal Transduction 7: 20-33.
231. Middeldorp JM, Pegtel DM (2008) Multiple roles of LMP1 in Epstein-Barr virus induced immune escape. Seminars in Cancer Biology, Manipulation of the immune response by Epstein-Barr virus and Kaposi's sarcoma-associated herpes virus: Consequences for tumor development. pp. 388-396.
232. Xu D, Brumm K, Zhang L (2006) The Latent Membrane Protein 1 of Epstein-Barr Virus (EBV) Primes EBV Latency Cells for Type I Interferon Production. Journal of Biological Chemistry 281: 9163-9169.
233. Richardson Cí, Fielding C, Rowe M, Brennan P (2003) Epstein-Barr Virus Regulates STAT1 through Latent Membrane Protein 1. Journal of Virology 77: 4439-4443.
234. Najjar I, Baran-Marszak F, Le Clorennec C, Laguillier C, Schischmanoff O, *et al.* (2005) Latent Membrane Protein 1 Regulates STAT1 through NF-kB-Dependent Interferon Secretion in Epstein-Barr Virus-Immortalized B Cells. Journal of Virology 79: 4936-4943.
235. Geiger TR, Martin JM (2006) The Epstein-Barr Virus-Encoded LMP-1 Oncoprotein Negatively Affects Tyk2 Phosphorylation and Interferon Signaling in Human B Cells. Journal of Virology 80: 11638-11650.
236. Zhang L, Pagano JS (2000) Interferon Regulatory Factor 7 Is Induced by Epstein-Barr Virus Latent Membrane Protein 1. Journal of Virology 74: 1061-1068.
237. Zhang L, Wu L, Hong K, Pagano JS (2001) Intracellular Signaling Molecules Activated by Epstein-Barr Virus for Induction of Interferon Regulatory Factor 7. Journal of Virology 75: 12393-12401.
238. Leslie E, Ning S, Kelliher M, Pagano JS (2007) Interferon Regulatory Factor 7 Is Activated by a Viral Oncoprotein through RIP-Dependent Ubiquitination. Molecular and Cellular Biology 27: 2910-2918.
239. Ning S, Campos AD, Darnay BG, Bentz GL, Pagano JS (2008) TRAF6 and the Three C-Terminal Lysine Sites on IRF7 Are Required for Its Ubiquitination-Mediated Activation by the Tumor Necrosis Factor Receptor Family Member Latent Membrane Protein 1. Molecular and Cellular Biology 28: 6536-6546.
240. Zhao Y, Xu D, Jiang Y, Zhang L (2010) Dual Functions of Interferon Regulatory Factors 7C in Epstein-Barr Virus–Mediated Transformation of Human B Lymphocytes. PLoS ONE 5: e9459.
241. Zhang J, Das SC, Kotalik C, Pattnaik AK, Zhang L (2004) The Latent Membrane Protein 1 of Epstein-Barr Virus Establishes an Antiviral State via Induction of Interferon-stimulated Genes. Journal of Biological Chemistry 279: 46335-46342.

242. Gires O, Kohlhuber F, Kilger E, Baumann M, Kieser A, *et al.* (1999) Latent membrane protein 1 of Epstein–Barr virus interacts with JAK3 and activates STAT proteins. The EMBO Journal 18: 3064-3073.
243. Fathallah I, Parroche P, Gruffat H, Zannetti C, Johansson H, *et al.* (2010) EBV Latent Membrane Protein 1 Is a Negative Regulator of TLR9. The Journal of Immunology 185: 6439-6447.
244. Cahir-McFarland ED, Davidson DM, Schauer SL, Duong J, Kieff E (2000) NF-kB inhibition causes spontaneous apoptosis in Epstein-Barr virus-transformed lymphoblastoid cells. Proceedings of the National Academy of Sciences 97: 6055-6060.
245. Izumi KM, Kieff ED (1997) The Epstein–Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to mediate B lymphocyte growth transformation and activate NF-kB. Proceedings of the National Academy of Sciences 94: 12592-12597.
246. Stewart S, Dawson CW, Takada K, Curnow J, Moody CA, *et al.* (2004) Epstein–Barr virus-encoded LMP2A regulates viral and cellular gene expression by modulation of the NF-kB transcription factor pathway. Proceedings of the National Academy of Sciences of the United States of America 101: 15730-15735.
247. Swanson-Mungerson M, Bultema R, Longnecker R (2010) Epstein–Barr virus LMP2A imposes sensitivity to apoptosis. Journal of General Virology 91: 2197-2202.
248. Shah KM, Stewart SE, Wei W, Woodman CBJ, O'Neil JD, *et al.* (2009) The EBV-encoded latent membrane proteins, LMP2A and LMP2B, limit the actions of interferon by targeting interferon receptors for degradation. Oncogene 28: 3903-3914.
249. Xia T, O'Hara A, Araujo I, Barreto J, Carvalho E, *et al.* (2008) EBV MicroRNAs in Primary Lymphomas and Targeting of CXCL-11 by ebv-mir-BHRF1-3. Cancer Res 68: 1436-1442.
250. Nachmani D, Stern-Ginossar N, Sarid R, Mandelboim O (2009) Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. Cell Host Microbe 5: 376-385.
251. Rathinam V, Fitzgerald K (2010) Inflammasomes and Anti-Viral Immunity. J Clin Immunol: Springer US. pp. 632-637.
252. Haneklaus M, Gerlic M, Kurowska-Stolarska M, Rainey AA, Pich D, *et al.* (2012) Cutting edge: miR-223 and EBV miR-BART15 regulate the NLRP3 inflammasome and IL-1beta production. J Immunol 189: 3795-3799.
253. Huang WT, Lin CW (2014) EBV-encoded miR-BART20-5p and miR-BART8 inhibit the IFN-gamma-STAT1 pathway associated with disease progression in nasal NK-cell lymphoma. Am J Pathol 184: 1185-1197.
254. Lin TC, Liu TY, Hsu SM, Lin CW (2013) Epstein-Barr virus-encoded miR-BART20-5p inhibits T-bet translation with secondary suppression of p53 in invasive nasal NK/T-cell lymphoma. Am J Pathol 182: 1865-1875.
255. Ambrosio MR, Navari M, Di LL, Leon EA, Onnis A, *et al.* (2014) The Epstein Barr-encoded BART-6-3p microRNA affects regulation of cell growth and immuno response in Burkitt lymphoma. Infect Agent Cancer 9: 12.

Chapter 2

Silencing the shutoff protein of Epstein-Barr virus in productively infected B cells points to (innate) targets for immune evasion

Michiel van Gent[1], Anna M. Gram[1,2], Ingrid G.J. Boer[1], Ruben J. Geerdink[1], Marthe F.S. Lindenbergh[1], Robert Jan Lebbink[1], Emmanuel J. Wiertz[1], Maaike E. Ressing[1,2]

[1]Department of Medical Microbiology, University Medical Center Utrecht, The Netherlands
[2]Department of Molecular Cell Biology, Leiden University Medical Center, The Netherlands

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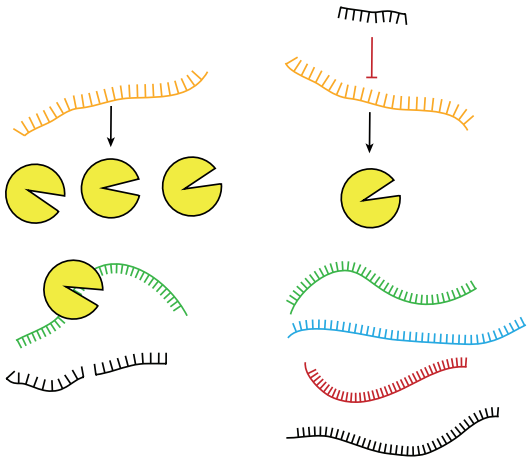


Summary

During productive infection with Epstein-Barr virus (EBV), a dramatic suppression of cellular protein expression is caused by the viral alkaline exonuclease BGLF5. Among the proteins downregulated by BGLF5 are multiple immune components. Here, we show that shutoff reduces expression of the innate EBV-sensing Toll-like receptor-2 and the lipid antigen-presenting CD1d molecule, thereby identifying these proteins as novel targets of BGLF5.

To silence BGLF5 expression in B cells undergoing productive EBV infection, we employed an shRNA approach. Viral replication still occurred in these cells, albeit with reduced late gene expression. Surface levels of a group of proteins, including immunologically relevant molecules such as CD1d and HLA class I and class II, were only partly rescued by depletion of BGLF5, suggesting that additional viral gene products interfere with their expression. Our combined approach thus provides a means to unmask novel EBV (innate) immune evasion strategies that may operate in productively infected B cells.

Herpesviruses are large enveloped DNA viruses that establish lifelong persistence in infected hosts. To achieve persistence, many herpesvirus gene products are dedicated to preventing elimination of virus-producing cells. For instance, members of all three herpesvirus subfamilies encode proteins that specifically interfere with antigen presentation to T cells (Griffin *et al.*, 2010). Prior to adaptive immunity, innate responses are elicited upon sensing of infection through pattern-recognition receptors, such as the Toll-like receptors (TLRs) (Iwasaki & Medzhitov, 2010;Paludan *et al.*, 2013). These innate antiviral responses are also subject to herpesvirus immune evasion (Feng *et al.*, 2013;Ning, 2011;Paludan *et al.*, 2013).

Productive infection by α- and γ-herpesviruses induces a global inhibition of protein synthesis resulting from enhanced mRNA degradation (Gaglia *et al.*, 2012). For the γ-herpesviruses, this shutoff is mediated by the viral alkaline exonuclease (AE) (Covarrubias *et al.*, 2009;Glaunsinger & Ganem, 2004;Rowe *et al.*, 2007). AE proteins are conserved throughout the herpesvirus family, reflecting their critical DNase function in processing of newly synthesized viral genomes; their additional RNase-based shutoff function is unique to γ-herpesviruses (Glaunsinger & Ganem, 2004;Rowe *et al.*, 2007). Shutoff appears broadly active and affects expression of most cellular proteins (Clyde & Glaunsinger, 2011). As such, herpesvirus-induced shutoff provides a general strategy to dampen anti-viral immune activation.

Additional, more specific, immune evasive mechanisms operating in herpesvirus-infected cells could be masked by the general effects of shutoff. Indeed, the first examples of T cell escape by dedicated herpesvirus immunoevasins were identified in the absence of shutoff: α-herpesvirus-encoded inhibitors of antigen presentation by HLA class I (HLA I) molecules were identified using shutoff-defective mutant viruses (Koppers-Lalic *et al.*, 2003;York *et al.*, 1994), and multiple HLA I evasion strategies were identified for the β-herpesvirus human cytomegalovirus that lacks a virus-encoded shutoff function (Barnes & Grundy, 1992). Cooperative targeting of a single immune pathway by multiple viral gene products has emerged as a common theme (Jones *et al.*, 1995;Ressing *et al.*, 2008). Compared to α-herpesviruses, shutoff by γ-herpesviruses has been discovered more recently. Consequently, less is known about the role of AE-mediated shutoff in immune evasion during productive γ-herpesvirus infection.

The prototypic human γ-herpesvirus, Epstein-Barr virus (EBV), naturally infects B cells, which form the latent virus reservoir in vivo (Rickinson & Kieff, 2007). For production of new viral progeny, EBV reactivates from a small percentage of latently infected B cells. The AE protein of EBV, BGLF5, is expressed during this lytic phase of infection. Earlier, we have reported that cellular expression of BGLF5 downregulates immunologically relevant proteins, such as HLA molecules and TLR9 (Rowe *et al.*, 2007;van Gent *et al.*, 2011;Zuo *et al.*, 2008), providing a means of general immune evasion. In this study, we aimed to evaluate BGLF5's effects in the context of productive EBV infection in B cells.

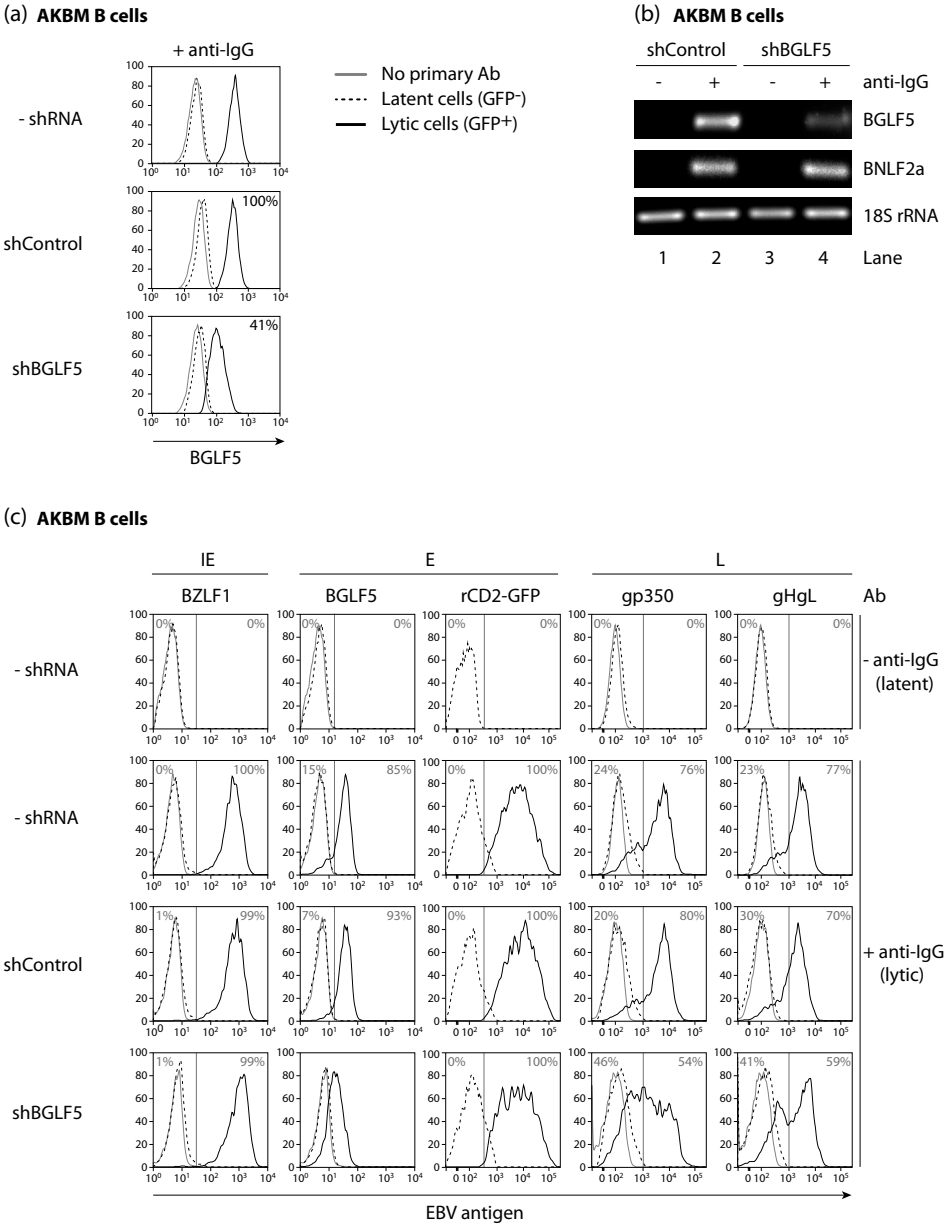


Figure 1 Silencing BGLF5 in productively EBV-infected B cells. AKBM B cells stably expressed no shRNA, a combination of two BGLF5-targeting shRNAs (shBGLF5), or an shRNA targeting Fas (shControl). By 20 hours of anti-human IgG treatment (+ anti-IgG), a population of cells had entered the EBV lytic cycle, with concomitant expression of the rCD2-GFP reporter. **(a)** Intracellular BGLF5 levels were determined by flow cytometry, with percentages indicating BGLF5 levels compared to those in shControl cells. In over 10 experiments, around 15-40% BGLF5 protein expression remained in lytic

AKBM-shBGLF5 cells. See **Figure S2** for further details. **(b)** Semi-quantitative RT-PCR analysis was performed to determine mRNA levels of BGLF5, an EBV control transcript (BNLF2a), or a cellular control RNA (18S ribosomal RNA). Almost pure populations of productively EBV-infected cells were obtained through magnetic sorting of lytically induced cells labeled with mouse-anti-rCD2 Ab and anti-mouse-magnetic beads (Miltenyi) (Ressing *et al.*, 2005). **(c)** Effects of shRNAs on progression through the replicative cycle were monitored by flow cytometric analysis of several EBV antigens (**Table S1, Figure S3**). Percentages indicate viral antigen-negative (left) and -positive cells (right) within the productively infected population (lytic cells); solid lines, lytic AKBM cells gated on expression of the rCD2-GFP reporter protein (GFP^+); dashed lines, latently EBV-infected cells (GFP^-); grey lines, no primary Ab.

Several approaches have been used to eliminate expression of individual herpesvirus genes from infected cells, one of which is based on the use of bacterial artificial chromosomes (BACs) (Delecluse *et al.*, 2008). Using this approach, deletion of BGLF5 was shown to perturb EBV replication in transfected 293T cells, resulting in reduced viral yields (Feederle *et al.*, 2009a). Studying EBV mutants during productive infection of B cells has been more difficult. Here, we have employed the EBV^+ Akata B cell line AKBM, in which cross-linking of the B cell receptor with anti-human IgG reactivates EBV in 10-40% of cells. Productively infected B cells can be identified and sorted on the basis of induced expression of a reporter protein, ratCD2-GFP (rCD2-GFP) (Ressing *et al.*, 2005). Using this system, we have elucidated several immune evasion mechanisms acting during the productive phase of EBV infection (Horst *et al.*, 2009;Ressing *et al.*, 2005;van Gent *et al.*, 2011;van Gent *et al.*, 2014). As our approach to suppress BGLF5-mediated shutoff during productive infection in B cells, lentivirus-delivered shRNAs were introduced into these EBV^+ AKBM cells.

Ten candidate shRNAs that target sites within the BGLF5 coding sequence were cloned into a lentiviral vector (**Figure S1**) (Lebbink *et al.*, 2011). Two of these considerably reduced BGLF5 levels in EBV-producing B cells, whereas the expression of a control protein, transferrin receptor (CD71), was not substantially affected (**Figure S2**). Combining the two shRNAs (referred to as shBGLF5) reduced BGLF5 protein levels by 60-75% compared to lytically induced control AKBM cells (**Figures 1a, S2**). Also mRNA levels of BGLF5 were markedly reduced in productively infected AKBM-shBGLF5 cells, whereas levels of another EBV transcript, BNLF2a, remained unchanged (**Figure 1b**). Thus, stable expression of specific shRNAs through lentiviral transduction substantially reduced BGLF5 levels during productive EBV infection in B cells.

AE proteins, through their conserved DNase function, are required for processing of replicated herpesvirus genomes in infected cells. We examined whether silencing of BGLF5 in B cells interfered with progression through the EBV replication cycle. Upon reactivation, immediate-early (IE), early, and late herpesvirus proteins are sequentially expressed (**Table S1**). Anti-IgG treatment of AKBM-shBGLF5 and control cells caused similar amounts of B cells to become positive for the IE transactivator BZLF1 and the early-expressed rCD2-GFP reporter (**Figures 1c, S3**), both of which precede expression of the BGLF5 protein. In

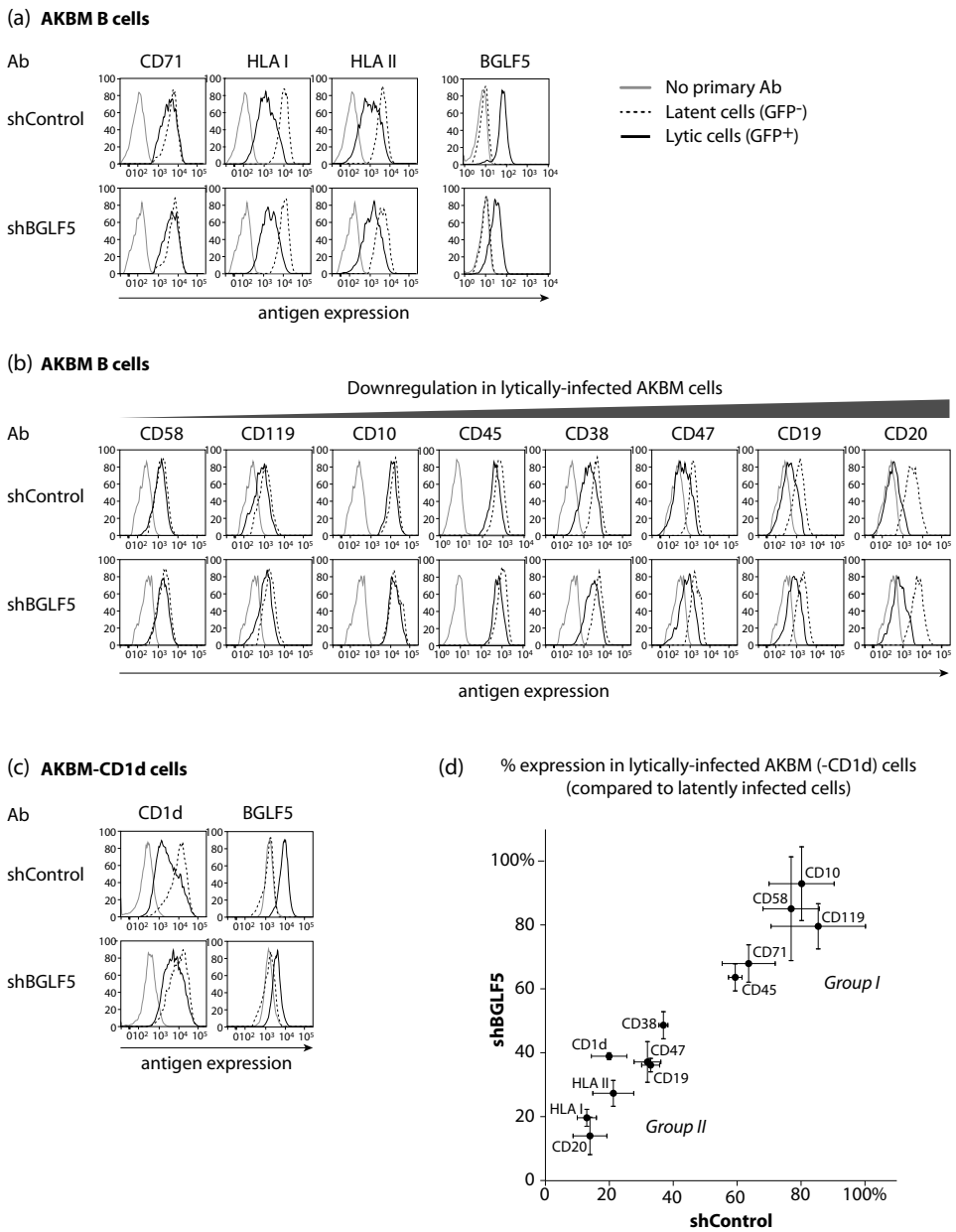


Figure 2 BGLF5 silencing rescues expression of surface proteins on EBV-producing B cells. EBV reactivation was induced in AKBM-shBGLF5 or -shControl cells. **(a,b)** Surface levels of the cellular antigens in **Table S2**, in **(b)** ordered by increasing downregulation from EBV-producing cells. **(c)** Surface levels of CD1d on AKBM cells that stably expressed human CD1d molecules after lentiviral transduction (AKBM-CD1d cells). The extent of BGLF5 silencing was visualized by intracellular staining. Solid lines,

productively EBV-infected (GFP^+) cells; dashed lines, latently infected (GFP^-) cells; grey, no primary Ab (dot plots in **Figure S4**). **(d)** Relative protein levels on productively versus latently EBV- infected shControl cells (horizontal axis) were plotted against those in shBGLF5 cells (vertical axis). Values were obtained by dividing geometric mean fluorescence intensities by background signals (isotype control or without primary antibody) and denoted as percentages expression in lytically compared to latently infected cells, as determined in at least four independent experiments (average ± SD; values for CD58, CD45, and CD119 based on two replicates).

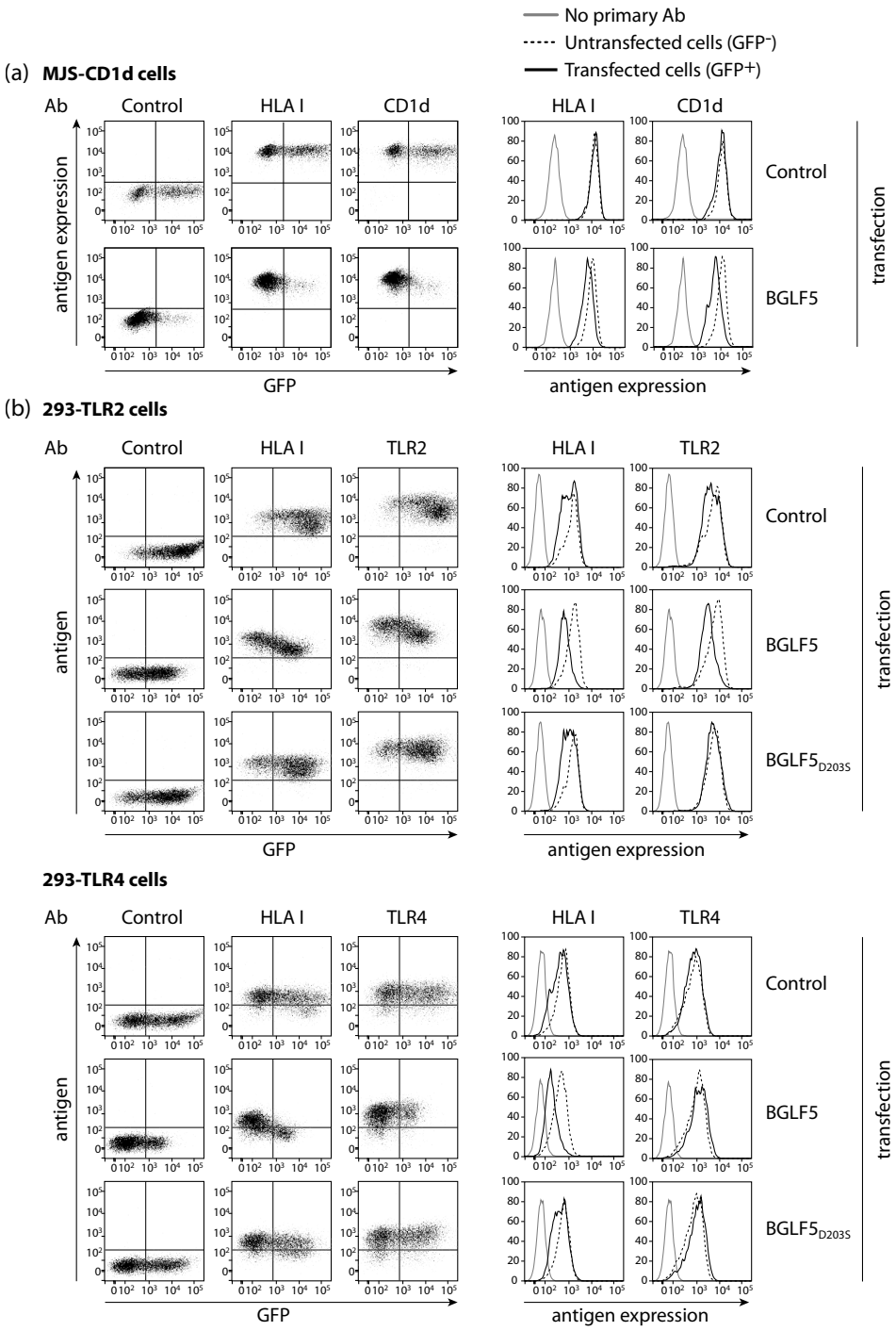
contrast, the proportion of AKBM-shBGLF5 cells expressing late proteins gp350, gH, and gL was substantially reduced. Thus, silencing BGLF5 appears to hamper entry into the late phase of productive EBV infection in B cells.

To evaluate the effects of shutoff, relying on BGLF5's RNase activity, in EBV-producing B cells, we examined the influence of BGLF5 silencing on the downregulation of various surface proteins. In control cells, EBV reactivation caused a minor reduction in CD71 surface levels, while HLA I and II were strongly downregulated (**Figures 2a and S4a**, upper panels), which is in line with earlier observations (Ressing *et al.*, 2005). In lytic AKBM-shBGLF5 cells, surface display of HLA I and II was partly rescued (**Figures 2a and S4a,** lower panels), supporting a contribution of shutoff to evasion from T cell detection during EBV replication in B cells. Still, levels of these antigen presenting molecules remained markedly reduced on BGLF5-silenced cells, which could reflect the specific effects on HLA I expression mediated by two dedicated EBV lytic cycle proteins, BNLF2a and BILF1 (Ressing *et al.*, 2008;Zuo *et al.*, 2009).

The analysis was extended to a panel of additional cellular proteins detectable at the surface of latently EBV-infected B cells (**Table S2**). Following viral reactivation, cellular display of the markers tested was reduced to varying degrees (**Figures 2b,d, S4b**). CD58, CD119, CD10, and CD45 were marginally affected and, therefore, the effect of BGLF5 silencing was difficult to evaluate (group I; **Figures 2b,d, S4b**), as was the case for CD71 (**Figure 2a**). Surface levels of another group of proteins, comprising CD38, CD47, CD19, and CD20, were strongly reduced during productive EBV infection of control cells and they remained downregulated in induced AKBM-shBGLF5 cells (group II, **Figures 2b,d, S4b)**. The phenotype for this latter group of proteins resembles that of the peptide-presenting HLA I and II complexes.

We also included the non-classical HLA molecule CD1d in this analysis. CD1d molecules present lipid antigens to invariant natural killer T (iNKT) cells that express a semi-invariant T cell receptor as well as NK cell markers. iNKT cells act at the interface of innate and adaptive immunity: they rapidly produce polarizing cytokines when activated, for instance in response to viral infection (Horst *et al.*, 2012a;Kinjo *et al.*, 2013). Induction of the EBV lytic cycle in AKBM-CD1d cells caused a dramatic decrease in surface appearance of human CD1d molecules (**Figures 2c,d, S4c**). Although CD1d expression was partly restored when BGLF5 was silenced, it remained far below the levels observed on latently infected cells. Thus, CD1d-restricted antigen presentation appears a novel target of EBV immune evasion, in part mediated by the shutoff protein BGLF5.

The combined data imply that B cell proteins whose surface display remains markedly


No primary Ab
Untransfected cells (GFP-)
Transfected cells (GFP+)
(a) MJS-CD1d cells
Ab
Control
HLA I
CD1d
antigen expression
GFP
antigen expression
Control
BGLF5
transfection
(b) 293-TLR2 cells
TLR2
antigen
BGLF5D203S
293-TLR4 cells
TLR4

reduced on BGLF5-silenced cells (**Figure 2d**, group II) are likely to be downregulated by additional EBV lytic phase proteins, for instance to effectuate reduced recognition of virus-producing B cells by the immune system.

To complement the studies performed in naturally infected AKBM-CD1d-shBGLF5 cells, we investigated BGLF5's effects on the non-classical antigen-presenting molecule CD1d in cells expressing BGLF5 in isolation. MJS-CD1d cells were transiently transfected with BGLF5 and reduction of GFP and surface HLA I levels cells confirmed induction of shutoff. The BGLF5-transfected cells displayed reduced surface expression of CD1d (**Figure 3a**). This CD1d downregulation was, however, less pronounced than that on B cells expressing all EBV gene products (**Figure 2c**), reminiscent of the phenotype for HLA I (**Figure 2a**) (Rowe *et al.*, 2007). These results show that BGLF5 reduces CD1d levels and that other viral factors, absent from the transfected MJS cells, are likely to add to the robust CD1d downregulation observed during productive EBV infection of B cells.

Earlier, we have found that expression of innate sensors, namely several TLRs, is reduced upon EBV reactivation in AKBM cells and that BGLF5 contributes to the downregulation of TLR9 (van Gent *et al.*, 2011). TLR2, 3, and 9 sense EBV particles (Gaudreault *et al.*, 2007;Iwakiri *et al.*, 2009;van Gent *et al.*, 2011), yet no evidence for TLR4-mediated recognition of EBV has been reported (Gaudreault *et al.*, 2007). Here, we monitored the influence of BGLF5 on TLR2 and TLR4. 293-TLR2 and 293-TLR4 cells were transiently transfected with the empty IRES-GFP vector, wild-type BGLF5, or a catalytically inactive mutant, $BGLF5_{D203S}$. TLR2 levels were reduced on wild-type BGLF5-expressing cells, but not on control cells (**Figure 3b**). In contrast, TLR4 levels were not affected by any of the transfected gene products. Thus, BGLF5-mediated shutoff appears to target TLR2, a pattern-recognition receptor sensing EBV.

To conclude, this study shows that lentivirus-delivered shRNAs can successfully be applied to our system for productive EBV infection of B cells to achieve stable silencing of BGLF5. A similar approach in EBV-transformed B-LCLs yielded around 75% knockdown of viral gene expression, which was sufficient to reveal a hierarchy in immune evasive properties of BNLF2a, BILF1, and BGLF5 (Quinn *et al.*, 2014).

In our system of EBV-producing AKBM cells, a comparable reduction of BGLF5 protein levels interfered with viral replication (**Figure 1**). While confirming that knockdown in EBV-producing B cells was sufficiently robust to observe a phenotype, this observation extends

Figure 3 BGLF5 contributes to downregulation of CD1d and TLR2. (a) MelJuSo cells were lentivirally transduced to stably express human CD1d molecules (MJS-CD1d). MJS-CD1d cells **(a)** and 293-TLR2/CD14 cells (Kurt-Jones *et al.*, 2002) or 293-TLR4/CD14/MD2 cells (Invitrogen) **(b)** were transiently transfected with a pcDNA3-IRES-nlsGFP vector without insert or encoding BGLF5 or the catalytic mutant $BGLF5_{D203S}$ (Horst *et al.*, 2012b). At 48 hours post-transfection, surface levels of HLA I, CD1d, TLR2, and TLR4 were determined by flow cytometry. Downregulation of GFP served as a measure for BGLF5's shutoff function. Dashed lines, untransfected GFP^- cells; solid lines, transfected GFP^+ cells; grey lines, secondary Ab only. Data shown are from one experiment representative of three independent experiments.

earlier studies in 293T cells transfected with a BGLF5 deletion mutant EBV BAC (Feederle *et al.*, 2009a). Within the EBV genome, *BGLF5* occurs in tandem with *BGLF4*, which codes for the EBV protein kinase that can regulate EBV late gene expression (El-Guindy *et al.*, 2014). Since BGLF4 is translated from a transcript encoding both BGLF4 and BGLF5, expression of both proteins was lost from BGLF5-deleted virus-producing 293T cells (Feederle *et al.*, 2009b). Likewise, the use of RNA interference to silence BGLF5 expression in B cells will target both BGLF5 and BGLF5+BGLF4 transcripts. When applying this approach to AKBM B cells, we have focused on the shutoff effects that are selectively induced by BGLF5.

During productive EBV infection, a broad range of B cell surface proteins is downregulated in the presence of BGLF5, and this effect is partly reversed upon silencing of BGLF5 (**Figure** 2). These findings are in agreement with promiscuous shutoff by γ-herpesvirus AE proteins, deduced from mRNA target analysis (Clyde & Glaunsinger, 2011) and metabolic labeling experiments (Rowe *et al.*, 2007). Still, some gene products escape shutoff and TLR4 appears to be one of them (**Figure 3**). Based on our current data, two groups of host surface proteins can be discriminated. The first group comprises proteins that are downregulated to a limited extent during EBV replication; the costimulatory molecules CD80 and CD86 can be included in this group (Ressing *et al.*, 2005). The second group is more strongly downregulated, likely by multiple EBV lytic proteins, and their surface levels remain substantially reduced when BGLF5 is silenced. For some proteins belonging to this latter group, a causative role for BGLF5 in their downregulation has been confirmed through transient transfection experiments, i.e. for HLA I and II (Rowe *et al.*, 2007), for TLR9 (van Gent *et al.*, 2011), and for CD1d and TLR2 (this study, **Figure 3**). These combined findings support the notion that BGLF5 contributes to EBV-induced immune evasion during productive infection of B cells.

A recent study in BGLF5-silenced B-LCLs revealed a minor role for BGLF5 in CD8+ T cell evasion when compared to BNLF2a and BILF1 (Quinn *et al.*, 2014). Along the same line, we observed only partially rescued surface display of B cell proteins upon BGLF5 knockdown during productive EBV infection (**Figure 2**). Residual downregulation could result from the ~25% BGLF5 protein expression that remained in induced AKBM-shBGLF5 cells and/or from additional EBV-encoded shutoff function(s), such as that recently reported to be exerted by BZLF1 (Park *et al.*, 2014). In vivo studies on the α-herpesvirus HSV-1 and the murine γ-herpesvirus MHV68 suggest that the immune evasive functions of shutoff mainly affect newly synthesized proteins induced by type I interferons (Murphy *et al.*, 2003;Pasieka *et al.*, 2008;Sheridan *et al.*, 2014). The above observations, together with the absence of shutoff from β-herpesviruses, point to a relatively small contribution of shutoff to immune evasion. This would provide a rationale as to why herpesviruses have acquired additional, specific immune evasion mechanisms to synergistically achieve the proper timing and extent of immune interference.

Analogous to all other herpesviruses studied, EBV encodes multiple gene products that act in concert to prevent T cell activation (Ressing *et al.*, 2008). Additional EBV strategies interfering with innate immunity continue to be identified (Ning, 2011). Here, we have added

CD1d and TLR2 to the target list of EBV BGLF5. Furthermore, we have identified a group of B cell surface proteins including CD1d whose expression is likely downregulated by EBV lytic phase proteins besides BGLF5.

Acknowledgements

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Supporting Information

Table S1. Viral antigens expressed during productive EBV infection

ORF	Name	Timing	Function	Ab clone
BZLF1	Z, Zta, ZEBRA	IE	Lytic cycle transactivator	BZ.1
BNLF2a		E	HLA I downregulation	N/A
BGLF5	EBV AE, DNase	E	Shutoff and exonuclease	311H
BLLF1	gp350	L	Major envelope glyco-protein, attachment to CR2	72A1
BXLF1	gp85/gH	L	Viral membrane fusion/entry	E1D1
BKRF2	gp25/gL	L	Chaperone of gH	E1D1
rCD2-GFP	rat CD2-GFP fusion protein	"E"	Lytic reporter, magnetic sorting	OX34 (α-ratCD2)

Table S2. Expression of cellular surface proteins by AKBM cells

CD#	Name	Function	Ab
Not expressed by latently EBV-infected AKBM cells			
CD1d		Non-classical HLA molecule, lipid presentation to iNKT cells	51.1.3
CD11b	Integrin alpha M	Complement receptor 3 subunit	BD550019
CD11c	Integrin alpha X	Complement receptor 4 subunit	BD559877
CD21	CR2	Complement receptor 2, B cell coreceptor component (with CD19), EBV B cell receptor	BC IM0473U
CD23	FcεRII	IgE receptor	BD347797
CD25		IL-2 receptor alpha-chain	BD341011
CD32		B cell coreceptor	RDI-CD32 abm-7PE
CD35	CR1	Complement receptor 1	BD559872
CD40		Costimulation	SC65263
CD44		Variety of lymphocyte functions	BD550989
CD54	ICAM-1	Intercellular adhesion molecule	BD555511
CD132	Common gamma chain (γc)	Common subunit of at least six interleukin receptors	BD555898
CD162	P-selectin glycoprotein ligand-1	Neutrophil recruitment	BD556055
CD181	CXCR1	IL-8 receptor	R&D-FAB330P
CD191	CCR1	Chemokine receptor, inflammatory responses	R&D-FAB145P
CD192	CCR2	Chemokine receptor, monocyte chemotaxis	R&D-FAB151P
CD282	Toll-Like Receptor 2	Pattern-recognition receptor	eBioscience 12-9024-82
CD284	Toll-Like Receptor 4	Pattern-recognition receptor	Biolegend 312802
Marginally downregulated during productive EBV infection (group I)			
CD10	Neprilysin	Zinc-dependent metalloprotease	BD340921
CD45	Leukocyte common antigen	Protein tyrosine phosphatase	BD555485
CD58	LFA-3	Adhesion molecule, antigen-presenting cell - T cell interaction	BD555921
CD71	Transferrin receptor	Iron uptake	BD555534
CD119	IFNγRα	Ligand binding α chain of IFNγ receptor	BD558934
Substantially downregulated during productive EBV infection (group II)			
CD19		B cell coreceptor component, reduces activation threshold	BD555415
CD20		Enables optimal B cell immune response	BD345793
CD38	Cyclic ADP ribose hydrolase	Multifunctional ectoenzyme, regulation of intracellular Ca2+ levels	BD347687
CD47	Integrin associated protein	Range of cellular processes	BD556047
	HLA I	Classical HLA molecule, peptide presentation to CD8+ T cells	W6/32
	HLA II	Classical HLA molecule, peptide presentation to CD4+ T cells	L243

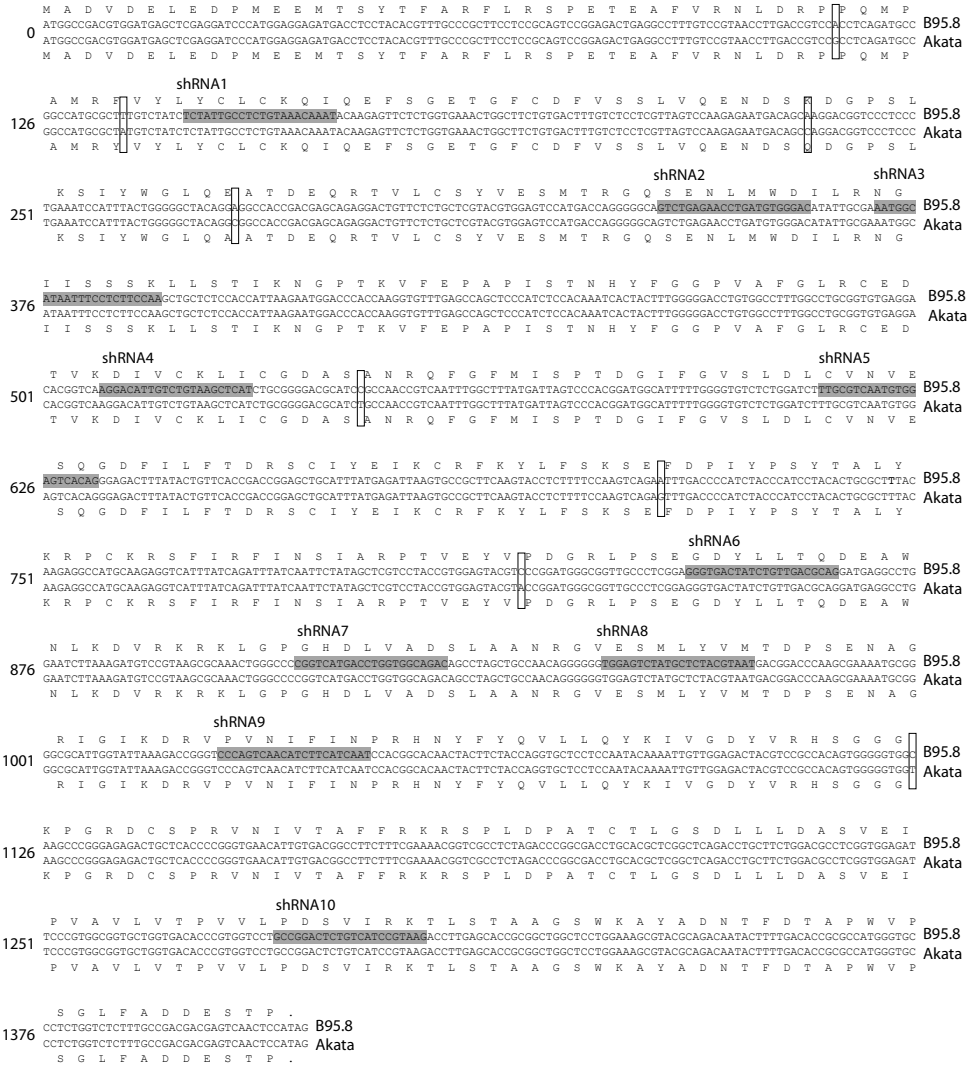

Figure S1 shRNA target sequences within EBV BGLF5. Nucleotide and amino acid alignments are depicted for the BGLF5 open reading frames from EBV strains B95.8 and Akata. Numbers indicate nucleotide positions and boxes show inter-strain differences. Ten candidate shRNA target sites within the BGLF5 coding sequence (in grey) were selected using a prediction algorithm (the Hannon lab website: http://cancan.cshl.edu/RNAi_central/main2.cgi) (Paddison *et al.*, 2004). As a control shRNA, a target sequence (tatgcagaggatgaaagattaa) within the cellular gene encoding the Fas receptor was used. shRNAs were cloned into a lentiviral vector derived from pSicoR (Jacks Lab, MIT), in which the U6 promoter was altered to allow for sticky cloning of shRNAs in between a BstXI and XhoI site, and in which an EF1α promoter was used to drive expression of a cassette encoding a puromycin resistance marker, the ribosome skipping peptide T2A, and mCherry. The resulting vectors were used for the generation of replication-deficient self-inactivating lentivirus stocks, as described (Lebbink *et al.*, 2011).

(a) AKBM B cells
+ anti-IgG
- shRNA
shRNA1
shRNA2
shRNA3
shRNA5
shRNA6
shRNA8
shRNA9
shRNA1+9
BGLF5
CD71
rCD2-GFP
No primary Ab
Latent cells (GFP-)
Lytic cells (GFP+)
(b) AKBM B cells
+ anti-IgG
- shRNA
shRNA1
shRNA2
shRNA3
shRNA5
shRNA6
shRNA8
shRNA9
shRNA1+9
0%
100%
37%
67%
75%
82%
131%
94%
54%
22%
0%
100%
105%
109%
94%
83%
87%
110%
100%
107%
BGLF5
CD71

Figure S2 Experimental selection of shRNA target sequences that silence BGLF5 protein expression. AKBM B cells were lentivirally transduced and puromycin selected (>95% mCherry-positive) to stably express single BGLF5-targeting shRNAs or a combination of two BGLF5 shRNAs (shRNAs #1 and #9, referred to as shBGLF5 in the remainder of the manuscript). Productive EBV infection was induced in these cells by treatment with anti-human IgG Abs (+ anti-IgG: 50 µg/mL; Cappel, MP Biochemicals) for 20 hours (Ressing *et al.*, 2005). Subsequently, cells were fixed, permeabilized, stained for intracellular expression of BGLF5 (Ab 311H; 'intracellular' staining; (Horst *et al.*, 2012b)), and analyzed by flow cytometry. The transferrin receptor (CD71; Ab BD555534) was taken along as a control protein and was visualized at the surface of unfixed and non-permeabilized cells ('surface' staining). Results of a representative experiment with seven out of the ten shRNAs tested are depicted as dot plots (a) and as overlay histograms (b). Solid lines, lytically induced AKBM cells gated on expression of the rCD2-GFP reporter protein (GFP+); dashed lines, latently EBV-infected cells (GFP-); grey lines, no primary Ab. Percentages denote BGLF5 protein expression levels in productively infected AKBM cells with BGLF5-targeting shRNAs compared to those of control cells without shRNA. Geometric mean fluorescence intensities of specifically stained cells (+ primary Ab + secondary APC-conjugated Ab) were divided by the background signal in the absence of primary Ab (second step only). The resulting fold inductions in lytic cells compared to latent cells were expressed as percentages with the expression in -shRNA control cells set at 100%. Over 10 such experiments have been performed with shBGLF5 (#1 + #9) yielding 60-85% silencing of BGLF5 protein expression. Of note, screening of the shRNAs specific for B95.8 BGLF5 by transient transfections in 293T cells (data not shown) had little predictive value for their efficacy during productive EBV infection in B cells, in our hands.

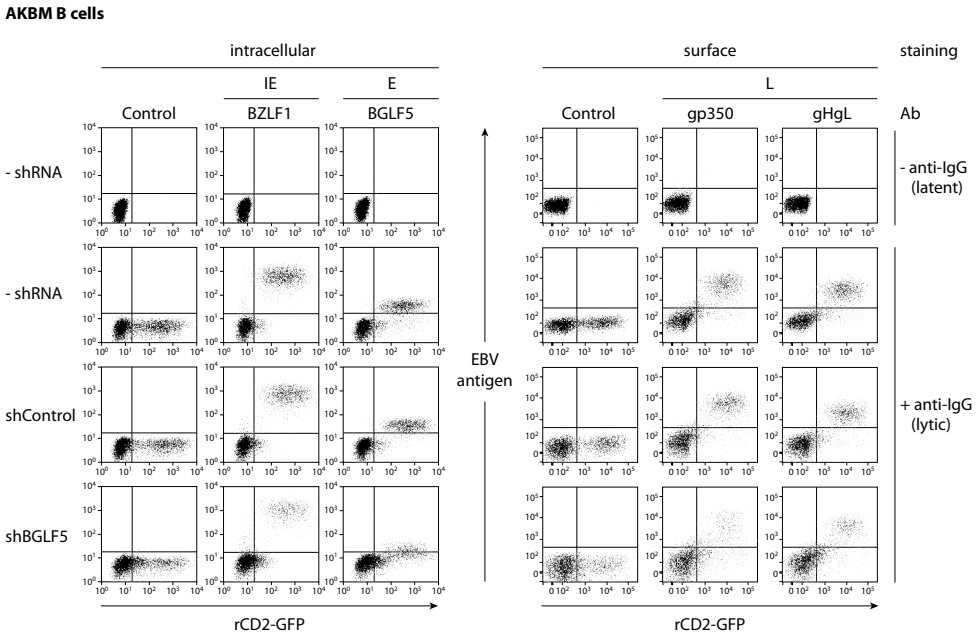

Figure S3 Silencing BGLF5 expression in AKBM B cells affects progression through the EBV replicative cycle. AKBM B cells stably expressing no shRNAs (-shRNA), a control Fas-targeting shRNA (shControl), or a combination of two BGLF5-specific shRNAs (shBGLF5) were treated with anti-human IgG for 20 hours. Entry into the EBV replicative cycle was monitored by flow cytometry. Abs used for intracellular stainings were directed against the IE transactivator BZLF1 and early (E)-expressed BGLF5. Abs used for surface stainings vizualised the late (L) EBV glycoproteins gp350 and gHgL. Dot plots depict the, early-expressed, rCD2-GFP lytic cycle reporter (horizontal axis) and the indicated viral antigens (vertical axis). Histograms of this experiment are presented in **Figure 1c.**

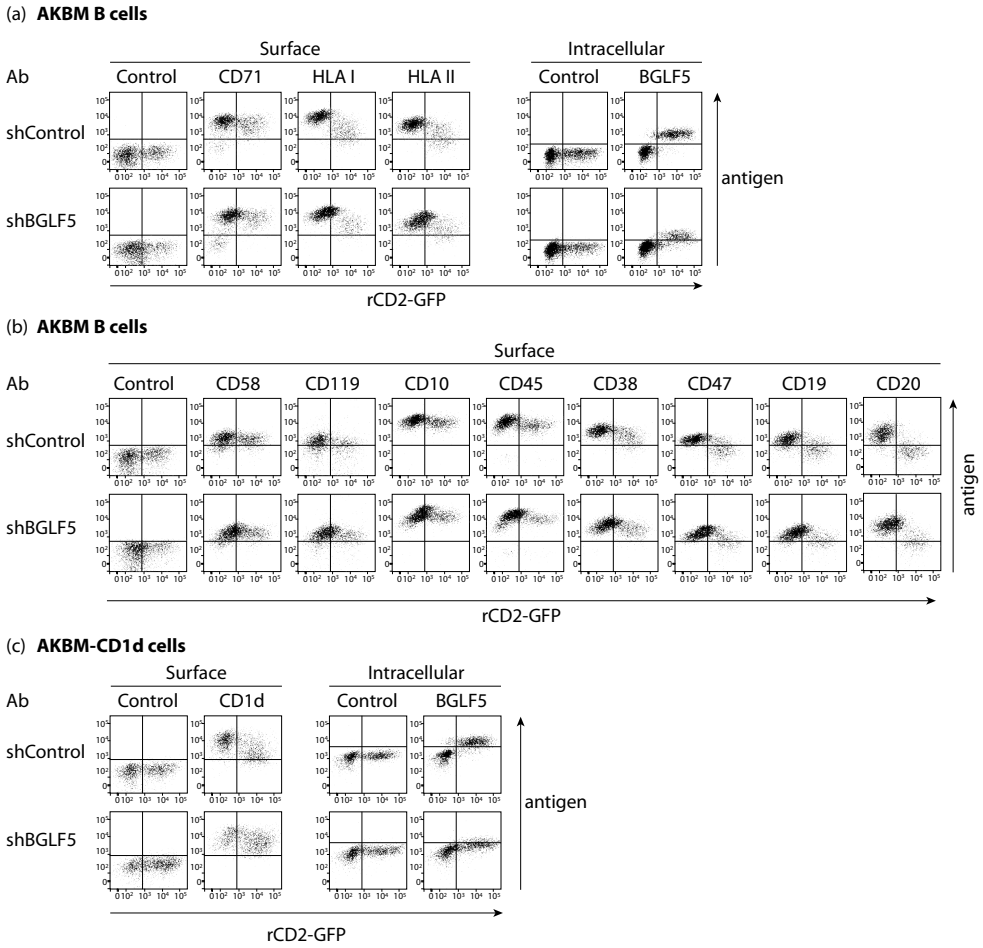

Figure S4 Silencing BGLF5 expression in AKBM B cells affects surface display of cellular antigens. AKBM B cells (a,b) and AKBM-CD1d cells (c) stably expressing a control shRNA targeting Fas (shControl) or a combination of two shRNAs targeting BGLF5 (shBGLF5) were treated with anti-human IgG for 20 hours to induce productive EBV infection. Intracellular BGLF5 levels and downregulation of various B cell surface markers were analyzed by flow cytometry and depicted as dot plots. Histograms of this experiment are presented in **Figure 2**.

References

Barnes, P. D. & Grundy, J. E. (1992).Down-regulation of the class I HLA heterodimer and B2-microglobulin on the surface of cells infected with cytomegalovirus. Journal of General Virology 73, 2395-2403.

Clyde, K. & Glaunsinger, B. A. (2011).Deep sequencing reveals direct targets of gammaherpesvirus-induced mRNA decay and suggests that multiple mechanisms govern cellular transcript escape. PLoS One 6, e19655.

Covarrubias, S., Richner, J. M., Clyde, K., Lee, Y. J. & Glaunsinger, B. A. (2009).Host shutoff is a conserved phenotype of gammaherpesvirus infection and is orchestrated exclusively from the cytoplasm. J Virol 83, 9554-9566.

Delecluse, H. J., Feederle, R., Behrends, U. & Mautner, J. (2008).Contribution of viral recombinants to the study of the immune response against the Epstein-Barr virus. Seminars in Cancer Biology 18, 409-415.

El-Guindy, A., Lopez-Giraldez, F., Delecluse, H. J., McKenzie, J. & Miller, G. (2014).A Locus Encompassing the Epstein-Barr Virus bglf4 Kinase Regulates Expression of Genes Encoding Viral Structural Proteins. PLoS Pathog 10, e1004307.

Feederle, R., Bannert, H., Lips, H., Muller-Lantzsch, N. & Delecluse, H. J. (2009a).The Epstein-Barr Virus Alkaline Exonuclease BGLF5 Serves Pleiotropic Functions in Virus Replication. Journal of Virology 83, 4952-4962.

Feederle, R., Mehl-Lautscham, A. M., Bannert, H. & Delecluse, H. J. (2009b).The Epstein-Barr virus protein kinase BGLF4 and the exonuclease BGLF5 have opposite effects on the regulation of viral protein production. J Virol 83, 10877-10891.

Feng, P., Moses, A. & Fruh, K. (2013).Evasion of adaptive and innate immune response mechanisms by y-herpesviruses. Current Opinion in Virology 3, 285-295.

Gaglia, M. M., Covarrubias, S., Wong, W. & Glaunsinger, B. A. (2012).A common strategy for host RNA degradation by divergent viruses. Journal of Virology 86, 9527.

Gaudreault, E., Fiola, S., Olivier, M. & Gosselin, J. (2007).Epstein-Barr Virus Induces MCP-1 Secretion by Human Monocytes via TLR2. Journal of Virology 81, 8016-8024.

Glaunsinger, B. & Ganem, D. (2004).Lytic KSHV Infection Inhibits Host Gene Expression by Accelerating Global mRNA Turnover. Molecular Cell 13, 713-723.

Griffin, B. D., Verweij, M. C. & Wiertz, E. J. H. J. (2010).Herpesviruses and immunity: The art of evasion. Veterinary Microbiology 143, 89-100.

Horst, D., Geerdink, R. J., Gram, A. M., Stoppelenburg, A. J. & Ressing, M. E. (2012a).Hiding Lipid Presentation: Viral Interference with CD1d-Restricted Invariant Natural Killer T (iNKT) Cell Activation. Viruses 4, 2379-2399.

Horst, D., Burmeister, W. P., Boer, I. G. J., van Leeuwen, D., Buisson, M., Gorbalenya, A. E., Wiertz, E. J. H. J. & Ressing, M. E. (2012b).The Bridge in the Epstein-Barr Virus Alkaline Exonuclease Protein

BGLF5 Contributes to Shutoff Activity during Productive Infection. Journal of Virology 86, 9175-9187.

Horst, D., van Leeuwen, D., Croft, N. P., Garstka, M. A., Hislop, A. D., Kremmer, E., Rickinson, A. B., Wiertz, E. J. H. J. & Ressing, M. E. (2009).Specific Targeting of the EBV Lytic Phase Protein BNLF2a to the Transporter Associated with Antigen Processing Results in Impairment of HLA Class I-Restricted Antigen Presentation. The Journal of Immunology 182, 2313-2324.

Iwakiri, D., Zhou, L., Samanta, M., Matsumoto, M., Ebihara, T., Seya, T., Imai, S., Fujieda, M., Kawa, K. & other authors (2009).Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from toll-like receptor 3. The Journal of Experimental Medicine 206, 2091-2099.

Iwasaki, A. & Medzhitov, R. (2010).Regulation of Adaptive Immunity by the Innate Immune System. Science 327, 291-295.

Jones, T. R., Hanson, L. K., Sun, L., Slater, J. S., Stenberg, R. M. & Campbell, A. E. (1995).Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains. Journal of Virology 69, 4830-4841.

Kinjo, Y., Kitano, N. & Kronenberg, M. (2013).The role of invariant natural killer T cells in microbial immunity. J Infect Chemother 19, 560-570.

Koppers-Lalic, D., Rychlowski, M., van Leeuwen, D., Rijsewijk, F. A. M., Ressing, M. E., Neefjes, J. J., Bienkowska-Szewczyk, K. & Wiertz, E. J. H. J. (2003).Bovine herpesvirus 1 interferes with TAP-dependent peptide transport and intracellular trafficking of MHC class I molecules in human cells. Arch Virol 148, 2023-2037.

Kurt-Jones, E. A., Mandell, L., Whitney, C., Padgett, A., Gosselin, K., Newburger, P. E. & Finberg, R. W. (2002).Role of Toll-like receptor 2 (TLR2) in neutrophil activation: GM-CSF enhances TLR2 expression and TLR2-mediated interleukin 8 responses in neutrophils. Blood 100, 1860-1868.

Lebbink, R. J., Lowe, M., Chan, T., Khine, H., Wang, X. & McManus, M. T. (2011).Polymerase II Promoter Strength Determines Efficacy of microRNA Adapted shRNAs. PLoS One 6, e26213.

Murphy, J. A., Duerst, R. J., Smith, T. J. & Morrison, L. A. (2003).Herpes Simplex Virus Type 2 Virion Host Shutoff Protein Regulates Alpha/Beta Interferon but Not Adaptive Immune Responses during Primary Infection In Vivo. Journal of Virology 77, 9337-9345.

Ning, S. (2011).Innate immune modulation in EBV infection. Herpesviridae 2, 1.

Paddison, P. J., Cleary, M., Silva, J. M., Chang, K., Sheth, N., Sachidanandam, R. & Hannon, G. J. (2004). Cloning of short hairpin RNAs for gene knockdown in mammalian cells. Nat Meth 1, 163-167.

Paludan, S. R., Bowie, A. G., Horan, K. A. & Fitzgerald, K. A. (2013).Recognition of herpesviruses by the innate immune system. Nat Rev Immunol 13, 614.

Park, R., El-Guindy, A., Heston, L., Lin, S. F., Yu, K. P., Nagy, M., Borah, S., Delecluse, H. J., Steitz, J. & other authors (2014).Nuclear Translocation and Regulation of Intranuclear Distribution of Cytoplasmic Poly(A)-Binding Protein Are Distinct Processes Mediated by Two Epstein Barr Virus Proteins. PLoS One 9, e92593.

Pasieka, T. J., Lu, B., Crosby, S. D., Wylie, K. M., Morrison, L. A., Alexander, D. E., Menachery, V. D. & Leib, D. A. (2008).Herpes Simplex Virus Virion Host Shutoff Attenuates Establishment of the Antiviral

State. Journal of Virology 82, 5527-5535.

Quinn, L. L., Zuo, J., Abbott, R. J. M., Shannon-Lowe, C., Tierney, R. J., Hislop, A. D. & Rowe, M. (2014). Cooperation between Epstein-Barr Virus Immune Evasion Proteins Spreads Protection from $CD8^+$ T Cell Recognition across All Three Phases of the Lytic Cycle. PLoS Pathog 10, e1004322.

Ressing, M. E., Horst, D. l., Griffin, B. D., Tellam, J., Zuo, J., Khanna, R., Rowe, M. & Wiertz, E. J. H. J. (2008).Epstein-Barr virus evasion of CD8+ and CD4+ T cell immunity via concerted actions of multiple gene products. Seminars in Cancer Biology 18, 397-408.

Ressing, M. E., Keating, S. E., van Leeuwen, D., Koppers-Lalic, D., Pappworth, I. Y., Wiertz, E. J. H. J. & Rowe, M. (2005).Impaired Transporter Associated with Antigen Processing-Dependent Peptide Transport during Productive EBV Infection. The Journal of Immunology 174, 6829-6838.

Rickinson, A. B. & Kieff, E. (2007). Epstein-Barr Virus. In Field's Virology, 5 edn, pp. 2655-2700. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Rowe, M., Glaunsinger, B., van, L. D., Zuo, J., Sweetman, D., Ganem, D., Middeldorp, J., Wiertz, E. J. & Ressing, M. E. (2007).Host shutoff during productive Epstein-Barr virus infection is mediated by BGLF5 and may contribute to immune evasion. Proc Natl Acad Sci U S A 104, 3366-3371.

Sheridan, V., Polychronopoulos, L., Dutia, B. M. & Ebrahimi, B. (2014).A Shutoff and Exonuclease Mutant of Murine Gammaherpesvirus-68 Yields Infectious Virus and Causes RNA loss in Type I Interferon Receptor Knock-Out cells. Journal of General Virology 95, 1135-1143.

van Gent, M., Griffin, B. D., Berkhoff, E. G., van, L. D., Boer, I. G., Buisson, M., Hartgers, F. C., Burmeister, W. P., Wiertz, E. J. & other authors (2011).EBV lytic-phase protein BGLF5 contributes to TLR9 downregulation during productive infection. J Immunol 186, 1694-1702.

van Gent, M., Braem, S. G. E., de Jong, A., Delagic, N., Peeters, J. G. C., Boer, I. G. J., Moynagh, P. N., Kremmer, E., Wiertz, E. J. & other authors (2014).Epstein-Barr Virus Large Tegument Protein BPLF1 Contributes to Innate Immune Evasion through Interference with Toll-Like Receptor Signaling. PLoS Pathog 10, e1003960.

York, I. A., Roop, C., Andrews, D. W., Riddell, S. R., Graham, F. L. & Johnson, D. C. (1994).A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell 77, 525-535.

Zuo, J., Currin, A., Griffin, B. D., Shannon-Lowe, C., Thomas, W. A., Ressing, M. E., Wiertz, E. J. H. J. & Rowe, M. (2009).The Epstein-Barr Virus G-Protein-Coupled Receptor Contributes to Immune Evasion by Targeting MHC Class I Molecules for Degradation. PLoS Pathog 5, e1000255.

Zuo, J., Thomas, W., van Leeuwen, D., Middeldorp, J. M., Wiertz, E. J. H. J., Ressing, M. E. & Rowe, M. (2008).The DNase of Gammaherpesviruses Impairs Recognition by Virus-Specific CD8+ T Cells through an Additional Host Shutoff Function. Journal of Virology 82, 2385-2393.

Chapter 3

The Epstein-Barr Virus glycoprotein gp150 forms an immune-evasive glycan shield at the surface of infected cells

Anna M. Gram[1,2], Timo Oosenbrug[1], Marthe F.S. Lindenbergh[2], Christian Büll[3], Anouskha Comvalius[1], Kathryn J.I. Dickson[4], Joop Wiegant[1], Hans Vrolijk[1], Robert Jan Lebbink[2], Ron Wolterbeek[5], Gosse J. Adema[3], Marieke Griffioen[6], Mirjam H.M. Heemskerk[6], David C. Tscharke[4], Lindsey M. Hutt-Fletcher[7], Emmanuel J.H.J. Wiertz[2], Rob C. Hoeben[1], Maaike E. Ressing[1]

[1] Department of Molecular Cell Biology, Leiden University Medical Center, The Netherlands; [2] Department Medical Microbiology, University Medical Center Utrecht, The Netherlands; [3] Department Tumor Immunology, Radboud Institute for Molecular Life Sciences, Nijmegen, The Netherlands; [4] John Curtin School of Medical Research, Australian National University, Canberra, Australia; [5] Department of Medical Statistics & Bioinformatics, Leiden University Medical Center, The Netherlands; [6] Department of Hematology, Leiden University Medical Center, The Netherlands; [7] Department Microbiology and Immunology, Louisiana State University Health Sciences Center, Shreveport, USA

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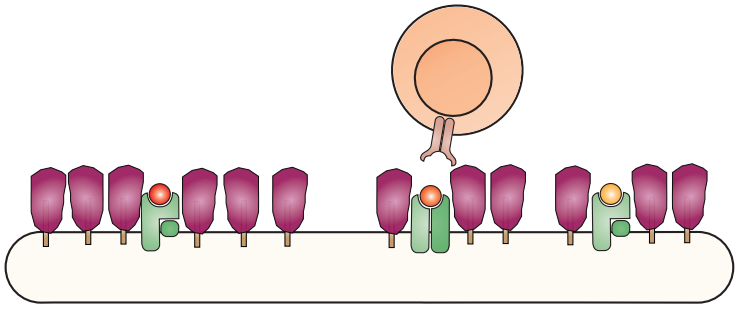


Abstract

Cell-mediated immunity plays a key role in host control of viral infection. This is exemplified by life-threatening reactivations of e.g. herpesviruses in individuals with impaired T- cell and/or iNKT cell responses. To allow lifelong persistence and virus production in the face of primed immunity, herpesviruses exploit immune evasion strategies. These include a reduction in viral antigen expression during latency and a number of escape mechanisms that target antigen presentation pathways. Given the plethora of foreign antigens expressed in virus-producing cells, herpesviruses are conceivably most vulnerable to elimination by cell-mediated immunity during the replicative phase of infection.

Here, we show that a prototypic herpesvirus, Epstein-Barr virus (EBV), encodes a novel, broadly acting immunoevasin, gp150, that is expressed during the late phase of viral replication. In particular, EBV gp150 inhibits antigen presentation by HLA class I, HLA class II, and the non-classical, lipid-presenting CD1d molecules. The mechanism of gp150-mediated T-cell escape does not depend on degradation of the antigen-presenting molecules nor does it require gp150's cytoplasmic tail. Through its abundant glycosylation, gp150 creates a shield that impedes surface presentation of antigen. This is an unprecedented immune evasion mechanism for herpesviruses. In view of its likely broader target range, gp150 could additionally have an impact beyond escape of T cell activation.

Importantly, B cells infected with a gp150-null mutant EBV displayed rescued levels of surface antigen presentation by HLA class I, HLA class II, and CD1d, supporting an important role for iNKT cells next to classical T cells in fighting EBV infection. At the same time, our results indicate that EBV gp150 prolongs the timespan for producing viral offspring at the most vulnerable stage of the viral life cycle.

Author summary

The human herpesvirus Epstein-Barr virus (EBV) is an important human pathogen involved in infectious mononucleosis and several malignant tumors, including lymphomas in the immunosuppressed. Upon primary infection, a balance between virus and host is established, to which EBV's capacity to dodge T cell-mediated attack contributes.

Here we identify the late protein EBV gp150 as a novel immunoevasin, frustrating antigen presentation by HLA class I, class II, and CD1d molecules. EBV gp150's many sialoglycans create a shield impeding surface detection of presented antigen. Interestingly, exploiting glycan shielding as a mechanism to mask surface exposed proteins on infected cells could permit EBV to additionally modulate other aspects of host antiviral defense. B cells producing wild-type EBV escaped immune recognition more efficiently than those infected with a gp150-null virus, pointing towards a role for gp150 in natural infection. Our results reveal a novel, broadly active strategy by which a herpesvirus glycoprotein, EBV gp150, blocks antigen presentation to T cells through glycan shielding, a new paradigm in herpesvirus immune evasion.

Introduction

Viruses are exceptionally well equipped to adjust processes in infected host cells to support their own replication and survival. Especially in persistent infections, they must withstand many layers of anti-viral activities exerted by the host immune system. Cell-mediated immunity, in particular that mediated by antigen (Ag)-specific T cells, is essential for elimination of virus-infected cells, reducing viral replication, and stimulating other immune effector functions.

$CD8^+$ and $CD4^+$ T cells are activated by peptide Ags presented at the cell surface in the context of HLA class I and class II (HLA I and II) molecules, respectively. In contrast, invariant natural killer T (iNKT) cells, a subset of specialized T cells characterized by a semi-invariant T cell receptor (TCR) in combination with NK cell receptors, recognize lipid Ags presented by CD1d, a non-classical, non-polymorphic HLA molecule. Upon activation, iNKT cells secrete a vast array of polarizing cytokines and they can also directly exert cytotoxicity [1]. In addition to the anti-viral cytotoxic and helper T cells, a role for iNKT cells in providing protection against viral infection has more recently been appreciated [2].

Herpesviruses are widespread viruses that establish lifelong persistent infections in their host, even in the face of virus-specific immunity [3]. Within their large (125-230 kb) DNA genomes, herpesviruses encode functions essential for viral replication, yet many additional gene products are non-essential for propagation *in vitro*. Evidence is accumulating that the latter are required for establishment of the delicate balance between viral replication and host responses *in vivo*. Among these gene products are immune evasion molecules that frustrate the activation of cell-mediated immunity by thwarting Ag presentation. The fact that such gene products have been independently acquired and maintained by multiple members of the herpesvirus family throughout millions of years of coevolution with their host testifies to the importance of T cell activation in the control of herpesvirus infection [4].

In humans, the in vivo contribution of cell-mediated immunity to fighting viral infection is probably best demonstrated for Epstein-Barr virus (EBV). This γ-herpesvirus was the first human tumor virus discovered [5], being associated with malignancies such as Burkitt's lymphoma and nasopharyngeal carcinoma. EBV is carried by more than 90% of adults worldwide, mostly asymptomatically. When primary EBV infection occurs during adolescence or adulthood, it presents in more than 50% of cases as self-limiting infectious mononucleosis [6], in which viral replication is eventually controlled by the EBV-specific immune response. This contrasts to patients with the primary immunodeficiency X-linked lymphoproliferative syndrome (XLP) who develop an often fatal mononucleosis when infected by EBV [6]. XLP patients have a genetic defect leading to nonfunctional T and NK cells and the absence of iNKT cells [7,8]. In addition, EBV can cause life-threatening lymphomas when cell-mediated

immunity is insufficient, for instance in transplantation or AIDS patients. Reconstitution of EBV-specific T cell immunity by adoptive transfer provides a cure for post-transplantation lymphoproliferative disease (PTLD) [9]. PTLD and XLP thus occur in two distinct patient groups having in common that they lack sufficient cell-mediated immunity to control EBV infection.

Like other herpesviruses, EBV has acquired ingenious strategies to escape elimination by cell-mediated immunity of the immunocompetent infected host. Among these, EBV downregulates its Ag expression in the latent stage of infection and exploits (viral) immune evasion gene products, in particular during its replicative phase, to interfere with immune activation [10]. In the early lytic phase, Ag presentation by HLA I is reduced by the concerted action of (at least) three EBV proteins: BNLF2a blocks antigenic peptide supply [11], BILF1 diverts HLA I complexes away from the cell surface [12], and the shutoff protein BGLF5 acts as an RNase that arrests synthesis of, for instance, HLA I, II, and CD1d [10]. Silencing expression of these early EBV proteins in B cells supporting productive infection yielded only partially rescued surface display of the Ag-presenting molecules [13,14]. This led to the hypothesis that EBV encodes additional immune evasion molecules targeting CD1d, HLA I, and HLA II, but their identity has remained elusive. In this study, we took a global look at Ag presentation by EBV-producing B cells with a focus on the late phase of infection, when most Ags are expressed that could activate T and iNKT cell immunity.

We identified the heavily glycosylated EBV protein gp150, encoded by the *BDLF3* gene, as a new viral immune evasion molecule. *BDLF3* is unique to EBV and the closely related Rhesus lymphocryptovirus (LCV). EBV gp150 interferes with immune recognition of HLA I, II, and CD1d/ Ag complexes at the cell surface. We provide evidence that gp150 shields the recognition of these surface molecules in a glycan-dependent manner. This represents a novel immune evasion mechanism in herpesviruses.

Results

Identification of EBV *BDLF3*-encoded gp150 as a novel immune evasion molecule

To study alterations in surface display of Ag-presenting molecules in the course of productive EBV infection of B cells, we employed our previously described EBV$^+$ AKBM Burkitt's lymphoma system [15]. Following anti-human IgG treatment, cells supporting replication of EBV are identified in flow cytometry by expression of a lytic cycle reporter, rat CD2-GFP, and/or EBV lytic proteins, such as the gHgL complex (**Figure 1A**, upper row). Surface display of the Ag-presenting molecules HLA I, II, and the non-classical HLA molecule CD1d is markedly reduced on EBV-producing cells in comparison to latently infected control

cells (**Figure 1A**, left panels), in line with our earlier reports [13,15]. Downregulation of surface HLA I complexes commenced around 8-10 hours post induction of the lytic cycle [15], preceding that of HLA II and CD1d. Surface levels of the latter were progressively reduced on EBV-producing cells with a delay of about 4-6 hours compared to HLA I (**S1A Figure** and [15]). To distinguish the contributions of late versus (immediate-)early EBV proteins to the downregulation of surface Ag-presenting molecules, phosphonoacetic acid (PAA) was used to arrest viral replication at the early phase. Upon anti-human IgG treatment of AKBM cells in the presence of PAA, late EBV protein expression of, for instance, gHgL complexes was inhibited, while the early rat CD2GFP reporter was still expressed (**Figure 1A**, upper row). At the surface of PAA-treated cells, CD1d and HLA II levels were mostly retained (**Figure 1A**, right panels), pointing towards involvement of late EBV protein(s) in T and iNKT cell immune evasion. HLA I downregulation was partly rescued upon PAA treatment indicating that, next to the early EBV proteins previously identified, also viral late proteins play a role in reducing the surface levels of HLA I (**Figure 1A**, right panels). In conclusion, one or several of the 30 late EBV proteins interfere with surface display of the CD1d, HLA I, and HLA II Ag-presenting molecules.

To identify the late EBV gene product(s) responsible for interference with Ag presentation observed during productive infection, we took the approach of expressing individual, or combinations of, late viral genes in 293T-CD1d cells through plasmid transfection (**Figures 1B and 1C**) and/or in MJS-CD1d cells via lentiviral transduction (**Figures 1D and S1**). Screening of late EBV genes in 293T-CD1d cells revealed that most glycoproteins did not affect surface levels of CD1d (**Figure 1B**), HLA I, or the control protein transferrin receptor (TfR) to a major extent (**S1B Figure**). Also expression of EBV gB did not target CD1d, even though its homologue in herpes simplex virus type 1 (HSV-1) was found to act in concert with the viral kinase US3 to mediate intracellular retention of CD1d molecules observed during HSV-1 infection [16]. EBV gp350 caused some downregulation of CD1d, yet the strongest effect was observed for gp150, encoded by the *BDLF3* gene (**Figure 1B**). Expression of EBV gp150 at levels comparable to those observed in virus-producing B cells (**Figure 1C,** left panels) resulted in marked downregulation of both Ag-presenting molecules tested (upper panels), which was not evident in control transfected cells. The dot plots show an apparent threshold to gp150 levels resulting in loss of HLA I and CD1d detection. These combined data demonstrate that expression of the *BDLF3*-encoded EBV gp150 reduces the detection of HLA I and CD1d at the cell surface.

Finally, we determined whether the timing of gp150 expression during productive EBV infection of human B cells would match the observed downregulation of Ag-presenting molecules. To visualize total gp150 protein levels in the course of EBV reactivation in B cells, we used an antibody (Ab) directed against its intracellular cytoplasmic tail [19]. Whereas the early EBV protein BGLF5 was detectable from 4 hours post induction [15] onwards, no gp150 protein was found before 8-10 hours into lytic infection with clear detection from 12 hours onwards (**Figure 1D**), leaving enough time to influence HLA I, II, and CD1d expression at

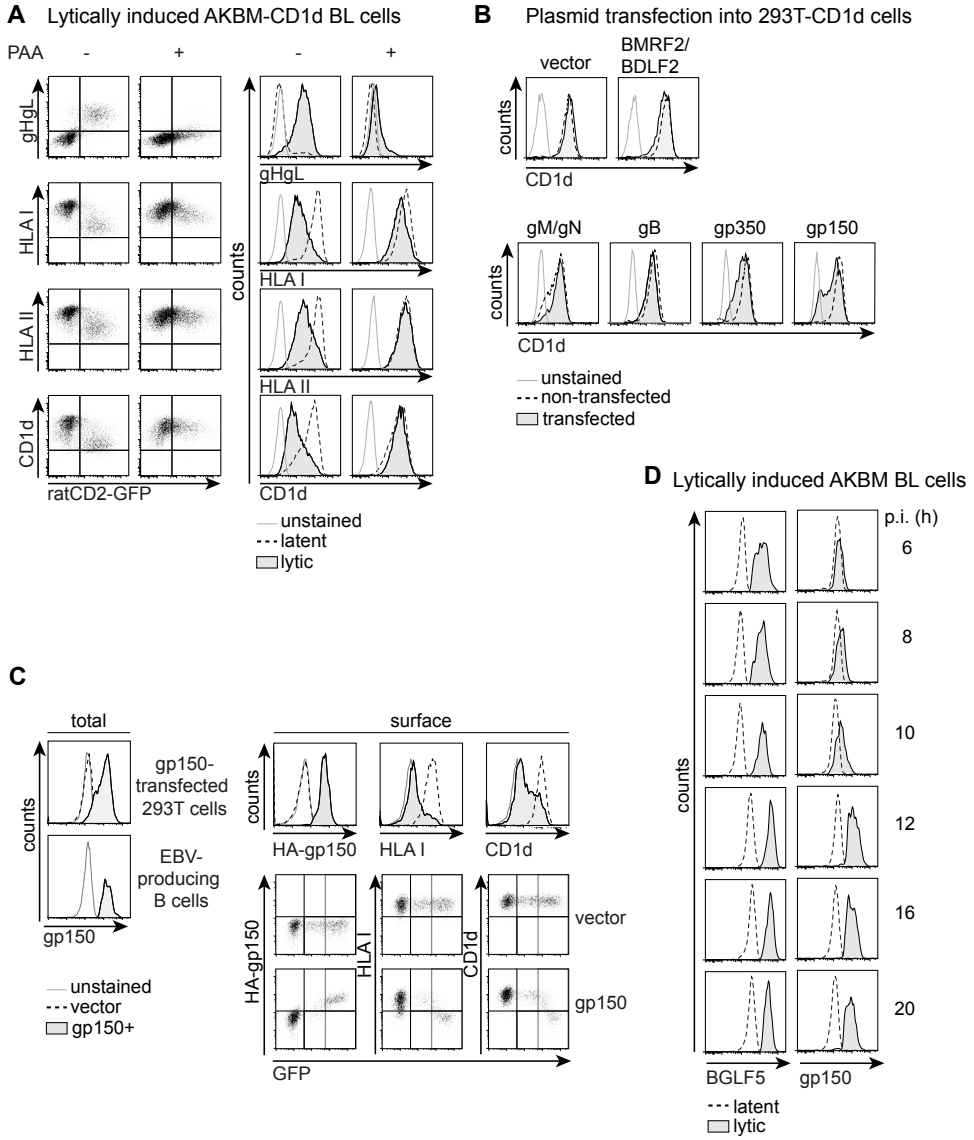


Figure 1 gp150 interferes with surface display of Ag-presenting molecules during productive EBV infection. A) EBV$^+$ AKBM-CD1d BL cells were treated for 20 hours with anti-human IgG Ab to induce viral replication. EBV-producing cells were identified by induced expression of the lytic cycle reporter rat CD2-GFP. In the presence of PAA, productive infection is arrested before late protein expression. Surface levels of the EBV late complex gHgL and of the Ag-presenting molecules HLA I, II, and CD1d were determined by flow cytometry. Histograms depict overlays to allow comparison of latently (GFP$^-$) and lytically (GFP$^+$) infected B cells. B) 293T-CD1d cells were transfected with expression vectors encoding late EBV glycoproteins. Glycoproteins known to require a viral interaction

partner were transfected together (BMRF2/BDLF2 and gM/gN). EBV protein expression was deduced from coexpression of GFP (BMRF2/BDLF2) or on the basis of a C-terminal tag (gM/gN, gB, gp350, gp150). Cell surface CD1d was stained prior to an intracellular staining for the tagged EBV proteins. Surface levels were compared between non-transfected and transfected cells. C) 293T-CD1d cells were transfected with N-terminally HA-tagged gp150 or an empty IRES-GFP vector control. Total gp150 expression was determined by intracellular staining and was compared to levels in EBV-producing B cells. The other histograms depict surface levels of HA (HA-gp150), HLA I, and CD1d staining for empty vector-transfected and $gp150^{+}GFP^{high}$ 293T-CD1d cells; GFP^{high} gating as indicated in the dot plots. D) Expression of gp150 was assessed at different time points during productive EBV infection of B cells; the early EBV antigen BGLF5 was taken along as a control (time points 6-10 hours and 12-20 hours are from different experiments).

late times. These combined data point to gp150 interfering with surface detection of Ag-presenting molecules on EBV-producing B cells. Since no function had hitherto been ascribed to the EBV glycoprotein gp150, its role in immune evasion of Ag-presenting molecules was further investigated.

EBV gp150 causes expression level-dependent interference with different cell surface molecules

To study gp150's phenotype and mechanism of action in more detail, we expressed the EBV protein in MJS-CD1d cells via lentiviral transduction, together with GFP as a marker for successful transduction. This approach was validated with known EBV evasion molecules [11,17,18] that specifically reduced surface display of HLA I (BNLF2a and BILF1) or HLA II (gp42gHgL). At the same time, this experiment showed that the known EBV T cell immunoevasins did not interfere with CD1d expression (**S2A Figure**).

HLA I and CD1d downregulation from the GFP^{+} MJS-CD1d-gp150 cells (Figs 2A and S2B) indicated that this immune evasive phenotype was not limited to transient overexpression in 293T cells (**Figure 1**). Furthermore, gp150 downregulated surface levels of HLA II and also affected other molecules such as CD10, CD54 (**Figure 2A**), and TfR (**S2B Figure**). To allow easier comparison of gp150's effects on various cell surface markers, we calculated the difference in logarithmically transformed mean fluorescence intensities (ΔlogMFI) for each surface marker to serve as a measure for the extent of gp150-mediated downregulation, by subtracting the logMFI values for cells that were non- (GFP^{-}) or successfully (GFP^{+}) transduced with either gp150-IRES-GFP or a control GFP lentivirus (**Figure 2B** right panel; the left panels show examples of the logMFI for CD1d and CD54). This approach visualized that the antigen-presenting molecules HLA I, II and CD1d were more sensitive to EBV gp150 downregulation than the CD10 and CD54 molecules (**Figure 2B**).

To investigate if gp150's effects reflect level-dependency, we transduced MJS-CD1d cells with a range of lentivirus amounts to achieve decreasing levels of gp150 expression. This resulted in a concomitant reduction in surface downregulation of the cellular proteins tested (**S2C Figure**). When using the higher dose of gp150 lentiviruses in the adherent MJS-CD1d cells, we observed that a proportion of cells detached from the culture dish by 3 days after

transduction. This detachment did not only occur as a consequence of lentiviral transduction or of gross gp150-induced cytotoxicity (see **S3A Figure**). Loss of attachment of the gp150^{high} GFP^{high} cells coincided with a further reduced surface detection of HLA I, II, and CD1d (compare Figs 2A and S3B). Very high levels of gp150 even downregulated the cell surface molecules CD10 and CD54, which were only marginally affected in the adherent cell fraction (Figs 2A and S3B). These results - together with the apparent threshold for gp150-induced downregulation (dot plots in Figs 1C and 2A) and the dose-dependent effect of gp150 expression (**S2C Figure**) - demonstrate that higher levels of gp150 cause a stronger phenotype.

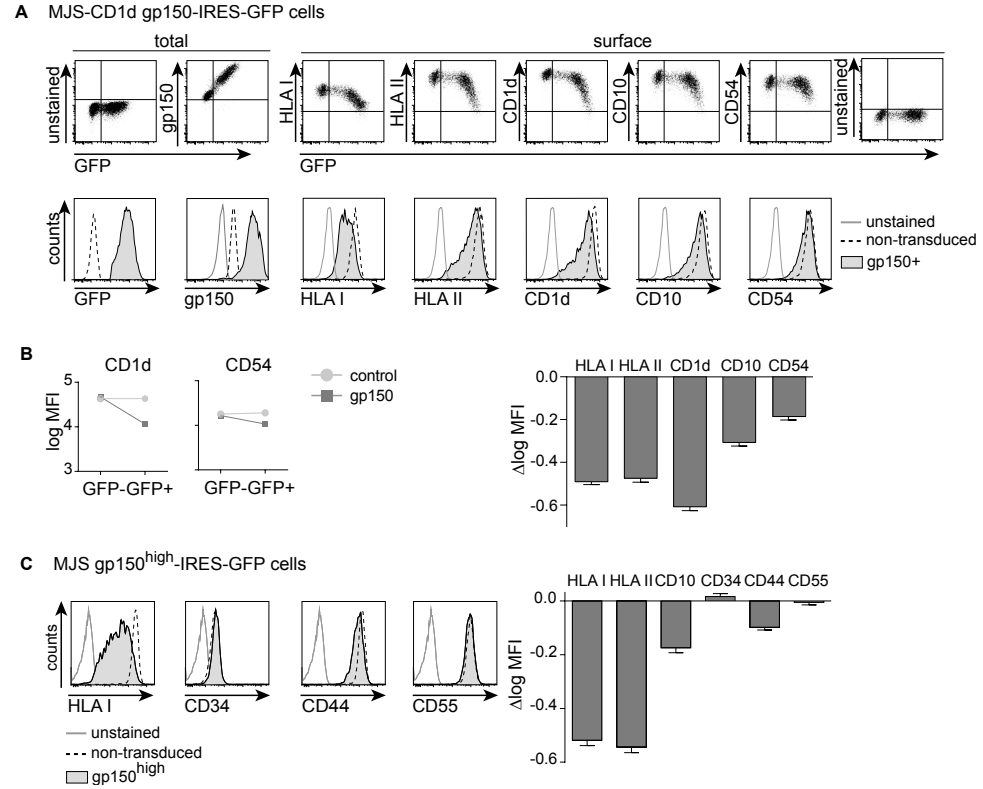


Figure 2 EBV gp150 is broadly acting, but displays a degree of specificity for antigen-presenting molecules. Flow cytometric analyses of MJS-CD1d cells (adherent fraction) three days post transduction with gp150-IRES-GFP lentiviruses. A) Total EBV gp150 levels were determined by intracellular staining of permeabilized cells. Surface levels of HLA I, II, CD1d, CD10, and CD54 were determined using Ab staining on non-permeabilized cells. B) Surface levels of CD1d and CD54 are depicted as log MFI values with 95% confidence intervals, for GFP$^+$ versus GFP$^-$ MJS-CD1d cells transduced with gp150 or a GFP control. The slopes of the connecting lines reflect the declines in fluorescence (Δlog MFI) and, thus, the downregulation of HLA I, II, CD1d, CD10, and CD54 induced by gp150 (for the GFP control, the Δlog MFI did not significantly differ from 0). C) Cell surface levels of HLA I, II, CD10, CD34, CD44, and CD54 were determined using surface Ab staining. EBV gp150-induced downregulation of these molecules was calculated as in B) and represented as Δlog MFI.

Thus, EBV gp150 acts in an expression level-dependent manner to downregulate multiple cell surface molecules, to different extents.

Finally, to obtain an impression of the target range of gp150, we looked for surface molecules on MJS-CD1d cells that were not downregulated by the EBV protein. We found that virtually equal levels of CD34, CD44, and CD55 were detected on cells that did or did not express gp150, even when gated on the GFP^{high} fractions of cells (**Figure 2C**). From these data we conclude that various cell surface molecules display differential sensitivity to EBV gp150, with CD34, CD44, and CD55 as examples of proteins that are resistant (**Figure 2**). Since gp150 has a strong effect on the Ag-presenting molecules HLA I, II, and CD1d, this EBV glycoprotein appears to be a broadly acting immune evasion molecule.

EBV gp150-induced downregulation of Ag-presenting molecules impairs T cell activation

Next, we intended to determine the functional consequences of EBV gp150-induced immune evasion. Reduced surface HLA I, II, and CD1d levels were detected with Abs that are capable of blocking many TCR – HLA/Ag interactions (Figs. 1 and 2), pointing towards functional relevance of gp150's blocking activity.

To assess if EBV gp150 expression in Ag-presenting cells affects T cells activation, the capacity of $HLA\text{-}A2^{+}DR3^{+}$ MJS-A2 cells to induce interferon (IFN)-γ production was monitored with well-defined T cell clones with different specificities. Activation of $CD8^{+}$ T cells specific for a human cytomegalovirus (HCMV) pp65 epitope presented in the context of HLA-A2 was analysed after stimulation with pp65-expressing MJS-A2 cells that did or did not co-express EBV gp150 (**Figure 3A**). Whereas GFP^{+} control cells efficiently activated the T cells, IFN-γ production was markedly reduced in T cells cultured with gp150-expressing Ag-presenting cells (**Figure 3A**). The use of another T cell clone, a $CD4^{+}$ T cell directed against a minor histocompatibility Ag (MiHa) endogenously expressed by MJS cells and presented in the context of HLA-DR3, allowed us to study inhibition of T cell responses restricted by HLA II. Diminished secretion of IFN-γ by the T-helper cells was observed in response to cells expressing gp150 compared to control cells (**Figure 3B**). These results show that not only detection of Ag-presenting molecules by Ab was blocked by gp150, but also T cell activation was hampered.

T cell activation upon target cell engagement involves formation of multiple receptor-ligand pairs, including TCR – HLA/Ag as well as costimulatory and adhesion molecule interactions. In view of the broader target range of EBV gp150, it could diminish multiple of these interactions. Here, we zoomed in on the effect of gp150 on MHC/peptide – TCR interactions. To this end, human 293T cells were used that express the mouse MHC molecule $H\text{-}2K^{b}$ to present the ovalbumin-derived SIINFEKL epitope. Display of SIINFEKL-loaded K^{b} molecules at the cell surface was detected with the 25D1.16 Ab, thereby recapitulating TCR binding (**Figure 3C**). Upon introduction of EBV gp150 into these cells, a significant reduction in surface levels of epitope-loaded K^{b} molecules was observed (Figs. 3C and 3D).

This further supports the conclusion that T cell activation is diminished upon gp150-induced downregulation of surface Ag-presenting complexes.

Our combined results indicate that EBV gp150 impairs Ag presentation to both HLA I- and II-restricted T cells.

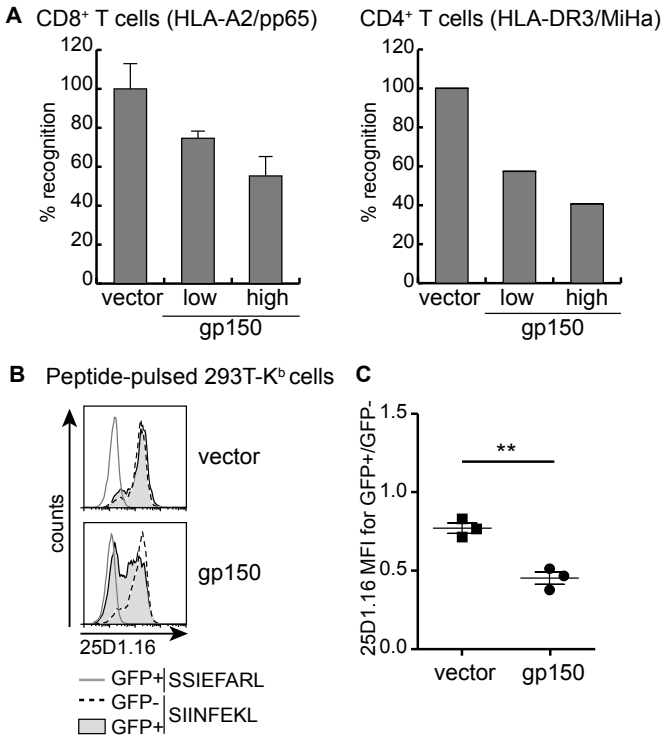


Figure 3 Cellular expression of EBV gp150 limits CD8+ and CD4+ T cell activation by disrupting Ag presentation. A,B) MJS-A2-pp65 cells were lentivirally transduced to express gp150 or control GFP (vector). Ag-presenting cells expressing low or high levels of gp150 protein were obtained by harvesting the floating cells and by FACS-sorting the adherent GFP+ fraction into GFPint and GFPhigh cells. Following overnight co-culture of these cells with either A2/pp65-specific CD8+ (A) or DR3/MiHa-specific CD4+ T cells (B), IFN-γ secretion into the culture supernatants was determined by ELISA. Relative T cell recognition is depicted with IFN-γ levels produced in response to stimulation with vector control cells set at 100%. C) 293T-Kb cells were transfected with an empty vector control or gp150. Cells were pulsed with SIINFEKL or a control peptide (SSIEFARL) and stained with Ab 25D1.16, which is specific for Kb/ SIINFEKL complexes. D) Statistical analysis of three independent experiments (performed as in C)) using the Student's t-test (two-tailed).

EBV gp150 does not induce the degradation of Ag-presenting molecules

Having demonstrated the functional impact of gp150-induced T cell evasion, we wanted to unravel its mechanism of action. In the remainder of these studies, we focused on the adherent fraction of gp150-expressing cells, although the effects might even be stronger for floating cells. In addition, we made use of constructs that encode gp150 with an N-terminal HA-tag (**Figure 4A**) to allow visualisation of the proportion of this EBV molecule expressed at the cell surface.

First, we investigated if reduced surface display of Ag-presenting molecules was due to gp150-induced protein degradation. Degradation of HLA molecules either by proteasomes residing in the cytoplasm or by endolysosomal proteases is an evasion strategy exploited by a number of herpesviruses, for instance by HCMV and by Kaposi's sarcoma herpesvirus [4]. For EBV gp150-induced effects, however, no difference in surface levels was observed for HLA I or CD1d in the presence either of the proteasome inhibitor epoxomicin or of chloroquine, an inhibitor of the endolysosomal degradation pathway (**Figure 4B**). Of note, HA-gp150 molecules arrived at the cell surface, irrespective of treatment with these inhibitors. Additionally, intracellular staining with the D5 Ab, which detects free CD1d heavy chains (not associated with β_2m) [20], revealed no reduction in this intracellular population of CD1d molecules in the presence of gp150 (**Figure 4B**, right panels), suggesting that gp150 does not induce degradation of newly synthesized CD1d molecules.

In an alternative approach, we compared gp150's effect on surface display directly to that on total cellular levels of HLA I in the form of an N-terminally eGFP-myc-tagged HLA-A2 molecule [21], which was introduced into the HLA-A2$^-$ cell line MJS (MJS-GFPmycA2 cells). In the non-permeabilized cells, GFP fluorescence reflected total levels of this fusion protein and, at the same time, surface levels were visualized with Abs directed to the extracellular myc-tag or HLA-A2 epitope (BB7.2). High levels of BB7.2 and anti-myc Ab staining as well as GFP fluorescence were detected in MJS-GFPmycA2 cells by flow cytometry (**Figure 4C,** non-transduced cells), indicating that the HLA-A2 fusion protein was properly expressed and reached the cell surface. Upon subsequent introduction of HA-gp150, we observed a strong reduction in surface detection of the chimeric HLA-A2 molecule, whereas total GFP levels remained unaffected (**Figure 4C**, gp150$^+$ cells). These data implicate that in gp150-expressing cells total levels of HLA molecules remain unaltered, yet their surface detection is reduced.

In the same experimental set-up, we studied which part(s) of gp150 are essential for immune evasion. EBV gp150 is a type I membrane protein with a short cytoplasmic tail (**Figure 4A**). Some herpesvirus proteins, such as EBV BILF1, depend on their cytoplasmic tail for interference with Ag presentation [22]. As deletion of 26 amino acids from the C-terminus of gp150 (HA-gp150ΔC) did not impair the apparent downregulation of HLA I (endogenous and A2-GFP; **Figure 4C**, lower panels) or of CD1d from the cell surface, we conclude that the immune evasion function of gp150 is independent of its cytoplasmic tail.

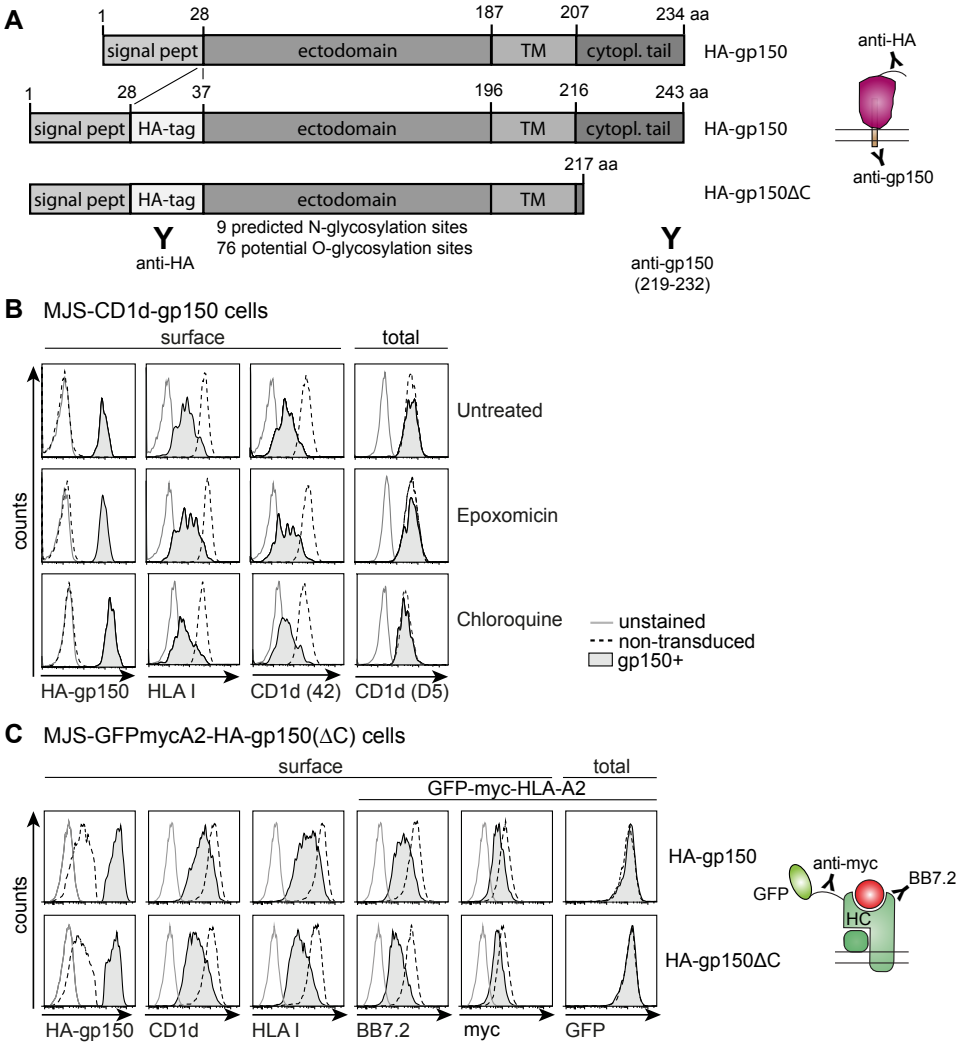


Figure 4 Immune evasion by EBV gp150 does not depend on degradation of Ag-presenting molecules nor on gp150's cytoplasmic tail. A) Schematic overview of the gp150 constructs and Abs used. Signal peptide, transmembrane domain, and cytoplasmic tail are abbreviated as signal pept, TM, and cytpl tail, respectively. B) Three days after lentiviral transduction, MJS-CD1d-gp150 cells were cultured overnight in the presence of an inhibitor of proteasomes (15nM epoxomicin) or of endolysosomal proteolysis (25μM chloroquine). Subsequently, flow cytometry was used to determine surface levels of HA-tagged gp150 (HA-gp150; detected with anti-HA Ab), HLA I, and mature CD1d (Ab #42) complexes on non-permeabilized cells and intracellular staining was performed to detect immature CD1d (Ab D5) molecules in permeabilized cells. C) MJS-GFPmycA2 cells were transduced with a lentivirus encoding HA-gp150 (upper) or HA-gp150ΔC (lower). Cell surface levels of CD1d, HLA I, and HLA-A2 (BB7.2 and anti-myc tag) and total HLA-A2 (GFP) levels were compared for $gp150^-$ and $gp150^+$ cells.

The combined data obtained upon protease inhibitor treatment and with the GFPmycA2 fusion protein demonstrate that gp150 does not promote degradation of Ag-presenting molecules as an immune evasive strategy.

EBV gp150 prevents detection of Ag-presenting molecules at the cell surface

Although the Ag-presenting molecules were not degraded in gp150^{+} cells, they were not detectable at the cell surface either. Therefore, we probed the intracellular location of these molecules both in gp150-expressing cells and controls.

As trafficking of newly synthesized glycoproteins from the ER onwards is reflected by the progressive maturation of their glycans, we compared the migration of the CD1d and HLA I heavy chains in Western blots using lysates of sorted populations of control and gp150-transduced cells (non-transduced, vector, and gp150$^{low/high}$, respectively; see **S4 Figure**). Differentially glycosylated maturation forms of these Ag-presenting molecules were identified upon glycosidase treatment. Endoglycosidase H (Endo H) removes high-mannose N-linked glycans, but not complex glycans that arise after the transport of glycoproteins beyond the *cis*-Golgi compartment. Peptide N-glycosidase F (PNGase F) treatment reveals the deglycosylated protein backbone, as it removes all N-linked glycans regardless of their maturation. Mature CD1d heavy chains carry 4 N-linked glycans, three of which become Endo H-resistant and one of which remains Endo H-sensitive [23]. In untreated control cells, two CD1d bands were detected that probably reflect different glycosylation stages of CD1d (**Figure 5A**,+CHO, lanes 1 and 4). PNGase F treatment resulted in one dominant band and a very faint band, reflecting virtually complete deglycosylation of CD1d heavy chains (-CHO, lanes 3 and 6). Endo H treatment revealed the deglycosylated CD1d heavy chain (-CHO, lanes 2 and 5); the two additional bands represent Endo H-resistant heavy chains, showing that these CD1d molecules have travelled beyond the *cis*-Golgi.

The HLA I heavy chain carries a single N-linked glycan and only one band is observed in untreated control cell lysates (lanes 1 and 3), which is removed upon digestion with both PNGase F (lanes 3 and 6) and Endo H (lanes 2 and 5). Only a minor fraction of the HLA I molecules apparently becomes Endo H-resistant in these MJS cells.

The pattern of maturation forms of CD1d and HLA I in gp150^{+} cells was virtually identical to that observed in control cells (compare lanes 1-6 to 7-12). If anything, the mature, endo H-resistant heavy chains were more prominent in gp150^{+} cells. From these data, we conclude that the ER-to-Golgi trafficking of new HLA I and CD1d molecules is not inhibited in the presence of EBV gp150. Furthermore, the presence of the heavily glycosylated viral protein in the ER does not appear to act as a "glycosylation sink" preventing the exit of newly synthesized Ag-presenting molecules.

Confocal microscopy allows the visualisation of Ag-presenting molecules within the cell as well as their potential colocalization with EBV gp150 [22]. For these experiments, we employed non-permeabilized, non-fixed MJS cells expressing a C-terminal GFP fusion

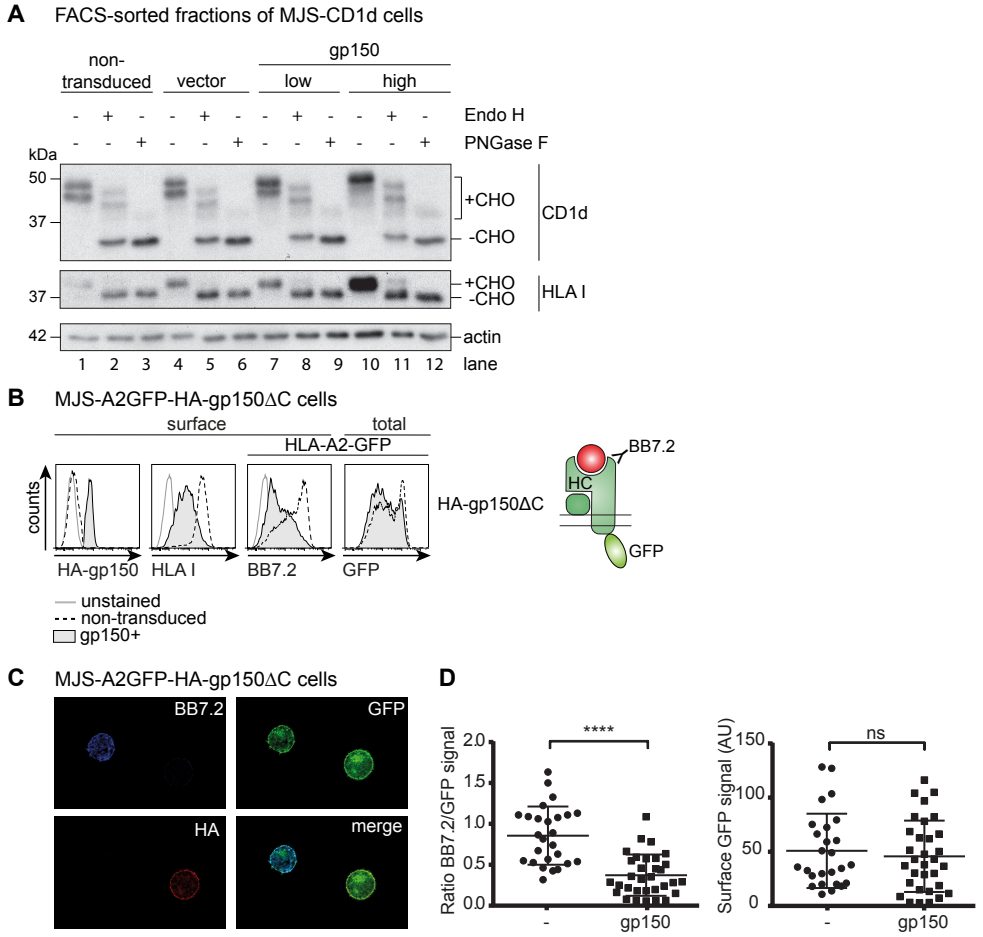


Figure 5 Immune evasion by EBV gp150 occurs at the cell surface. A) The glycosylation status of CD1d and HLA I molecules in lysates of MJS-CD1d-gp150 and control cells was analysed by Western blot. To this end, denatured post-nuclear lysates of different FACS sorted cell populations (see **S4 Figure**) were treated with Endo H or PNGase F to remove N-linked glycans. Endo H digestions served to examine protein transport beyond the *cis*-Golgi compartment; PNGase F digestions revealed the deglycosylated protein backbone (minus carbohydrates, -CHO). Actin was a loading control. B) Using the same experimental setup as in **Figure 4C**, MJS-A2-GFP cells were transduced with a lentivirus encoding HA-gp150ΔC. Cell surface levels of CD1d, HLA I, and HLA-A2 (BB7.2) and total levels of HLA-A2 (GFP) were compared for (non-permeabilized) $gp150^-$ and $gp150^+$ cells. C) Confocal microscopy of non-permeabilized MJS-A2GFP-HA-gp150ΔC cells. Cell surface stains were performed for HLA-A2 (BB7.2) and gp150ΔC (HA). D) Quantification of surface expression of the indicated molecules based on multiple microscopy pictures.

molecule of HLA-A2 (A2GFP), which had been used before in microscopy studies on HLA I maturation and trafficking [24]. Flow cytometry analysis confirmed that this HLA-A2 fusion protein behaved similarly to the other Ag-presenting molecules tested. Introduction of gp150 in MJS-A2GFP cells decreased surface detection by HLA I-specific Abs, while GFP fluorescence indicated that the total amount of cellular A2GFP molecules was not altered by gp150 (**Figure 5B**). In confocal microscopy, gp150$^+$ MJS-A2-GFP cells were identified based on positive staining for the HA-tag attached to gp150 (in red; **Figure 5C**, lower left panel), which confirmed that HA-gp150 molecules arrived at the cell surface. Focusing on the non-transduced (HA$^-$) control cells, the GFP signal shows localization of A2GFP fusion proteins at the plasma membrane (**Figure 5C**, upper right panel). Accordingly, the surface-exposed A2GFP molecules were detectable with Ab BB7.2 (**Figure 5C**, upper left panel), yet only in the non-transduced fraction of cells. This costaining demonstrated the integrity of the chimeric HLA-A2 molecule. Using Ab-staining (BB7.2), surface A2GFP molecules were not detected on the gp150$^+$ fraction of cells by confocal microscopy (Fig 5C, upper left panel), as may have been anticipated from the flow cytometry data described above (Figs 1,2 and 4). However, GFP fluorescence revealed the presence of the A2GFP molecules also at the surface of gp150$^+$ cells, where they colocalized with gp150 (**Figure 5C**, right panels). The arrival of HLA I molecules at the surface of both gp150$^+$ and control cells, indicates that gp150 does not alter the localization of these Ag-presenting molecules. Quantification of multiple microscopy pictures supports that the Ab-based detection of HLA-A2-GFP is significantly reduced for gp150-expressing cells compared to control cells and this was not due to reduced GFP fluorescence intensity at the cell surface (**Figure 5D**).

Together, these data indicate that cellular expression of EBV gp150 does not alter the total protein levels or localization of HLA molecules, but prevents their Ab-based detection on the cell surface.

EBV gp150 shields surface molecules via sialoglycans on its ectodomain

Since the cytoplasmic tail of gp150 is not essential for immune modulation, it is likely that the extracellular domain of gp150 is mechanistically involved. The most peculiar characteristic of EBV gp150 is its highly abundant glycosylation, with about half of the amino acid residues in the N-terminal domain being potential substrates for N- or O-linked glycosylation (**Figure 4A**). Its extensive glycosylation is reflected by the observation that in SDS-PAGE gels, gp150 migrates as a diffuse band with a molecular weight of 100-150 kDa, whereas the unglycosylated protein backbone is around 30 kDa [25,26]. Although the exact glycan composition is not known, gp150 carries many negatively-charged sialic acid moieties [25,26]. To directly assess whether the glycan decoration on gp150 plays a role in the shielding of surface Ag-presenting molecules, flow cytometry analysis was performed on gp150 expressing cells from which sialic acids had been depleted using three different approaches.

First, we introduced human CD1d, HLA-A2, and β_2m both into Lec2 cells, which are Chinese hamster ovary (CHO) cells with a mutated CMP-sialic acid Golgi transporter resulting

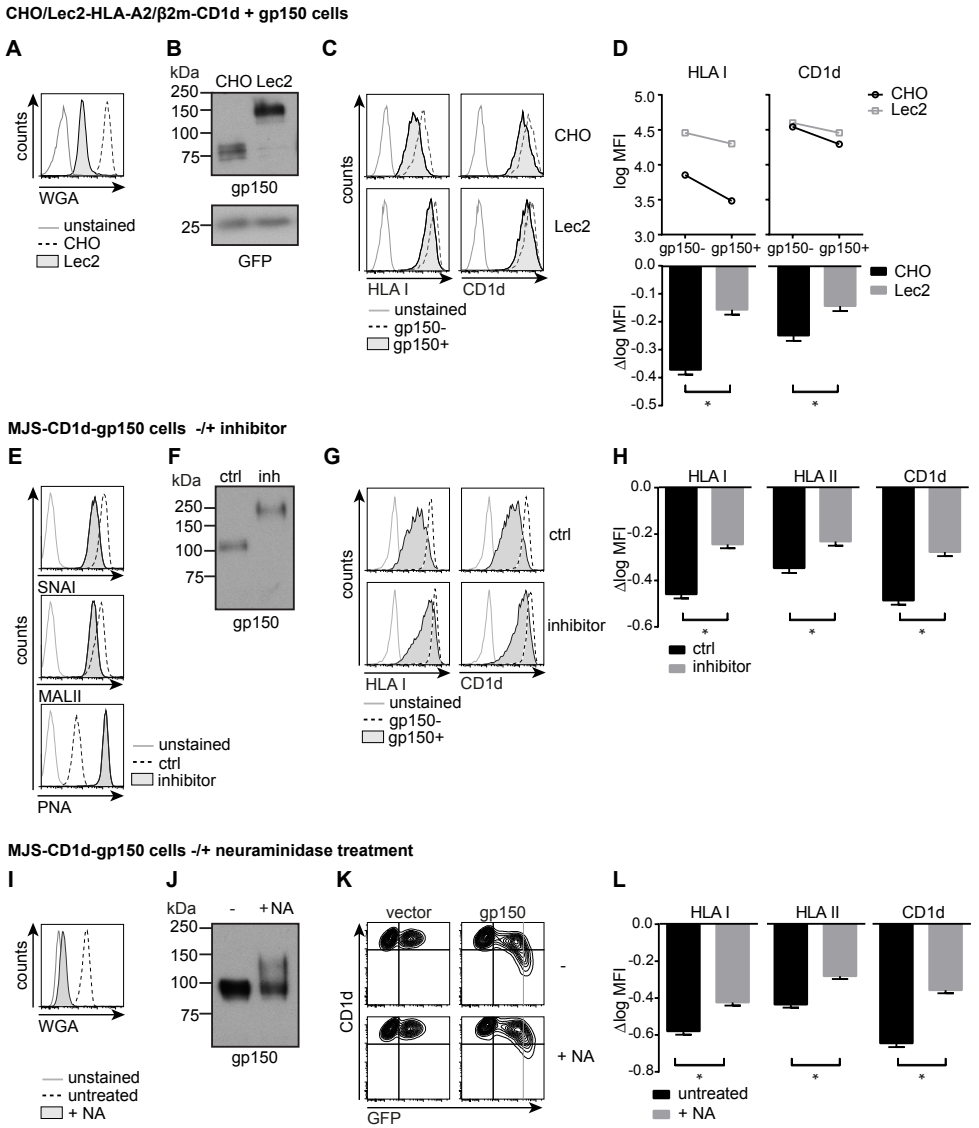


Figure 6 The mechanism of gp150-induced immune evasion relies on sialoglycans shielding surface-exposed Ag-presenting molecules. A-D) CHO and Lec2 cells expressing human β_2m, HLA I, and CD1d were lentivirally transduced to co-express gp150 or HA-gp150ΔC and GFP (from EF1a and PGK promoters, respectively). A) Lectin (WGA-FITC) binding confirmed the glycosylation defect in Lec2 cells compared to parental CHO cells. B) Migration height of HA-gp150 was visualised by Western blot analysis. C-D) Levels of HLA I and CD1d at the surface of HA-gp150ΔC$^+$GFP$^+$ (gp150$^+$) cells were compared to those on control, non-transduced GFP- (gp150-) cells. D) Surface levels of HLA I and CD1d are depicted as log MFI values with 95% confidence intervals, for gp150$^+$ versus gp150$^-$ wt CHO cells or sialylation-defective Lec2 cells. The slopes of the connecting lines (Δlog MFI) reflect

the downregulation induced by gp150. Statistical analysis was performed using two-way ANOVAs and significance of the interaction term was assessed, as described in the Material and Methods section. One representative experiment of at least six is depicted. * p<0.01. E-L) MJS-CD1d-gp150 cells were generated by transduction with a lentivirus encoding both gp150 and GFP (from a CMV promoter and an IRES sequence, respectively) and were analysed 3 days post-transduction by Western blot and by flow cytometry, as for A-D with the modifications indicated below. E-H) To prevent sialylation, cells were treated with the sialic acid transferase inhibitor (500 μM inhibitor) fluorinated P-3Fax-Neu5Ac. As a control, cells were treated with the non-fluorinated compound (500 μM, ctrl) for 4 days, starting 1 day prior to transduction. This control treatment was comparable to when cells were left untreated. E) Lectins SNAI, MALII, or PNA were used to detect sialoglycans or desialylated glycans, respectively. G) gp150+GFPhigh cells were compared to non-transduced cells. H) One representative experiment of two is depicted. * p<0.01. I-L) To remove surface sialylation, intact cells were treated with neuraminidase (1U/μl, 60 min, 37°C) prior to cell lysis or lectin/Ab staining. L) One representative experiment of three is depicted. * p<0.01.

in decreased sialylation of glycans [27], and into control CHO cells. Defective sialylation in the Lec2 cells was confirmed by decreased binding of wheat germ agglutinin (WGA), a lectin that binds sialic acid and N-acetylglucosamine structures (chitobiose) (**Figure 6A**). The effect of defective glycosylation on EBV gp150 expressed in Lec2 cells was visible by Western blot: compared to lysates from control CHO cells, gp150 from Lec2 cell lysates migrated at a higher apparent MW, likely due to the absence of negatively charged sialic acids (**Figure 6B**). Staining for surface HLA I and CD1d on Lec2 cells was somewhat increased compared to parental CHO cells, probably reflecting a better accessibility of the Ab binding sites when the Ag-presenting molecules are less sialylated (**Figure 6C,D**). In the CHO cells, expression of EBV gp150 decreased HLA I and CD1d staining, which was partly reversed in Lec2 cells. This indicates that sialic acids contribute to the phenotype caused by gp150 (**Figure 6C,D**).

Second, to address the role of sialylation in protein downmodulation in human cells and, thereby, also to be able to test a broader panel of surface markers, sialylation was inhibited by pretreating MJS-CD1d cells with the fluorinated sialic acid analogue P-3F_{ax}-Neu5Ac [28]. This analogue blocks sialyltransferases, enzymes that transfer sialic acids onto glycans. Inhibition of sialylation was detected by the reduced binding of the sialic acid-binding lectins SNAI and MALII to treated cells (**Figure 6E**). Strongly enhanced binding of PNA, a lectin that binds to glycan structures that become accessible upon desialylation, further confirmed successful inhibition of sialyltransferases following cell culture in the presence of this competitive inhibitor (**Figure 6E**). Similar to the sialylation-defective hamster cells, sialyltransferase inhibitor treatment of MJS-CD1d cells expressing gp150 was sufficient to cause the viral protein to migrate differently in SDS-PAGE (**Figure 6F**). Cells transduced to express gp150 that were pre-incubated with P-3F_{ax}-Neu5Ac showed increased surface staining for HLA I, II, and CD1d compared to control cells (**Figure 6G,H**), consistent with a crucial role for sialylation in the shielding of Ab binding sites by gp150.

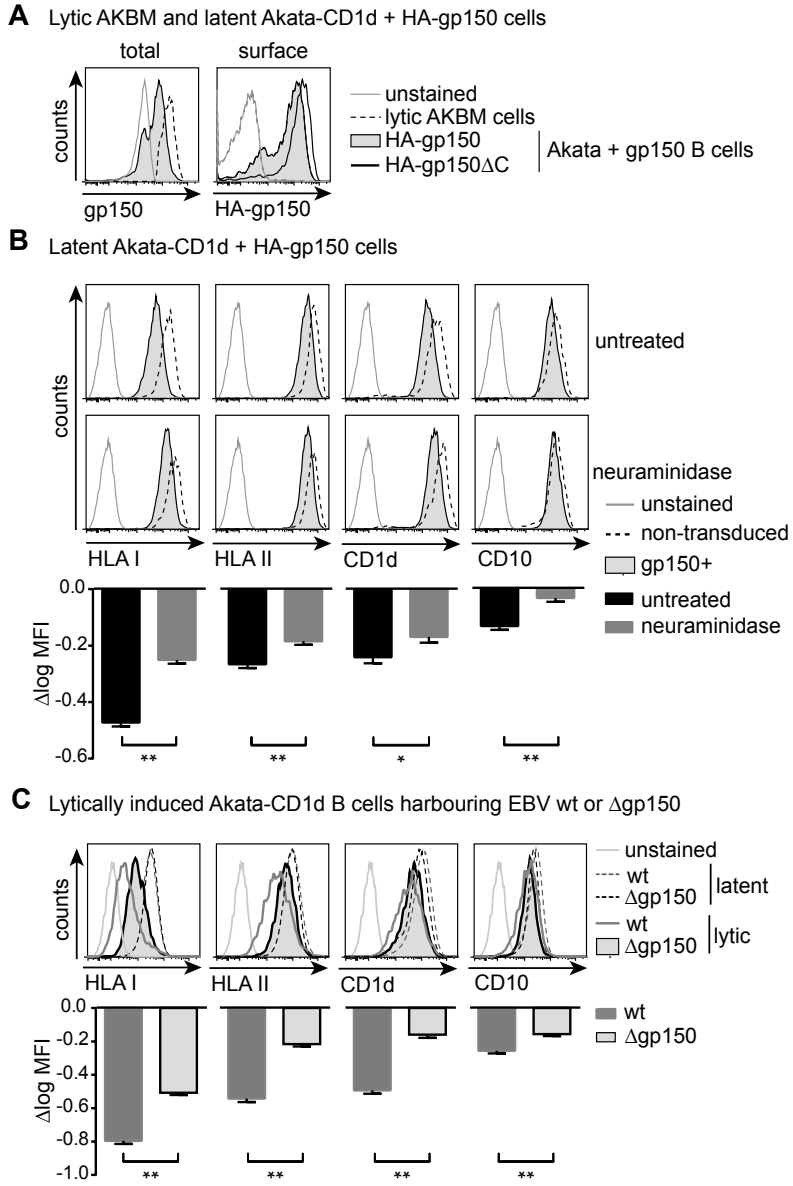


Figure 7 Glycan shielding of surface Ag-presenting molecules by gp150 occurs in human B cells and is reversed during productive EBV infection when gp150 is deleted. A-B) Latent AkataΔgp150 cells were lentivirally transduced to co-express either HA-gp150 or HA-gp150ΔC and GFP (from EF1a and PGK promoters, respectively) and were puromycin-selected to obtain pure populations of gp150^{+} cells. B cells already grow in suspension and gp150-positive B cells were maintained in culture for several weeks, indicating that gp150 expression was not toxic to the cells. A) Flow cytometry was used to assess total (intracellular staining with anti-gp150 Ab on permeabilized cells) and surface (anti-HA Ab on non-

permeabilized cells) levels of EBVgp150. Expression levels of gp150 were compared to lytically induced AKBM cells (20 hours anti-human IgG treatment, rat CD2GFP$^+$ cells). B) Akata+gp150 and non-transduced control cells were left untreated or were treated with neuraminidase (1U/μl, 60 min, 37°C). Surface levels of Ag-presenting molecules and cellular CD10 as a control were compared to gp150$^-$ non-transduced cells. One representative experiment of three is depicted. C) Viral reactivation was induced in Akata wt and Δgp150 B cells by overnight culture with anti-human IgG and EBV-producing cells were identified by staining for the late viral protein gp350. Surface levels of Ag-presenting molecules and CD10 on lytic (gp350$^+$) and latent (gp350$^-$) cells are depicted in overlay histograms. One representative experiment of at least four is depicted. Statistical analysis was performed for B and C) as described for **Figure 6.** * $p<0.05$, ** $p<0.01$.

Third, to selectively cleave off cell surface sialic acids, we treated cells with neuraminidase. Enzymatic desialylation occurred in a dose-dependent fashion, as witnessed by progressive loss of WGA lectin binding at the cell surface (Figs 6I and S5). Selectivity of this neuraminidase treatment for surface-exposed glycans was supported by Western blot analysis, in which a proportion of gp150 molecules from treated cells displayed reduced mobility, whereas the remaining, likely intracellular, EBV proteins had an apparent MW comparable to that in untreated cells (**Figure 6J**). At the surface of gp150-transduced MJS-CD1d cells, CD1d detection improved with increasing doses of neuraminidase (Figs 6K and S5), pointing to a direct involvement of sialic acid-modified glycans in interference with CD1d detection. Likewise, surface display of the host cell molecules HLA I and HLA II was improved by neuraminidase treatment (**Figure 6K,L**) to the same range as observed for the inhibitor treatment (**Figure 6G,H**), suggesting that primarily sialic acids at the cell surface played a role.

In the three settings above (**Figure 6**), a rescue of 15-25% in the surface detection of Ag-presenting molecules was observed in the presence of desialylated versus fully sialylated gp150. This rescue being modest is perhaps due to the remaining glycan structures on gp150. Still, a highly significant increase in display was detected in all abovementioned experimental approaches. Therefore, these combined results provide evidence that surface sialic acid-carrying N- and O-linked glycans contribute to EBV gp150's ability to hide certain host cell surface molecules, including those involved in Ag presentation, thereby providing a mechanistic basis for T and iNKT cell evasion.

EBV gp150 mediates glycan shielding of Ag-presenting molecules on human B cells

Natural EBV infection *in vivo* targets human B cells and epithelial cells and the main reservoir of persistent infection is the memory B cell. To produce new offspring, EBV reactivates from latently infected B cells carrying the viral genome. Whereas the 293T and MJS cells could reflect epithelial infection, we specifically addressed the question if gp150-mediated immune evasion was also operative in human B cells.

As relevant human B cells for this study, we chose Akata BL cells in latency, which upon induction are capable of supporting productive EBV infection, implying that all host cell factors

essential for EBV replication are present. These Akata BL cells are the parental equivalent of the AKBM cells described above (without the rat CD2GFP lytic cycle reporter). In the Akata BL background, Borza and Hutt-Fletcher [19] had previously generated a knockout mutant EBV that lacks the *BDLF3* open-reading frame coding for gp150 (AkataΔgp150 cells, see below). For immune evasion studies, we introduced a constitutively expressed EBV gp150 gene into these latent AkataΔgp150 B cells, thereby avoiding potential interference by any endogenous gp150 expression (referred to as Akata+gp150 cells).

To achieve gp150 expression at levels similar to those occurring in EBV-producing B cells, we compared gp150 protein levels in Akata cells transduced with HA-gp150 or HA-gp150ΔC lentiviruses to those in lytically induced AKBM cells. In flow cytometry using an Ab against the gp150 C-terminus, levels of gp150 in productively infected B cells appeared to exceed those in cells transduced to express full-length gp150 in isolation (**Figure 7A**, left panel). The C-terminally truncated gp150 lacks the epitope recognized by the anti-gp150 Ab. Therefore, we next compared full-length and truncated gp150 expression levels in transduced B cells by means of their HA-tag and found that levels of the cytoplasmic tailless gp150 were slightly higher than those of the full-length protein (**Figure 7A**, right panel). Based on this, we reasoned that B cells expressing gp150ΔC in isolation likely reflect the levels occurring in the context of natural EBV-infection of B cells and we have, therefore, studied these cells in more detail.

Detection of HA-tag on non-permeabilized HA-gp150 Akata cells (**Figure 7B**) also shows that the gp150 protein arrives at the surface of B cells as it did on 293T (**Figure 1C**) and MJS (Figs 2A, 4B and C, 5B and C) cells. At the surface of HA-gp150ΔC-transduced Akata B cells, detection of HLA I, II, CD1d, and to a lesser extent CD10 was reduced in comparison to untransduced control cells (**Figure 7B**, upper panels). This shows that gp150 induces an immune evasive phenotype in human B cells as it did in the other cell types studied. Neuraminidase treatment of Akata+gp150 cells increased surface binding of Abs specific for the three Ag-presenting molecules and CD10 (**Figure 7B**, lower panels and bar graphs), pointing towards direct involvement of sialic acids in gp150-induced shielding of B cell surface markers.

These data combined with those in **Figure 6** of gp150 masking surface HLA I, II, and CD1d via a glycan shield indicate that the same mechanism of action can operate both in epithelium-like cells and in B cells, the natural targets for EBV infection *in vivo*.

EBV gp150 contributes to evasion of Ag presentation during productive infection

To assess the contribution of gp150-mediated interference with Ag presentation in the full viral context, we studied the productive phase of infection in B cells that harbour either wildtype EBV (Akata wt) or the *BDLF3* deletion mutant AkataΔgp150. Based on the use of these cells, gp150 was earlier reported not to be essential for EBV replication *in vitro*, as the recombinant virus had no defects in binding, infectivity, assembly, or egress [19]. In line with

this, we found no apparent differences in progression through the replicative cycle between lytically induced Akata wt and Δgp150 cells: both expressed the EBV immediate-early BZLF1, early BGLF5, and late gp350 proteins, but only Akata wt cells produced gp150, as did control induced AKBM cells (**S6A Figure**).

To determine surface levels of the host cell molecules HLA I, II, CD1d, and CD10 by flow cytometry, both types of induced Akata cells were counter-stained for gp350, to identify the EBV-producing B cells. As observed for AKBM cells (**Figure 1A**), induction of the lytic cycle caused a downregulation of surface display of the Ag-presenting molecules HLA I, II, and CD1d in (gp350$^+$) Akata wt cells, when compared to the latently infected cells (**Figure 7C**). In the absence of gp150, however, HLA I, II, and CD1d surface levels were significantly higher during productive infection, as visible when comparing gp350$^+$ AkataΔgp150 to Akata wt cells (**Figure 7C**, compare tinted with non-filled grey histograms). Likewise, downregulation of the B cell surface markers CD10 and CD86 was less pronounced in lytic cells devoid of gp150 (**Figure 7C** and **S6B Figure**). These results indicate that gp150 contributes to the apparent downregulation of Ag-presenting molecules and – to a lesser extent- other surface markers in a model for natural EBV infection of human B cells.

Our combined data support a model in which surface molecules, including Ag-presenting molecules, are shielded from recognition by the extensive glycosylation of gp150, pointing towards a contribution of this novel EBV evasion molecule to interference with cell-mediated immunity against EBV (**Figure 8**).

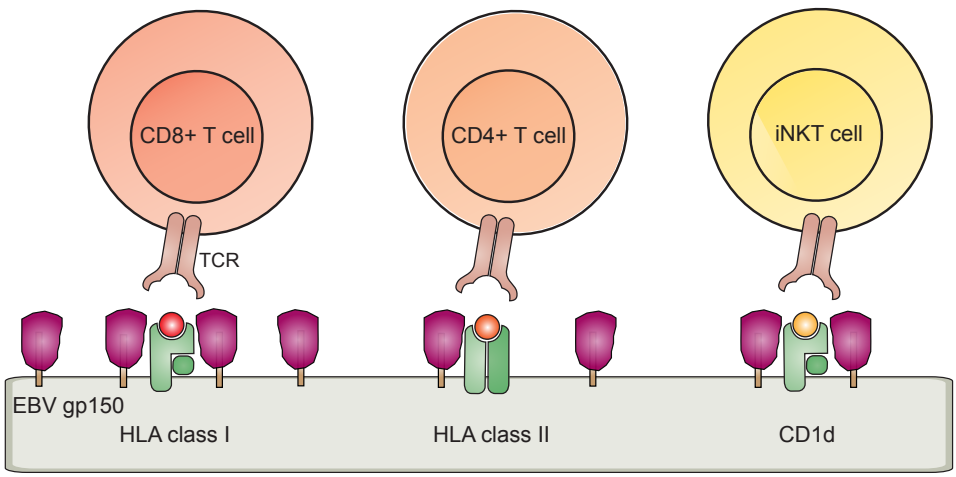


Figure 8 Schematic model of a glycan shielding mechanism for T and iNKT cell immune evasion mediated by EBV gp150.

Discussion

Cell-mediated immunity by HLA I and II-restricted cytotoxic and helper T cells as well as by CD1d-restricted iNKT cells is critical in the defence against many viruses [1]. Especially persistent viruses, such as herpesviruses, have acquired strategies to permit their escape from elimination by these immune cells. In this study, we report that EBV infection, even in its late productive phase, substantially reduced display of CD1d, HLA I, and HLA II complexes at the surface of human B cells. Reduced detection of these antigen-presenting molecules was observed with Abs that can block TCR binding. This effect is functionally important, because HLA downregulation results in reduced T cell activation. For transmission to other target cells or hosts, new viral particles need to be produced through EBV reactivation from latently infected B cells, the primary natural targets for EBV infection. At the late stage of viral replication, up to 100 EBV genes are expressed, thus resulting in a wide array of Ags presented at the surface of infected cells to T cells. EBV-specific T cells are generated upon primary infection [6]. Therefore, immune escape strategies targeting Ag-presenting molecules including HLA I, II, and CD1d might allow EBV-producing B cells to effectively evade T and iNKT cell surveillance *in vivo*, prolonging the timespan for the production of viral offspring. Indeed, patients with reduced functional T and iNKT cell numbers are known to have life-threatening complications upon encountering EBV [6].

Our finding that gp150 acts as a novel EBV-encoded immune evasion molecule causing reduced detection of surface CD1d, HLA I, and HLA II complexes is intriguing as it is not only the first function ascribed to this viral glycoprotein, it also reveals a novel mechanism employed by herpesviruses to dodge immune recognition. In contrast to most herpesvirus structural glycoproteins, EBV gp150 was found to be non-essential for viral entry, assembly, or egress [19] and the function of this late viral protein has remained enigmatic until now. Here, we provide evidence in support of a model in which gp150 is able to shield surface HLA molecules, through its abundantly sialylated glycans, in order to escape T cell activation (**Figure 8**). Cellular expression of gp150 in isolation was sufficient to reduce detection of multiple cellular surface molecules, for example interfering with binding of an Ab detecting the H-2 K^b/SIINFEKL antigenic complex. Moreover, we show that B cells producing a gp150-deleted mutant virus display more Ag-presenting molecules as compared to wild-type EBV, indicating that gp150 contributes to immune evasion in the full viral context. EBV gp150 did not appear to promote degradation, retention, internalization, or altered glycosylation of HLA molecules. Rather, HLA molecules arrived at the cell surface, yet failed to be recognized, a defect which was restored in part by preventing sialylation.

To date, only the Ebola virus glycoprotein (EBOV GP) has been described to shield host cell surface molecules, namely HLA I and β1 integrin molecules, via its extensively glycosylated mucin-like domain [29]. This suggests that similar mechanisms have evolved independently in different viruses belonging to different families. Both EBOV GP's mucin-like domain and

the ectodomain of EBV gp150 contain around 10 potential N-glycosylation and more than 70 potential O-glycosylation sites. The mucin-like domain of EBOV GP consists of mainly core 2 O-linked glycans [30]. The 'core' type of O-linked glycans on gp150 is, at present, unknown, but could also consist of core 2 glycans in view of its resistance to O-glycanase that removes core 1 and core 3 O-glycans [25]. Thus, it remains to be investigated whether the glycans of EBV gp150 and EBOV GP share key features or whether they evolved similarly to shield host surface molecules. Detailed characterization of the glycans of EBOV GP and EBV gp150 is likely required to fully comprehend the molecular necessities of viral glycoproteins to shield cell surface molecules and thereby facilitate further identification of such proteins.

It would be interesting to know whether other (herpes)viruses have incorporated similar strategies to escape from immune recognition, as this would provide further insight into the significance and requirements of this mechanism. The BDLF3 ORF is also present in Rhesus LCV. Rhesus LCV BDLF3 protein displays only 46% amino acid homology to EBV gp150, but shares the feature of the serine/threonine-rich ectodomain indicating that it too might be heavily glycosylated. In view of the low sequence homology between the BDLF3 proteins of these closely related γ-herpesviruses, glycosylation potential rather than amino acid sequence might provide immune evasive properties. It will be interesting to analyse the immune evasive properties of MHV-68 ORF28, the positional homologue of EBV BDLF3. MHV-68 ORF28 also appears to be non-essential for viral replication in vitro and in vivo [31]. Compared to gp150, the ectodomain of ORF28 is reduced in size, yet highly glycosylated and could therefore provide insights into the structural requirements for glycan shielding. It is tempting to speculate that other proteins or different (herpes)viruses present functional homologues of EBV gp150 and EBOV GP that do not share amino acid similarity, yet retain the feature of a heavily glycosylated extracellular domain. Intriguingly, we observed that the major envelope glycoprotein of EBV, gp350, also interfered with surface detection of CD1d (**Figure 1B**) and TfR (**S2B Figure**).

Interestingly, not only do viruses appear to exploit a glycan shielding strategy, but also cancer cells show aberrant expression of sialic acid-modified glycans that are thought to play a role in tumor immune evasion [32], which may in part be due to the reported sialylation-dependent inhibition of NK cells by masking ligands of activating NK cell receptors [33]. Relevance of sialylation-dependent immune interference by tumors is demonstrated by the fact that hypersialylation of tumor cells is associated with poor prognosis for patients [34]. Interestingly, when sialic acid decoration on tumor cells was inhibited with the same compound as we have used (**Figure 6E-H**), it resulted in reduced metastases in an *in vivo* mouse model [35].

Our results show a direct effect of gp150 on the presentation of antigenic peptides to T cells. In addition to Ag-presenting molecules, EBV gp150 could also affect the function of various other cell surface molecules. Potential targets include receptor-ligand pairs involved

in formation of the immunological synapse. An immunological synapse is formed at the interface of the T cell and the virus-infected cell through interactions of several receptor-ligand pairs [36]. In the centre of this synapse, HLA/Ag complexes reside, engaged by specific TCRs. Costimulatory molecules locate in proximity of the Ag-presenting molecules, and are surrounded by a ring of adhesion molecules. We have observed that cellular expression of gp150 also appears to mask the adhesion molecule CD54/ICAM1 (**Figure 2A**), the costimulatory molecule CD86 (**S6B Figure**), as well as TfR (**S2B Figure**), and CD10 (**Figure 2A**). Of note, shielding of these molecules appears to require higher levels of gp150 expression than was sufficient for shielding of these Ag-presenting molecules (**S2C Figure**, and **Figure 2** & **S3B Figure**). In contrast, CD33, CD44, and CD55 were not sensitive to downregulation by gp150, even when expressed at high levels (**Figure 2C**), showing that gp150 does not mask all cell surface molecules. The broad target range of gp150 suggests that other receptor-ligand interactions, such as receptor-induced apoptosis by FasL-Fas interactions, or other (non)-immune cell-cell contacts could be disturbed by gp150. Intriguingly, cell adherence and cell-cell contact were lost in floating MJS cells expressing high levels of gp150 (**S3A Figure**), as was also observed for EBOV GP [29], suggesting that gp150 can uncouple, at least partially, actively EBV-producing B cells from environmental cues. One could speculate that the EBV-producing B cell could thus become mobile by interfering with signals that restrain B cells at a particular site in the body, allowing them to localize for instance to tonsils, facilitating viral shedding in saliva.

Besides the host cell molecules described above, expression of the heavily glycosylated gp150 protein could also shield the recognition of *viral* membrane-expressed proteins that could otherwise by recognized by EBV-specific Abs. EBV exploits the endogenous glycosylation machinery of the infected cell to form glycan structures, which are, by nature, not foreign to the immune system. When these "self-structures" are placed on a - foreign - viral protein, it could be shielded from recognition by Ab-producing B cells. In line with this, no good Abs against the extracellular part of gp150 are available. Furthermore, when the glycosylation is abundant as is the case for gp150, it could also lead to the masking of other viral proteins that are expressed at the cell/ virus surface. So far, we have not observed strongly reduced detection of gHgL, gp350, or gB on wild-type versus gp150-null EBV-producing B cells, and it would not be advantageous to viral entry if these receptors were fully shielded. Yet, both for EBOV GP [37] as well as for some herpesviruses, glycosylation-mediated evasion of Ab neutralization has been reported, for example for the proteins gN of HCMV and the bovine herpesvirus 4 (BoHV-4) gp180, which is the functional homologue of EBV gp350. Truncated HCMV gN lacking part of its glycosylated ectodomain as well as absence of BoHV-4 gp180 renders viral particles more sensitive to Ab neutralization [38,39]. In addition, cells infected with BoHV-4 lacking gp180 show increased Ab binding to viral proteins, pointing to the role of glycans in shielding viral proteins from immune recognition.

Persistent viruses dedicate a large part of their genome to modulating host antiviral

activities. Among these, herpesviruses have acquired a very diverse set of strategies to downregulate Ag-presenting molecules and, thereby, T cell activation in a temporal fashion. Viral strategies include blocking protein synthesis, depletion of antigenic peptides, dislocalization to the cytoplasm followed by proteasomal degradation, re-routing and/or accelerated internalization, or steric hindrance of TCR binding [4]. In this study, we report on a novel EBV strategy: by expressing the heavily glycosylated, late EBV protein gp150, Ag-presenting molecules on productively infected cells are shielded from interaction with TCRs, thereby allowing replication and production of viral particles that would otherwise by interrupted by a cellular immune response. This mechanism is novel in herpesvirus biology, and further supports the notion that herpesviruses use very diverse ways to achieve escape not only from T cells, but also from iNKT cell immunity. Evasion from CD1d-mediated Ag presentation supports an essential role for iNKT cells in anti-EBV immunity, which was already inferred from the devastating effects of EBV infection in XLP patients. Combining multiple strategies allows them to escape various layers of immune elimination during various stages of infection. This underscores the notion that EBV, as other herpesviruses, is extremely well adapted to persist in the face of functional immunity.

Material and Methods

Ethics Statement

For T-helper cell assays, peripheral blood samples from patients and healthy individuals were obtained after approval by the LUMC institutional review board and written informed consent according to the Declaration of Helsinki.

Cells

All media were supplemented with 10% FCS, 100 U/ml penicillin, and 100µg/ml streptomycin. The EBV$^+$ BL cell lines AKBM [15], Akata wt, and Akata Δgp150 [19] were cultured in supplemented RPMI medium. Productive EBV infection and inhibition of late protein expression by phosphonoacetic acid were performed as previously described [22]. Human embryonic kidney (HEK) 293T, HEK 293H-2Kb, and the human melanoma cell line MelJuSo (MJS, HLA typing A*01, B*08, DR3) and MJS-derived cell lines were cultured in supplemented DMEM medium. MJS-A2-GFP cells were obtained from Dr. E Reits [24]. Chinese hamster ovary (CHO) and Lec2 cells [40] were cultured in supplemented MEM-α medium.

Antibodies and streptavidin conjugates

Primary antibodies used in this study were mouse α-CD1d (CD1d42, CD1d51, CD1d55, D5; a kind gift by S. Porcelli, NY, USA), mouse α-CD1d-PE (clone CD1d42, BD Pharmingen), mouse α-HLA-ABC (W6/32), mouse α-HLA-ABC-PE (W6/32, Serotec), mouse α-HLA-A2 (BB7.2), mouse α-HLA-DR (L243), mouse α-HLA-DR-PE (L243, BD Biosciences), mouse α-TfR (CD71, clone M-A721, BD Pharmingen), mouse α-CD10-PE (clone Hl10a, BD Pharmingen), mouse α-CD34-PE (clone 8G12, BD Biosciences), rat α-CD44-PE (clone IM7, BD Pharmingen), mouse α-CD54-PE (ICAM-1, clone HA58, BD Pharmingen), mouse α-CD55-PE (clone IA10, BD Pharmingen), mouse α-BZLF1 (clone BZ.1, kindly provided by Dr. M. Rowe, Birmingham, UK), mouse α-BGLF5 (clone 311H, kindly provided by Dr. J. Chen, Taipei, Taiwan), mouse α-gHgL (E1D1), rabbit α-gp150 (V8), biotinylated mouse α-gp350 (72A1), rat α-HA-tag (3F10, Roche), rat α-EBNA1-tag (1H4, kindly provided by Dr. J. Mautner), mouse α-myc-tag (9E10). Secondary antibodies used in flow cytometry were goat α-mouse-PE (eBioscience), donkey α-rat-APC or –FITC (Jackson Immunoresearch), and goat α-rabbit-PE (Jackson Immunoresearch). To detect biotinylated antibodies or lectins, streptavidin-BV421 or streptavidin-PE (both BioLegend) were used, respectively. For Western blot analysis, an HRP-conjugated goat α-rabbit (Southern Biotech) antibody was used. For confocal microscopy, goat α-mouse-Atto647 (Sigma) and goat α-rat-Alexa594 (Invitrogen) antibodies were used.

Plasmids

Plasmids encoding His- and EBNA1 (aa435-445, recognized by 1H4 Ab)-tagged EBV glycoproteins (gM, gN, gp78, gp350, and gp150) were a generous gift from Dr J. Mautner and were described previously [41].

In this study, different lentiviral vectors were used to create third generation replication-deficient lentiviruses. Expression of the human CD1d or HLA-A2 gene fused to a zeocin resistance gene by means of the ribosomal skipping peptide P2A was driven by an EF1a promoter [22]. C-terminally Flag (DYKDDDDK)-tagged BMRF2, N-terminally Flag-tagged BDLF2, and *BDLF3* EBV genes were PCR-amplified from EBV B95.8 genome. PCR products were cloned into the lentiviral vector pLV-CMV-IRES-GFP. We generated an in-frame N-terminal HA-tagged (YPYDVPDYA) gp150 full-length variant and a mutant lacking the last 26 aa (209-234) of the cytoplasmic tail (HA-gp150ΔC). The PCR products were cloned behind an EF1a promoter into the dual promotor lentiviral vector EF1a-/pGK-GFP-T2A-puro, described elsewhere [21], and into the lentiviral vectors pLV-CMV-IRES-GFP and pLV-CMV-IRES-puroR. The lentiviral vector encoding eGFP-myc-HLA-A2 has been described elsewhere [21].

Codon-optimized human β_2m was encoded in a variant of the abovementioned dual promoter lentiviral vector containing a zeocin resistance gene and mAmetrine fluorescence gene fused by means of the ribosomal skipping peptide T2A.

Replication-deficient retroviruses and retroviral transductions

Third generation replication-deficient SIN recombinant lentiviruses were generated by PEI transfection of 293T cells with pCMV-VSVG, pMDLg-RRE, and pRSV-REV and a lentiviral vector coding for the genes of interest. Target cells were transduced with lentivirus supernatant in the presence of 4 μg/ml polybrene by spin infection (1000x*g*, 2h, 33°C). AKBM and Akata B cells, MJS, and 293T cells were transduced with human CD1d and selected using zeocin (200-500 μg/ml).

MJS cells were retrovirally transduced in the presence of 12μg/ml retronectin with the pLZRS-HCMV pp65-IRES-trNGFR vector to generate MJS-pp65 cells. Cells were FACS sorted for surface trNGFR expression. MJS or MJS-pp65 cells were transduced with eGFP-myc-tagged HLA-A2 or HLA-A2 to generate MJS-GFPmycA2 and MJS-A2/pp65 cells, respectively.

To generate CHO-A2-CD1d and Lec2-A2-CD1d cells, CHO and Lec2 cells were first transduced with human codon-optimized β_2m and selected with zeocin (500 μg/ml). Subsequently, cells were transduced to express HLA-A2 and human CD1d. HLA-A2$^+$CD1d$^+$ cells were FACS sorted using a FACS Aria III (BD).

To study gp150 in MJS cell lines, gp150 or the variants HA-gp150 or HA-gp150ΔC were expressed from a lentiviral pCMV-IRES-GFP or pCMV-IRES-puro^R vector. The empty pCMV-IRES-GFP vector served as a control. MJS cell lines were transduced and analysed 3 to 5 days post transduction. Floating MJS cells were harvested and analysed 3-4 days post transduction. CHO and AkataΔgp150 cell lines were transduced, as indicated, with gp150, HA-gp150, or HA-gp150ΔC, which were expressed from the dual promotor lentiviral vector pEF1a-/pGK-GFP-T2A-puro^R. CHO cells were analysed up to 10 days post transduction. AkataΔgp150 cells were selected using puromycin and analysed up to 4 weeks post transduction.

Transient transfection

For transient transfections, 293T cells were seeded in 12w plates one day prior to transfection. Cells were transfected with 1 μg plasmid DNA coding for EBV proteins using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. Cells were analysed by flow cytometry two days post transfection.

Flow cytometry, cell sorting, and cell viability assay

To assess cell surface levels of indicated molecules, cells were stained with antibodies of indicated specificity in PBS supplemented with 0,5% BSA and 0,02% sodium azide. For intracellular stains (ICS), cells were fixed in 2% PFA and permeabilized in ICS-FACS buffer (PBS, 2% FCS, 0,5% saponine). Subsequent staining and washing steps were performed in ICS-FACS buffer. All stainings were performed at 4°C. Cells were fixed prior to analysis in FACS buffer containing 2% PFA. Samples were subjected to flow cytometry using a LSR II or Canto II (BD Biosciences). Flow cytometry data were analysed using FlowJo (Tree star)

software.

For cell sorting, cells were stained with indicated antibodies in PBS supplemented with 0,5% BSA for 1 hour on ice. Cells were washed in supplemented PBS and subjected to sorting by flow cytometry using an Aria III (BD Biosciences). Sorted cells were washed and prepared for use in T cell assays or Western blot analysis.

To determine cell viability by flow cytometry, unfixed cells were incubated with 7-AAD for 5 min at RT and subjected to flow cytometry. Live cells excluded the dye, whereas dead cells become 7-AAD positive.

Functional assays

MJS-HLA-A2/pp65 cells were transduced with two different doses of lentivirus for gp150 expression or an empty vector control. Three days post-transduction, floating and adherent cells were harvested and adherent cells were FACS sorted for GFPhigh and GFPint cells. In 96w U-bottom plates, triplicates of 5000 cells were incubated with equal numbers of A2/pp65-specific $CD8^+$ T cells or DR3/MiHa-specific $CD4^+$ T cells overnight. Culture supernatants were collected to determine IFN-γ secretion by ELISA (Affymetrix) according to manufacturer's protocol. Standard deviation was determined over biological triplicates.

For MHC/peptide-TCR interactions, $293K^b$ cells were transfected with gp150 or an empty vector control. One day post-transfection, cells were peptide-pulsed for 2h with either 1 μM ovalbumin-derived SIINFEKL peptide or the control peptide SSIEFARL. Cells were harvested and cell surface-stained using the 25D1.16 Ab, which is specific for K^b/SIINFEKL complexes. Samples were subjected to flow cytometry. Mean fluorescence intensity was determined for triplicates in each experiment. Statistical analysis was performed on three independent experiments using Student's t-test (two-tailed).

Confocal microscopy

The localization of C-terminally GFP-tagged HLA-A2 expressed in MJS cells was determined by confocal microscopy. HA-gp150ΔC-transduced cells were harvested and cell surface stained for HLA-A2 (BB7.2) and gp150 (anti-HA) as performed for flow cytometry. Cell suspension was applied on a glass coverslip. Cells were analysed using a Leica TCS SP5 confocal microscope. Microscopy images were adjusted in levels in a linear fashion. For quantification, around 30 single cells were analysed using a composite colour image of the three different channels. Images of single-stained cells (BB7.2 or anti-HA) were used to define the amount of fluorescence leakage into the regarding other channel. Prior to image quantification of double-stained cells, measured pixel intensity was corrected for the determined leakage. Pixel intensity was determined for the outer 8 pixels of the selected objects. Statistical analysis was performed using an unpaired t-test (two-tailed).

Western blot

Post-nuclear cell lysates were prepared using NP40 lysis buffer (50 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.5% Igepal-CA630) containing protease inhibitors (Roche, Protease cocktail), as described previously [15]. For treatment with glycosidases, lysates were denatured at 95°C for 5 min prior to incubation with Endo H or PNGase F (both NEB) at 37°C for 1h. Proteins were separated by SDS-PAGE and blotted onto PVDF membranes. Membranes were blocked using 5% milk in PBS. The indicated primary antibodies were used to probe membranes overnight at 4°C in 1% milk in PBST (PBS, 0,1% Tween 20). Incubation with secondary antibody was performed for 2 hours at RT. Antibody-reactive bands were detected by ECL and signal was captured by film.

Protease inhibitor treatment

Cells were cultured overnight in the presence of 25 μM chloroquine (provided by Dr. A de Wilde) or 15 nM epoxomicin (provided by Dr. H Overkleeft) to inhibit proteolysis by endolysosomal proteases or cytoplasmic proteasomes, respecitively.

Inhibition of sialylation

Optimal concentration of the fluorinated P-3F$_{ax}$-Neu5Ac inhibitor was determined and effectiveness of treatment was monitored by binding of the biotinylated lectins SNAI (*Sambucus nigra*), MALII (*Maackia amurensis*), and PNA (*Ara chishypogaea*, all purchased by EY Laboratories). Lectin stains were performed in carbo-free blocking solution (Vector Laboratories) as described previously [42]. Cells were treated with 500 μM fluorinated P-3F$_{ax}$-Neu5Ac inhibitor or with the control compound P-Neu5Ac 1 day prior to lentiviral transduction. Cells were cultured in presence of inhibitor for additional four days and analysed by flow cytometry.

Neuraminidase treatment

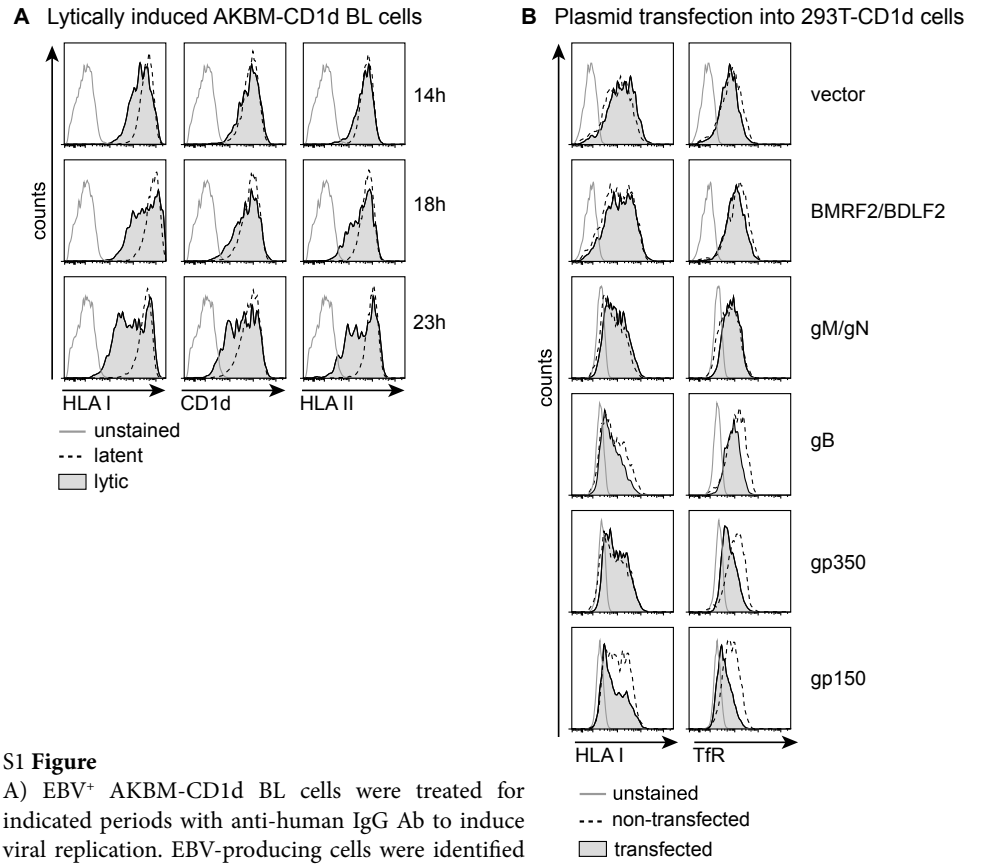
Neuraminidase treatment was performed in FACS buffer containing 1U/μl neuraminidase (NEB), at 37°C for 45-60 min. Subsequently, cells were washed and stained in cold FACS buffer. Neuraminidase treatment was monitored by loss of binding of the biotinylated or FITC-conjugated lectin WGA (*Triticum vulgaris*). Stained cells were subjected to flow cytometry.

Statistical analysis

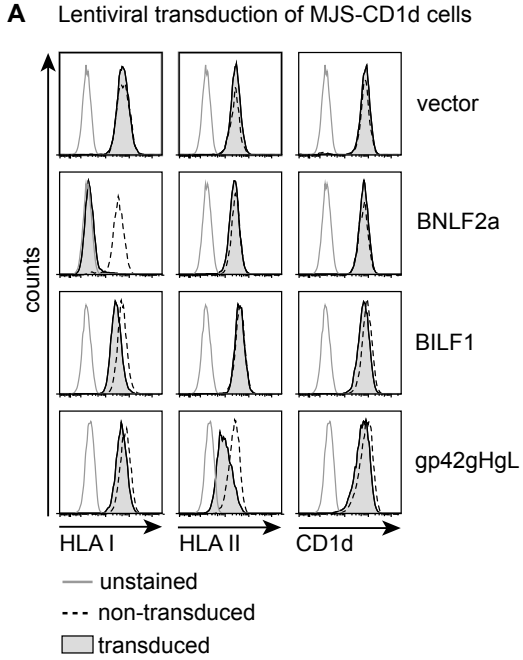
Fluorescence intensity signals of each analysed cell were logarithmically transformed and 1000 cells per parameter (**Figure 6** and **7B**, gp150^{-}/gp150^{+}, **Figure 7C**, latent/lytic) were randomly picked. For each parameter, two "treatments" were compared (**Figure 6D**, CHO vs Lec2, 6H, control vs inhibitor, 6L and 7B, untreated vs neuraminidase, 7C, WT vs Δgp150). Whether treatment differences vary over gp150 expression has been explored by means of

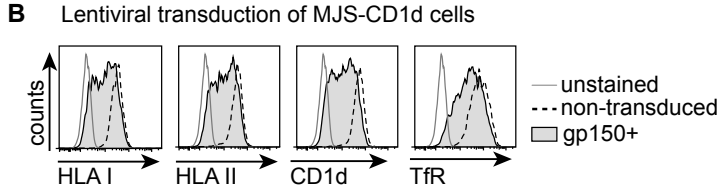
evaluating a statistical interaction between these factors in an analysis of variance model. Statistical analysis was performed using two-way ANOVAs and significance of the interaction terms (ctrl/gp150*untreated/treated) was assessed using SPSS (version 20.0.0.2). p-values < 0.05 were considered statistically significant.

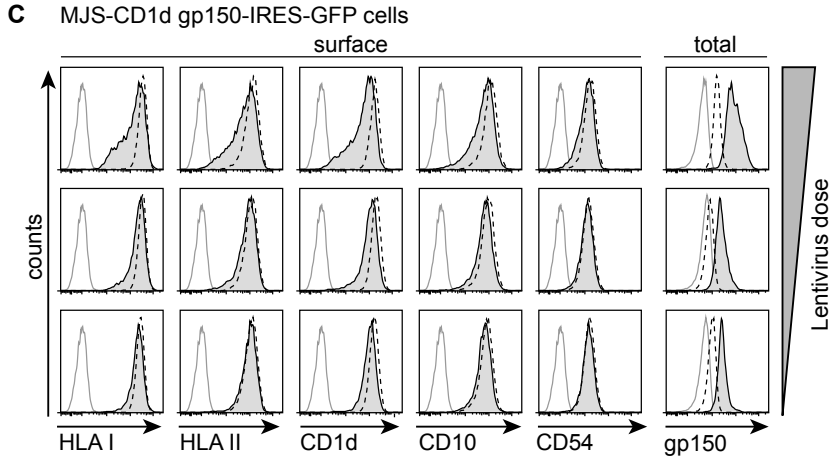
Supporting Information



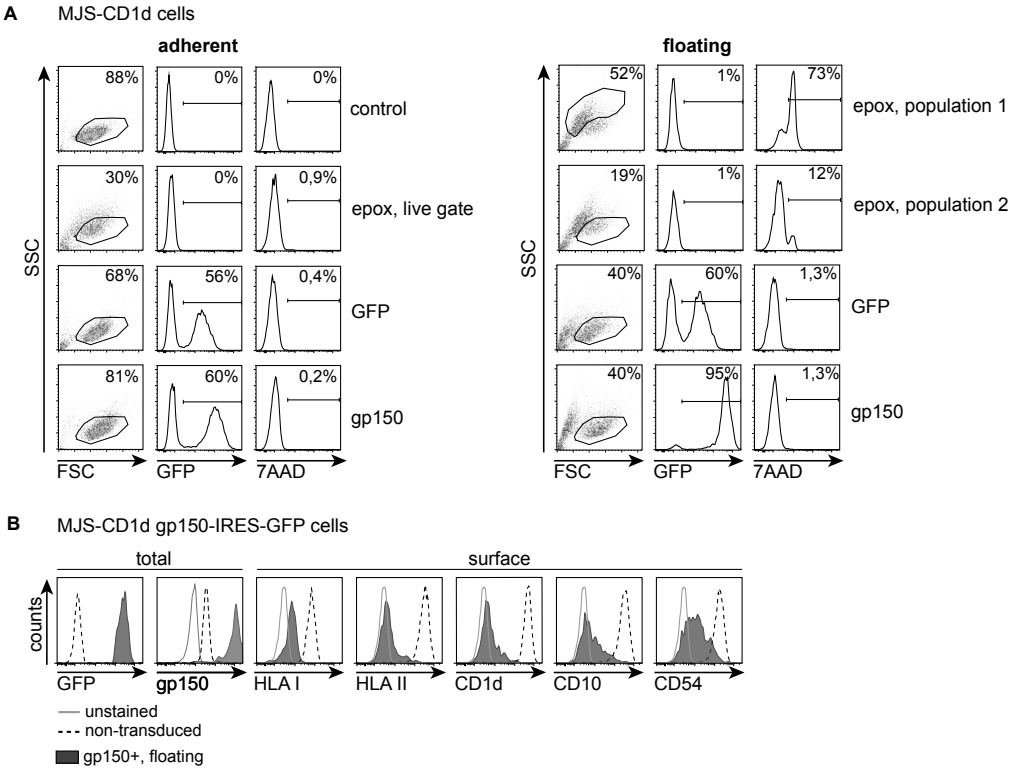
S1 **Figure**
A) EBV$^+$ AKBM-CD1d BL cells were treated for indicated periods with anti-human IgG Ab to induce viral replication. EBV-producing cells were identified by induced expression of the lytic cycle reporter rat CD2-GFP. Surface levels of the Ag-presenting molecules HLA I, II, and CD1d were determined by flow cytometry. Histograms depict overlays to allow comparison of latently (rat CD2-GFP$^-$) and lytically (rat CD2-GFP$^+$) infected B cells. B) 293T-CD1d cells were transfected with expression vectors encoding late EBV glycoproteins. Glycoproteins known to require a viral interaction partner were transfected together (BMRF2/BDLF2 and gM/gN). EBV protein expression was deduced from coexpression of GFP (BMRF2/BDLF2) or on the basis of a C-terminal tag (gM/gN, gB, gp350, gp150). Cell surface HLA I or TfR was stained prior to an intracellular staining for the tagged EBV proteins. Surface levels were compared between non-transfected and transfected cells.







S2 Figure
MJS-CD1d cells were transduced with lentiviruses encoding (A) the indicated EBV immune evasion gene products (BNLF2a, BILF1, gp42+gH+gL) or only IRES-GFP (vector) and (B,C) gp150-IRES-GFP. Surface levels of HLA I, HLA II, and CD1d (A) as well as TfR (B) or CD10 and CD54 (C) were determined by flow cytometry on non-permeabilized cells. Histograms depict a comparison of GFP- control (non-transduced) and GFP$^+$ EBV protein-expressing (transduced) cells. C) A dose range of pCMV-gp150-IRES-GFP lentivirus was used for transduction. Total gp150 expression levels in permeabilized cells were determined by intracellular staining with an Ab specific for gp150's cytoplasmic tail.

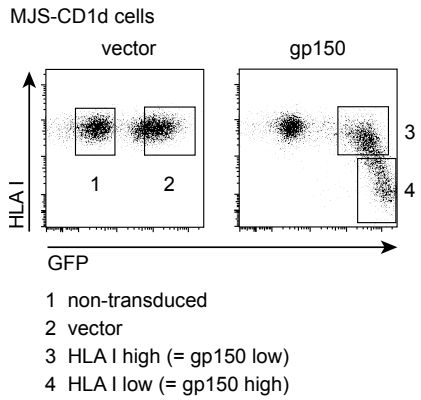


S3 Figure

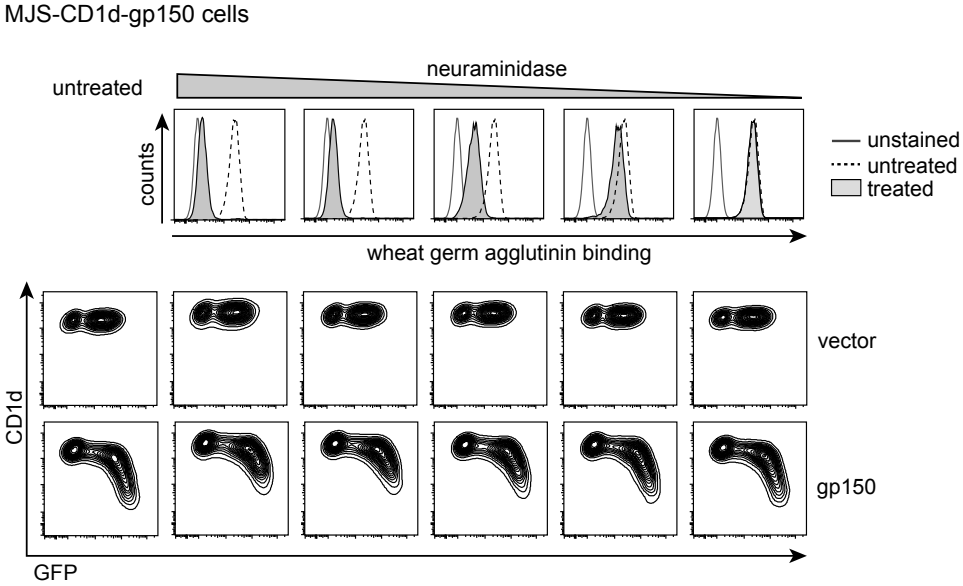
A) The adherent MJS cell line was transduced either with the gp150-IRES-GFP lentivirus or an IRES-GFP control. Three days post transduction, both the floating and adherent fractions of transduced cells were subjected to flow cytometry. The proportion of floating cells was larger for gp150-transduced cells than for control cells and, additionally, the gp150$^+$ floating cells were enriched for gp150 expression (reflected by higher GFP levels compared to the adherent cells). These observations suggested that high levels of gp150 expression induce loss of cell adherence. To exclude that the higher gp150 levels were cytotoxic, the viability of floating and adherent fractions of transduced cells from the same culture dish was determined by incubation with the live exclusion dye 7-aminoactinomycin D (7AAD) followed by flow cytometry analysis. As controls served the adherent fraction of untreated MJS-CD1d cells (control) and the adherent and floating fractions of cells treated with toxic concentrations of the proteasome inhibitor epoxomicin (epox; 200 nM) for 24h. In the FSCxSSC dot plots, the live gates are depicted for the cell populations analyzed for GFP levels (transduction efficiencies) and 7AAD exclusion (viability). An additional gate on population 1 in the floating epoxomicin-treated cells shows that the 7AAD was effective in penetrating dead cells.

Among both the adherent and the floating cells transduced with either control or gp150 lentivirus, only very few 7AAD$^+$ (dead) cells were present, indicating that gp150 does not cause gross cytotoxicity.

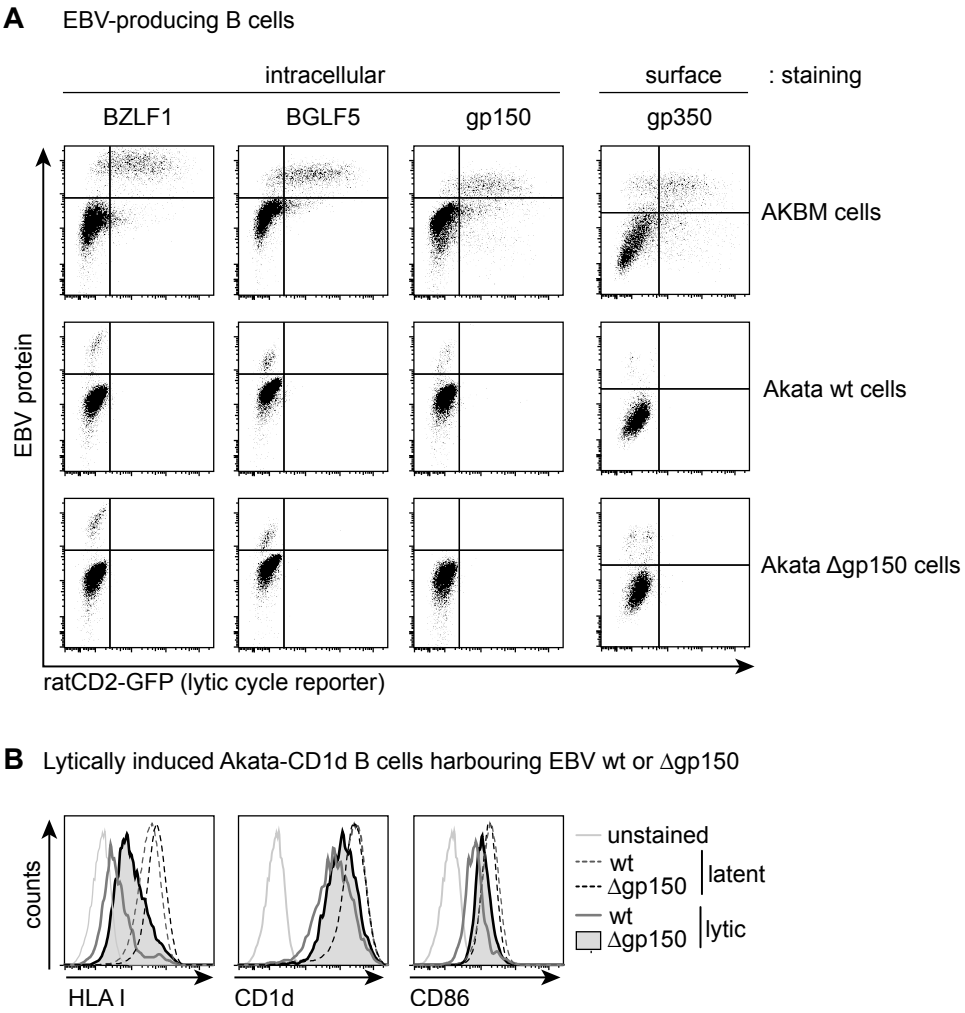
B) The floating cell fraction from the experiment depicted in **Figure 2A** was analyzed by flow cytometry, as described in the legend to **Figure 2A.**



S4 Figure
Three days after lentiviral transduction, four different populations were FACS sorted from MJS-CD1d cells that were transduced with gp150-IRES-GFP or control IRES-GFP viruses: GFP$^-$ (1, non-transduced) and GFP$^+$ (2, vector) cells were isolated from control cells and the GFP$^+$ cells from the gp150-transduction were further separated into gp150^{low} (3, HLA Ihigh) cells and gp150^{high} (4, HLA Ilow) cells on the basis of the extent of HLA I downregulation. The sorted cell populations were lysed for analysis by immunoblot (see **Figure 4B**).



S5 Figure
Intact MJS-CD1d-gp150 and control-IRES-GFP cells were treated with decreasing amounts of neuraminidase (5-0,008U/ml) for 60 min at 37°C and compared to untreated cells. Effectiveness of neuraminidase treatment was monitored by WGA-FITC binding. CD1d surface levels were determined by flow cytometry.



S6 Figure

A) EBV$^+$ AKBM, Akata wt and Δgp150 BL cells were treated with anti-human IgG to induce the viral lytic cycle. Twenty hours later, expression of several EBV proteins - BZLF1 (immediate-early), BGLF5 (early), and gp150 and surface gp350 (both late) - was determined using flow cytometry. B) Akata wt and Δgp150 BL cells were treated with anti-human IgG for 20 hours. Surface expression of the cellular proteins HLA I, CD1d, and CD86 was determined using flow cytometry as described in **Figure 7C**.

Acknowledgements

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References

1. Salio M, Silk JD, Jones EY, Cerundolo V (2014) Biology of CD1- and MR1-restricted T cells. Annu Rev Immunol 32: 323-366.
2. Horst D, Geerdink RJ, Gram AM, Stoppelenburg AJ, Ressing ME (2012) Hiding lipid presentation: viral interference with CD1d-restricted invariant natural killer T (iNKT) cell activation. Viruses 4: 2379-2399.
3. Pellett PE, Roizman B, editors (2007) The Family Herpesviridae: A Brief Introduction.
4. Horst D, Verweij MC, Davison AJ, Ressing ME, Wiertz EJ (2011) Viral evasion of T cell immunity: ancient mechanisms offering new applications. Curr Opin Immunol 23: 96-103.
5. Epstein MA, Achong BG, Barr YM (1964) Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma. Lancet 1: 702-703.
6. Taylor GS, Long HM, Brooks JM, Rickinson AB, Hislop AD (2015) The immunology of Epstein-Barr virus-induced disease. Annu Rev Immunol 33: 787-821.
7. Pasquier B, Yin L, Fondaneche MC, Relouzat F, Bloch-Queyrat C, *et al.* (2005) Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. J Exp Med 201: 695-701.
8. Rigaud S, Fondaneche MC, Lambert N, Pasquier B, Mateo V, *et al.* (2006) XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. Nature 444: 110-114.
9. Rooney CM, Smith CA, Ng CY, Loftin S, Li C, *et al.* (1995) Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. Lancet 345: 9-13.
10. Ressing ME, van Gent M, Gram AM, Hooykaas M, Piersma S, *et al.*, editors (2015) Immune evasion by Epstein-Barr virus.
11. Hislop AD, Ressing ME, van Leeuwen D, Pudney VA, Horst D, *et al.* (2007) A CD8+ T cell immune evasion protein specific to Epstein-Barr virus and its close relatives in Old World primates. J Exp Med 204: 1863-1873.
12. Zuo J, Quinn LL, Tamblyn J, Thomas WA, Feederle R, *et al.* (2011) The Epstein-Barr virus-encoded BILF1 protein modulates immune recognition of endogenously processed antigen by targeting major histocompatibility complex class I molecules trafficking on both the exocytic and endocytic pathways. J Virol 85: 1604-1614.
13. van Gent M, Gram AM, Boer IG, Geerdink RJ, Lindenbergh MF, *et al.* (2015) Silencing the shutoff protein of Epstein-Barr virus in productively infected B cells points to (innate) targets for immune evasion. J Gen Virol 96: 858-865.
14. Quinn LL, Zuo J, Abbott RJ, Shannon-Lowe C, Tierney RJ, *et al.* (2014) Cooperation between Epstein-Barr virus immune evasion proteins spreads protection from CD8+ T cell recognition across all three phases of the lytic cycle. PLoS Pathog 10: e1004322.
15. Ressing ME, Keating SE, van Leeuwen D, Koppers-Lalic D, Pappworth IY, *et al.* (2005) Impaired transporter associated with antigen processing-dependent peptide transport during productive

EBV infection. J Immunol 174: 6829-6838.
16. Rao P, Pham HT, Kulkarni A, Yang Y, Liu X, *et al.* (2011) Herpes simplex virus 1 glycoprotein B and US3 collaborate to inhibit CD1d antigen presentation and NKT cell function. J Virol 85: 8093-8104.
17. Zuo J, Currin A, Griffin BD, Shannon-Lowe C, Thomas WA, *et al.* (2009) The Epstein-Barr virus G-protein-coupled receptor contributes to immune evasion by targeting MHC class I molecules for degradation. PLoS Pathog 5: e1000255.
18. Ressing ME, van Leeuwen D, Verreck FA, Gomez R, Heemskerk B, *et al.* (2003) Interference with T cell receptor-HLA-DR interactions by Epstein-Barr virus gp42 results in reduced T helper cell recognition. Proc Natl Acad Sci U S A 100: 11583-11588.
19. Borza CM, Hutt-Fletcher LM (1998) Epstein-Barr virus recombinant lacking expression of glycoprotein gp150 infects B cells normally but is enhanced for infection of epithelial cells. J Virol 72: 7577-7582.
20. Exley M, Garcia J, Wilson SB, Spada F, Gerdes D, *et al.* (2000) CD1d structure and regulation on human thymocytes, peripheral blood T cells, B cells and monocytes. Immunology 100: 37-47.
21. van de Weijer ML, Bassik MC, Luteijn RD, Voorburg CM, Lohuis MA, *et al.* (2014) A high-coverage shRNA screen identifies TMEM129 as an E3 ligase involved in ER-associated protein degradation. Nat Commun 5: 3832.
22. Griffin BD, Gram AM, Mulder A, Van Leeuwen D, Claas FH, *et al.* (2013) EBV BILF1 evolved to downregulate cell surface display of a wide range of HLA class I molecules through their cytoplasmic tail. J Immunol 190: 1672-1684.
23. Paduraru C, Spiridon L, Yuan W, Bricard G, Valencia X, *et al.* (2006) An N-linked glycan modulates the interaction between the CD1d heavy chain and beta 2-microglobulin. J Biol Chem 281: 40369-40378.
24. Gromme M, Uytdehaag FG, Janssen H, Calafat J, van Binnendijk RS, *et al.* (1999) Recycling MHC class I molecules and endosomal peptide loading. Proc Natl Acad Sci U S A 96: 10326-10331.
25. Kurilla MG, Heineman T, Davenport LC, Kieff E, Hutt-Fletcher LM (1995) A novel Epstein-Barr virus glycoprotein gp150 expressed from the BDLF3 open reading frame. Virology 209: 108-121.
26. Nolan LA, Morgan AJ (1995) The Epstein-Barr virus open reading frame BDLF3 codes for a 100-150 kDa glycoprotein. J Gen Virol 76 (Pt 6): 1381-1392.
27. Eckhardt M, Gotza B, Gerardy-Schahn R (1998) Mutants of the CMP-sialic acid transporter causing the Lec2 phenotype. J Biol Chem 273: 20189-20195.
28. Rillahan CD, Antonopoulos A, Lefort CT, Sonon R, Azadi P, *et al.* (2012) Global metabolic inhibitors of sialyl- and fucosyltransferases remodel the glycome. Nat Chem Biol 8: 661-668.
29. Francica JR, Varela-Rohena A, Medvec A, Plesa G, Riley JL, *et al.* (2010) Steric shielding of surface epitopes and impaired immune recognition induced by the ebola virus glycoprotein. PLoS Pathog 6: e1001098.
30. Ritchie G, Harvey DJ, Stroeher U, Feldmann F, Feldmann H, *et al.* (2010) Identification of N-glycans from Ebola virus glycoproteins by matrix-assisted laser desorption/ionisation time-of-flight and negative ion electrospray tandem mass spectrometry. Rapid Commun Mass Spectrom 24: 571-585.
31. May JS, Coleman HM, Boname JM, Stevenson PG (2005) Murine gammaherpesvirus-68 ORF28 encodes a non-essential virion glycoprotein. J Gen Virol 86: 919-928.
32. Büll C, den Brok MH, Adema GJ (2014) Sweet escape: sialic acids in tumor immune evasion. Biochim Biophys Acta 1846: 238-246.
33. Cohen M, Elkabets M, Perlmutter M, Porgador A, Voronov E, *et al.* (2010) Sialylation of 3-methylcholanthrene-induced fibrosarcoma determines antitumor immune responses during immunoediting. J Immunol 185: 5869-5878.
34. Schneider F, Kemmner W, Haensch W, Franke G, Gretschel S, *et al.* (2001) Overexpression of sialyltransferase CMP-sialic acid:Galbeta1,3GalNAc-R alpha6-Sialyltransferase is related to poor

patient survival in human colorectal carcinomas. Cancer Res 61: 4605-4611.
35. Büll C, Boltje TJ, van Dinther EA, Peters T, de Graaf AM, *et al.* (2015) Targeted delivery of a sialic acid-blocking glycomimetic to cancer cells inhibits metastatic spread. ACS Nano 9: 733-745.
36. Huppa JB, Davis MM (2013) The interdisciplinary science of T-cell recognition. Adv Immunol 119: 1-50.
37. Cook JD, Lee JE (2013) The secret life of viral entry glycoproteins: moonlighting in immune evasion. PLoS Pathog 9: e1003258.
38. Kropff B, Burkhardt C, Schott J, Nentwich J, Fisch T, *et al.* (2012) Glycoprotein N of human cytomegalovirus protects the virus from neutralizing antibodies. PLoS Pathog 8: e1002999.
39. Machiels B, Lete C, Guillaume A, Mast J, Stevenson PG, *et al.* (2011) Antibody evasion by a gammaherpesvirus O-glycan shield. PLoS Pathog 7: e1002387.
40. van den Wollenberg DJ, Dautzenberg IJ, van den Hengel SK, Cramer SJ, de Groot RJ, *et al.* (2012) Isolation of reovirus T3D mutants capable of infecting human tumor cells independent of junction adhesion molecule-A. PLoS One 7: e48064.
41. Adhikary D, Behrends U, Boerschmann H, Pfunder A, Burdach S, *et al.* (2007) Immunodominance of lytic cycle antigens in Epstein-Barr virus-specific CD4+ T cell preparations for therapy. PLoS One 2: e583.
42. Büll C, Boltje TJ, Wassink M, de Graaf AM, van Delft FL, *et al.* (2013) Targeting aberrant sialylation in cancer cells using a fluorinated sialic acid analog impairs adhesion, migration, and in vivo tumor growth. Mol Cancer Ther 12: 1935-1946.

Chapter 4

Epstein-Barr virus BILF1 evolved to downregulate cell surface display of a wide range of HLA class I molecules through their cytoplasmic tail

Bryan D. Griffin[1,2,*], Anna M. Gram[2,*], Arend Mulder[3], Daphne Van Leeuwen[2], Frans H.J. Claas[3], Fred Wang[4], Maaike E. Ressing[1,2,#], and Emmanuel Wiertz[1,2,#]

*,# These authors contributed equally to this work

[1]Department of Medical Microbiology, University Medical Center Utrecht, The Netherlands, [2]Department of Medical Microbiology, Leiden University Medical Center, The Netherlands, [3]Department of Immunohaematology and Blood Transfusion, Leiden University Medical Center, The Netherlands, [4]Department of Medicine, Brigham & Women's Hospital, Harvard Medical School, Boston, USA

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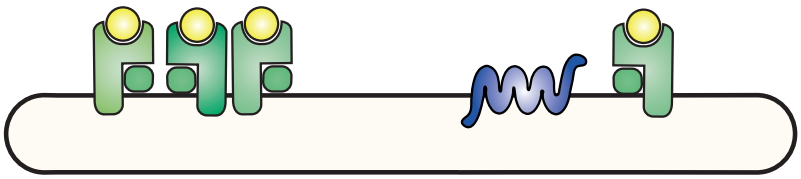

Abstract

Co-evolution of herpesviruses and their hosts has driven the development of both host antiviral mechanisms to detect and eliminate infected cells, and viral ploys to escape immune surveillance. Among the immune evasion strategies employed by the lymphocryptovirus (g_1-herpesvirus) EBV is the downregulation of surface HLA class I expression by the virally-encoded G-protein coupled receptor BILF1, thereby impeding presentation of viral antigens and cytotoxic T cell recognition of the infected cell. Here, we show EBV BILF1 to be expressed early in the viral lytic cycle. BILF1 targets a broad range of HLA class I molecules, including multiple HLA-A and -B types, and HLA-E. In contrast, HLA-C was only marginally affected. We advance the mechanistic understanding of the process by showing the cytoplasmic C-terminal tail of EBV BILF1 to be required for reducing surface HLA class I expression. Susceptibility to BILF1-mediated downregulation is in turn conferred by specific residues in the intracellular tail of the HLA class I heavy chain. Finally, we explore the evolution of BILF1 within the lymphocryptovirus genus. While the homolog of BILF1 encoded by the lymphocryptovirus infecting Old World rhesus primates shares the ability of EBV to downregulate cell surface HLA class I expression, this function is not possessed by New World marmoset lymphocryptovirus BILF1. This study therefore furthers our knowledge on the evolution of immunoevasive functions by the lymphocryptovirus genus of herpesviruses.

Introduction

Lymphocryptoviruses (LCVs) comprise a genus of the γ-herpesvirus subfamily whose members are only found in primates [1]. The LCV targeting humans, Epstein-Barr virus (EBV), is carried by more than 90% of adults worldwide [2, 3]. While infection is usually asymptomatic, primary encounter with the virus can present as infectious mononucleosis. EBV infection is also strongly associated with tumours of lymphoid and epithelial origin, reflecting the tropism of the virus for B cells and epithelial cells, and its potential for oncogenic transformation [4].

Despite the activation of a robust host T cell response upon primary infection, and the capacity for a memory T cell response thereafter, the virus persists for life even in immunocompetent individuals [5]. This is partly due to the ability of EBV, like all herpesviruses, to enter a state of latency in which protein expression is minimized, thereby limiting viral antigen display by the infected cell. Yet in order for EBV to spread to a new host, it must produce infectious virions by entering the replicative, or lytic, phase of its life cycle. In this phase, over 80 EBV-encoded proteins are expressed in a temporal cascade, with initial production of immediate early (IE) transactivators triggering induction of early genes, including enzymes required for viral replication [2]. This is followed by expression of late genes encoding virion structural components. The lytic phase thus creates ample viral antigens for proteasomal processing and HLA class I cell surface presentation, which can then recruit memory $CD8^+$ T cells capable of eliminating the infected cell. However, millions of years of co-evolution with their hosts have seen herpesviruses acquire active immune evasion mechanisms to thwart this host response and permit sufficient time for the lytically infected cell to generate new virus particles [6-8].

In the case of EBV, several such immune evasion strategies target the HLA class I antigen processing and presentation pathways [9]. The EBV host shutoff protein BGLF5 degrades mRNA, thus obstructing synthesis of new HLA class I molecules [10, 11]. Meanwhile, BNLF2a blocks entry of proteasome-generated peptides into the ER and subsequent loading onto HLA class I molecules by inhibiting the heterodimeric TAP complex [12, 13]. TAP transport is also hindered by the viral chemokine homolog, vIL-10, which reduces expression of the TAP1 subunit [14]. Another manner in which EBV can downregulate cell surface HLA class I expression and inhibit T cell recognition of infected cells is through BILF1, a viral G-protein coupled receptor (vGPCR) [15, 16].

Several poxviruses and herpesviruses encode GPCRs that were most likely pirated from their host by retrotransposition [17-19]. vGPCRs serve many functions, including the scavenging of host chemokines [20], cell-to-cell-adhesion [21] and the re-programming of intracellular signalling networks to promote efficient viral replication [22]. BILF1 is expressed during the EBV lytic cycle and was first identified as a potential vGPCR due to the presence of seven membrane-spanning domains and (limited) homology with known herpesviral GPCRs [23-25]. While it displays both structural and functional similarities to chemokine receptors,

EBV BILF1 modulates intracellular signalling pathways constitutively as an orphan receptor [24, 25].

Initial immune evasion ability was ascribed to EBV BILF1 as it reduced phosphorylation of the dsRNA-dependent protein kinase R [24]. It has also been found to heterodimerize with human chemokine receptors [26]. In the case of CXCR4 this results in impairment of ligand-induced receptor signalling [27]. Finally, EBV BILF1 decreases cell-surface levels of HLA class I [15]. This can be achieved by BILF1-mediated acceleration of endocytosis and subsequent lysosomal degradation of HLA class I molecules from the cell surface, or by diversion of newly synthesized HLA class I molecules from the normal exocytic pathway that allows proteins to travel from the ER to the cell surface ([16]). In turn, this leads to the inhibition of $CD8^+$ T cell recognition of infected cells.

Here, we further investigate the ability of EBV BILF1 to subvert the antigen presentation pathway. We assess the ability of BILF1 to target a range of specific HLA class I alleles and identify the protein domain present in BILF1 and specific amino acid residues in the HLA class I heavy chain (HC) that are required to facilitate downregulation of HLA class I from the cell surface. Finally, we examine the evolution of BILF1 by comparing the immunoevasive ability of EBV BILF1 with that of homologs expressed by LCVs infecting Old World rhesus and New World marmoset primates.

Results

EBV BILF1 is an early lytic cycle gene

Previous reports present conflicting information on the expression of BILF1 during the productive phase of EBV infection [24, 25, 35]. As the absence of a working anti-BILF1 antibody precluded the detection of BILF1 protein, we monitored the appearance of BILF1 mRNA in an EBV^+ Akata-derived B cell line following induction of the viral lytic cycle by cross-linking of the B cell receptor. BILF1 expression was strongly induced by 4 h and remained elevated up to 16 h post-induction (**Figure 1A**). The faint band observed for uninduced cells may indicate a low level of BILF1 expression, as has been described for other EBV^+ B cell lines under strict latency [24]. PAA inhibits the viral DNA polymerase and transcription of late lytic genes, and can thus be used to dissect the temporal profile of lytic gene expression. While expression of the gp42-encoding late gene BZLF2 was detectable after overnight lytic cycle induction, it was completely blocked by prior addition of PAA (**Figure 1B** upper panel). In contrast, BILF1 mRNA was still induced in the presence of PAA (middle panel). Together, these data show EBV BILF1 to be an early lytic cycle gene.

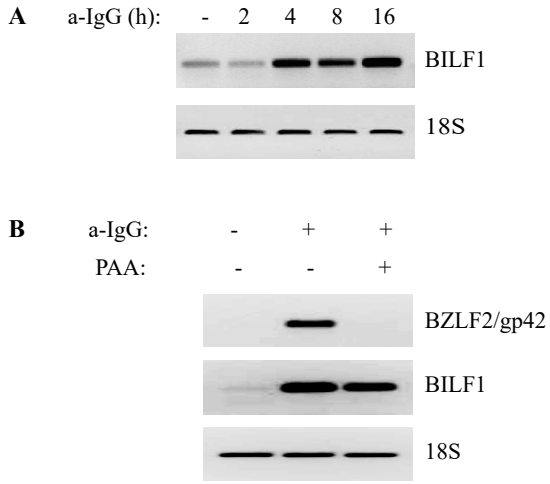

Figure 1 The BILF1 gene is expressed early in the EBV lytic cycle. EBV lytic cycle gene expression was induced in AKBM cells by cross-linking of the B cell receptor with α-IgG for the indicated time periods (hours) (A), or for 16 h in the presence or absence of PAA (B). RNA was isolated, cDNA generated, and expression levels of the indicated gene products determined by PCR and EtBr gel electrophoresis.

EBV BILF1 can selectively modulate cell surface expression of HLA class I alleles

To examine its effect on individual HLA class I alleles, cells with different HLA haplotypes were transduced to express EBV BILF1. In a lentiviral vector, the EBV BILF1 gene was cloned upstream of an internal ribosomal entry site that is followed by the gene encoding enhanced GFP. Transduced cells could thus be identified easily as a GFP^+ population. In addition, a FLAG-tag added to the BILF1 N-terminus made it possible to confirm surface expression of the viral protein. Cells were transduced with control GFP or EBV BILF1/GFP-encoding lentivirus and the presence of surface markers was analysed by flow cytometry after 7 days. Prior to staining, transduced and untransduced cells were mixed to allow comparison in a single assay.

Melanoma-derived Mel JuSo (MJS) cells [30], widely used in antigen presentation studies due to the expression of both HLA class I and II, displayed strong cell surface BILF1 expression after transduction with BILF1/GFP lentivirus (**Figure 2A**, upper row). Total surface HLA class I expression was reduced in BILF1/GFP-expressing cells compared to untransduced cells and cells expressing GFP alone (second row). However, BILF1 did not decrease cell surface HLA class II expression (third row). Through the use of alloantigen-specific antibodies, the effect of BILF1 on individual HLA class I types expressed by MJS cells was then evaluated. BILF1 reduced expression of HLA-A1 (fourth row) and mediated a particularly strong decrease in surface HLA-B8 expression (bottom row).

HeLa cells expressing EBV BILF1 after lentiviral transduction also displayed reduced surface levels of W6/32-reactive HLA class I (**Figure 2B**). Specific staining of both HLA-A68 and HLA-B15 showed that expression of both was decreased in $BILF1^+$ HeLa cells. Similarly, surface HLA class I levels in $BILF1^+$ HEK 293T cells were reduced compared to control cells (**Figure 2C**). Downregulation of HLA-B7 and a moderate decrease in HLA-A2 levels contributed to this effect (**Figure 2C**). Taking these data together, EBV BILF1 can thus target

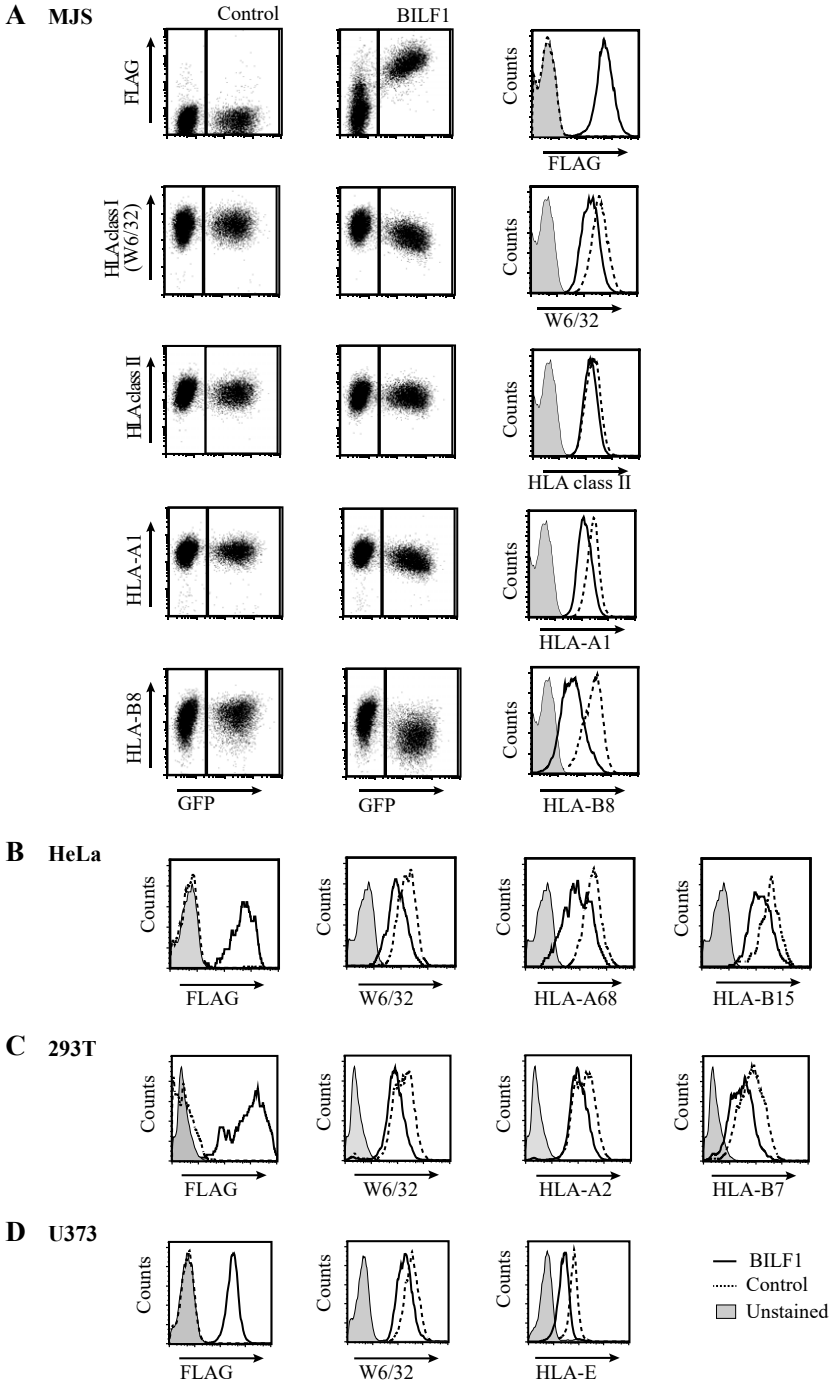
A MJS
Control
BILF1
FLAG
Counts
FLAG
HLA class I (W6/32)
W6/32
HLA class II
HLA class II
HLA-A1
HLA-A1
HLA-B8
GFP
GFP
HLA-B8
B HeLa
FLAG
W6/32
HLA-A68
HLA-B15
C 293T
FLAG
W6/32
HLA-A2
HLA-B7
D U373
FLAG
W6/32
HLA-E
BILF1
Control
Unstained

Figure 2 EBV BILF1 reduces surface expression of a broad range of HLA class I alleles. *A*, MJS cells were transduced with GFP-encoding (control) or EBV BILF1/GFP-encoding (BILF1) replication-deficient lentivirus. After 7 days, surface expression of FLAG-tagged EBV BILF1, total HLA class I, HLA class II, HLA-A1 and HLA-B8 was determined by flow cytometry. Transduced and untransduced MJS cells were mixed before antibody staining to allow comparison in a single assay (left and middle columns). Surface levels of the indicated proteins were also compared between GFP^+ control cells and $BILF1/GFP^+$ cells (histograms, right column). *B*, HeLa; *C*, 293T and *D*, U373 cells were transduced with GFP-encoding (control) or EBV BILF1/GFP-encoding (BILF1) lentivirus. Surface expression of FLAG-tagged EBV BILF1, total HLA class I or of the indicated HLA class I alleles was examined by flow cytometry after 7 days.

HLA-A and -B gene products, giving a reduction in expression of W6/32-reactive HLA class I molecules at the cell surface.

We next examined whether expression of the non-classical HLA-E is also affected by BILF1. U373 cells expressing endogenous HLA-E were lentivirally transduced to achieve BILF1 expression. BILF1 caused a decrease in cell surface levels of W6/32-reactive HLA class I, molecules (**Figure 2D**). Interestingly, $BILF1^+$ cells also displayed reduced levels of HLA-E, demonstrating that EBV BILF1 can target a wide range of HLA class I molecules to bring about a reduction in cell surface HLA class I expression.

Finally, we assessed whether EBV BILF1 can also downregulate cell surface levels of HLA-C. Although poorly expressed by many cell types, HLA-C is expressed at the surface of U937 myeloid cells and can be specifically detected using the human antibody WK4C11 [36]. N-terminally HA-tagged BILF1 was efficiently expressed in U937 cells following retroviral transduction (**Figure 3**, upper row), which led to a reduction in total surface HLA class I expression (second row). While surface expression of HLA-A3 (third row) and HLA-B18 (fourth row) was strongly reduced by BILF1, HLA-C was only marginally downregulated (bottom row). EBV BILF1 can therefore selectively decrease cell surface levels of HLA-A, -B and -E alleles, while only slightly affecting HLA-C.

The C-terminal tail of EBV BILF1 is required for downregulation of surface HLA class I

The C-terminal domain of GPCRs is known to interact with intracellular proteins including endocytic adaptors [37]. To assess whether the C-terminal domain of EBV BILF1 is involved in downregulation of surface HLA class I molecules, we generated a C-terminal deletion mutant, BILF1-ΔC19, which lacks the 19 most C-terminally-located amino acid residues (**Figure 4A**). This C-terminal deletion mutant retained some of the characteristics of its wt counterpart. EBV BILF1wt activates the transcription factor NFκB in a manner dependent on the integrity of the EKT motif in its third transmembrane domain [15]. BILF-ΔC19 expression also activated NFκB in HEK 293-kB luc cells following transient transfection (**Figure 4B**).

BILF1-ΔC19, containing an N-terminal FLAG-tag, was cloned into a lentiviral expression vector. MJS cells were then transduced with control, BILF1wt, or BILF1-ΔC19 lentivirus. Anti-FLAG staining showed that similar levels of BILF1wt and BILF1-ΔC19 were expressed at

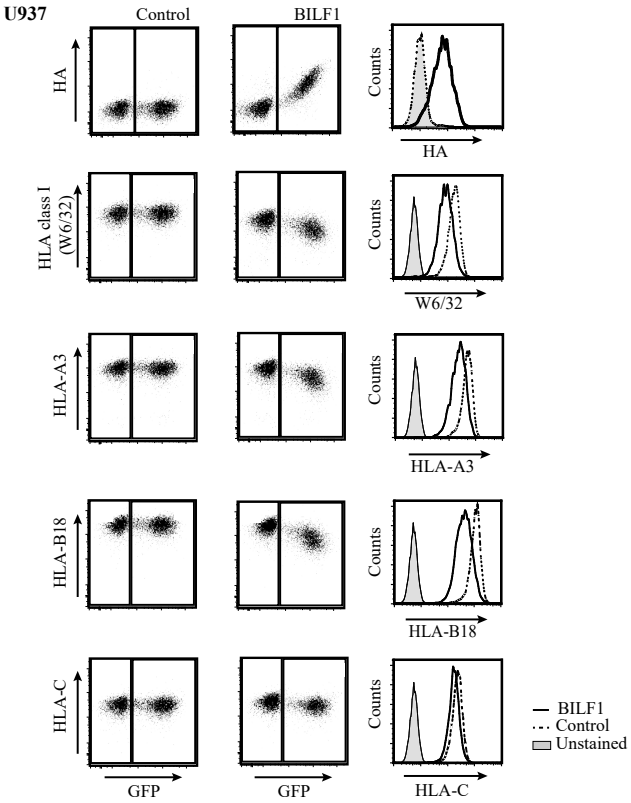

Figure 3 EBV BILF1 only marginally downregulates cell surface HLA-C expression. U937 cells were transduced with GFP-encoding (control) or EBV BILF1/GFP-encoding (BILF1) replication-deficient retrovirus. Surface expression of HA-tagged EBV BILF1, total HLA class I, HLA-A3 and HLA-B18 and HLA-C was determined by flow cytometry. Surface levels of the indicated proteins were compared between BILF1$^-$/GFP$^-$ cells and BILF1$^+$/GFP$^+$ cells (histograms, right column).

the surface of transduced cells (**Figure 4C**). However, whereas MJS cells expressing BILF1 wt displayed a concomitant decrease in surface HLA-B8 expression, BILF1-ΔC19 was severely diminished in its ability to downregulate HLA-B8. Meanwhile, neither BILF1 wt nor BILF1-ΔC19 significantly altered the surface levels of HLA class II. This indicates that the C-terminal domain of EBV BILF1, specifically the 19 last C-terminal residues, is required to bring about the downregulation of HLA class I from the cell surface.

Inspection of the sequence of the BILF1 C-terminus identified two potential internalization motifs (highlighted in dashed-line boxes in **Figure 4A**). The first was QVTV, a putative Type II PDZ ligand sequence, conforming to the consensus sequence X-Φ-X-Φ, where X is any amino acid and Φ is a bulky hydrophobic residue [37, 38]. The second was a potential non–classical tyrosine-based motif, YFRRV, conforming to the consensus sequence Y-X-X-X-Φ [39, 40]. We thus generated two independent BILF1 point mutant proteins, substituting an alanine residue for either V299 of the putative Type II PDZ ligand sequence or Y303 of the proposed tyrosine based motif. The resulting point mutants were both expressed at the cell surface. However, the ability to downregulate HLA class I was retained in both cases, demonstrating that neither putative motif contributes to EBV BILF1-mediated downregulation of surface HLA class I (**Supplementary Figure I**).

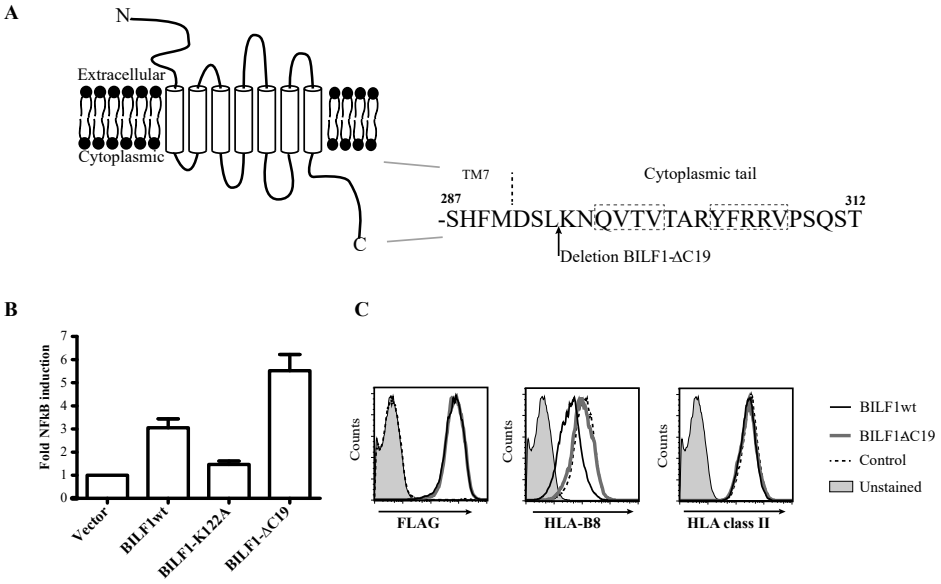

Figure 4 The EBV BILF1 C-terminal tail is required for HLA class I downregulation. *A*, Schematic representation of the 7-transmembrane EBV BILF1 vGPCR with the sequence of the 26 most C-terminally located amino acid residues described. The arrow indicates the point of truncation used to generate EBV BILF1-ΔC19. Dashed-line boxes indicate putative endocytosis/sorting motifs. *B*, EBV BILF1-ΔC19 activates intracellular signalling pathways. 293-NFκB-luciferase cells were co-transfected with control expression vector or constructs encoding EBV BILF1wt, EBV BILF1-K122A or EBV BILF1-ΔC19, and pGL3-*Renilla* (for normalizing transfection efficiency) luciferase. At 24 h post-transfection, cell lysates were assayed for firefly and *Renilla* luciferase activity. Data are presented as fold NFκB induction relative to cells transfected with vector alone. Results represent mean +/- SEM of a representative experiment performed in triplicate. *C*, MJS cells were transduced with GFP-encoding (control), EBV BILF1wt/GFP-encoding (BILF1wt) or EBV BILF1-ΔC19/GFP-encoding (ΔC19) replication-deficient lentivirus. After 7 days, surface expression of FLAG-tagged EBV BILF1, HLA-B8 and HLA class II was determined by flow cytometry.

The intracellular region of the HLA class I HC is required for downregulation by BILF1

Given the requirement for the C-terminal domain of BILF1 in bringing about a reduction of surface HLA class I, we further hypothesized that the intracellular region of the HLA class I HC might also be necessary. We therefore generated a form of the HLA-B8 HC from which the 24 most C-terminal amino acid residues were deleted, leaving only 6 intracellular residues (HLA-B8 short; **Figure 5A**). Full-length HLA-B8 and HLA-B8 short were cloned into a lentiviral expression vector that co-expressed the control protein truncated nerve growth factor receptor (trNGFR). The U373 cell line, which is susceptible to the effects of BILF1 on HLA class I (**Figure 2D**) and does not express endogenous HLA-B8 (**Figure 5B**), was then transduced with either HLA-B8 wt/trNGFR or HLA-B8 short/trNGFR lentivirus. Equivalent

surface levels of trNGFR and of wt and short HLA-B8 were observed in the respective U373 transductants (**Figure 5B**).

The U373-HLA-B8wt and U373-HLA-B8 short cell lines were next transduced with either control or BILF1 lentivirus. BILF1 functionality in both cell lines was confirmed by its ability to downregulate surface expression of the endogenously expressed HLA-B18 allele to a similar extent (**Figure 5C**).

Interestingly, BILF1 caused a decrease in HLA-B8wt levels in U373 cells, but failed to reduce surface levels of HLA-B8 short. Surface levels of trNGFR were not affected by BILF1, while surface expression of BILF1 itself was equivalent in U373-HLA-B8wt and U373-HLA-B8 short cells (**Supplementary Figure II**). These data therefore show that in addition to the C-terminal domain of BILF1, the intracellular region of the HLA class I HC is required for BILF1-mediated downregulation of HLA class I surface expression.

Having assessed the effect of BILF1 on a wide range of HLA class I alleles (**Figures 2** and **3**), we next examined whether the selective targeting of surface HLA-A, -B and -E, but not HLA-C, molecules could help pinpoint key residues in the HLA class I HC tail that facilitate BILF1-mediated downregulation (**Figure 6A**). Comparing the amino acid sequence of the BILF1-sensitive HLA-B intracellular tails with those of BILF1-resistant HLA-C showed that discriminating differences exist at positions 344, 351, 358 and 361 of the HLA-B alleles as indicated in **Figure 6A**. We therefore generated point mutations in HLA-B8 to examine whether the replacement of HLA-B8 residues with those found in the corresponding positions of the HLA-C intracellular tail would render the mutant HLA protein resistant to the effects of BILF1. The C-tail sequence of the HLA-B8 point mutants used is indicated in **Figure 6A**, lower panel.

U937 cells, which do not express endogenous HLA-B8 (data not shown), were transduced to express HLA-B8 wt or HLA-B8 point mutant proteins. Surface levels of endogenously expressed HLA-B18 were reduced by BILF1 to a similar extent in U937-HLA-B8 wt and U937-HLA-B8 point mutant cell lines (**Figure 6B**). However, while BILF1 downregulated cell surface HLA-B8 wt, it did not decrease cell surface HLA-B8 $Y_{344}C/D_{351}N/V_{358}E/T_{361}I$ expression (**Figure 6B**). This strongly indicated that one, some, or all of the HLA-B8 residues mutated in HLA-B8 $Y_{344}C/D_{351}N/V_{358}E/T_{361}I$ are required to facilitate targeting by BILF1.

In further analysis of HLA-B8 point mutants, surface expression of HLA-B8 $T_{361}I$ was efficiently decreased by BILF1, demonstrating that residue T361 is not essential for BILF1-mediated HLA-B8 downregulation. Indeed, as with HLA-B8 $Y_{344}C/D_{351}N/V_{358}E/T_{361}I$, surface levels of HLA-B8 $Y_{344}C/D_{351}N/V_{358}E$ remained unaffected by BILF1. Individual mutation of residues Y344, D351 and V358 has not allowed a single amino acid to be identified as being essential to targeting of HLA-B8 by BILF1 (data not shown). Rather, the possibility exists that each could play a contributory role.

In conclusion, the BILF1-mediated downregulation of HLA class I molecules requires their cytoplasmic domains; the amino acid residues at position 344, 351 and 358 appear to contribute essentially to the observed phenotype in the case of HLA-B8.

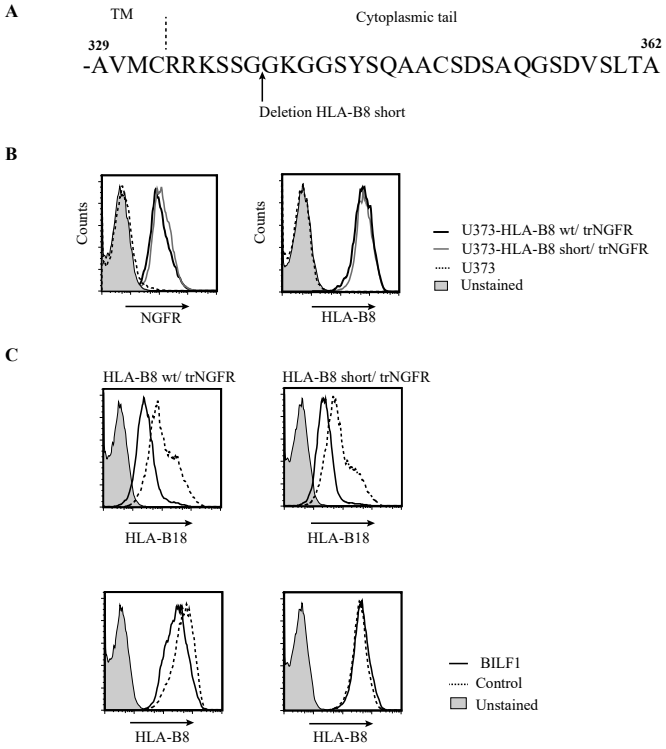

Figure 5 The cytoplasmic region of HLA class I HC is required for BILF1-mediated cell surface downregulation. *A*, Sequence of the HLA-B8 C-terminus. The arrow denotes the point of truncation used to generate HLA-B8 short. *B*, U373 cells were transduced with HLA-B8wt/trNGFR-encoding or HLA-B8 short/trNGFR-encoding replication-deficient lentivirus. Surface expression of HLA-B8 and trNGFR on transduced and untransduced cells was determined by flow cytometry. *C*, U373-HLA-B8wt and U373-HLA-B8 short cells were further transduced with GFP-encoding (control) or EBV BILF1/GFP-encoding (BILF1) replication-deficient lentivirus. After 7 days, surface expression of HLA-B8 and the endogenous HLA-B18 allele was measured by flow cytometry.

Marmoset LCV BILF1 fails to downregulate cell surface MHC class I

BILF1 homologs are encoded by rhesus LCV (80.4% amino acid identity to EBV BILF1 in an NCBI blastp two-sequence comparison) and marmoset LCV (41% identity to EBV BILF1) (**Figure 7A**). Rhesus LCV BILF1 has previously been shown to mediate MHC class I downregulation [15], but the functionality of marmoset LCV BILF1 in this regard is unknown. Cloning the rhesus and marmoset LCV BILF1 genes into a lentiviral expression vector allowed us to directly compare the ability of the three LCV BILF1 homologs to downregulate surface HLA class I in MJS cells. Both EBV BILF1 and rhesus LCV BILF1 strongly decreased surface HLA-B8 in lentivirally transduced MJS cells, but marmoset LCV BILF1 failed to do so (**Figure 7B**). HLA class II was not affected by expression of any of the BILF1 proteins (data not shown).

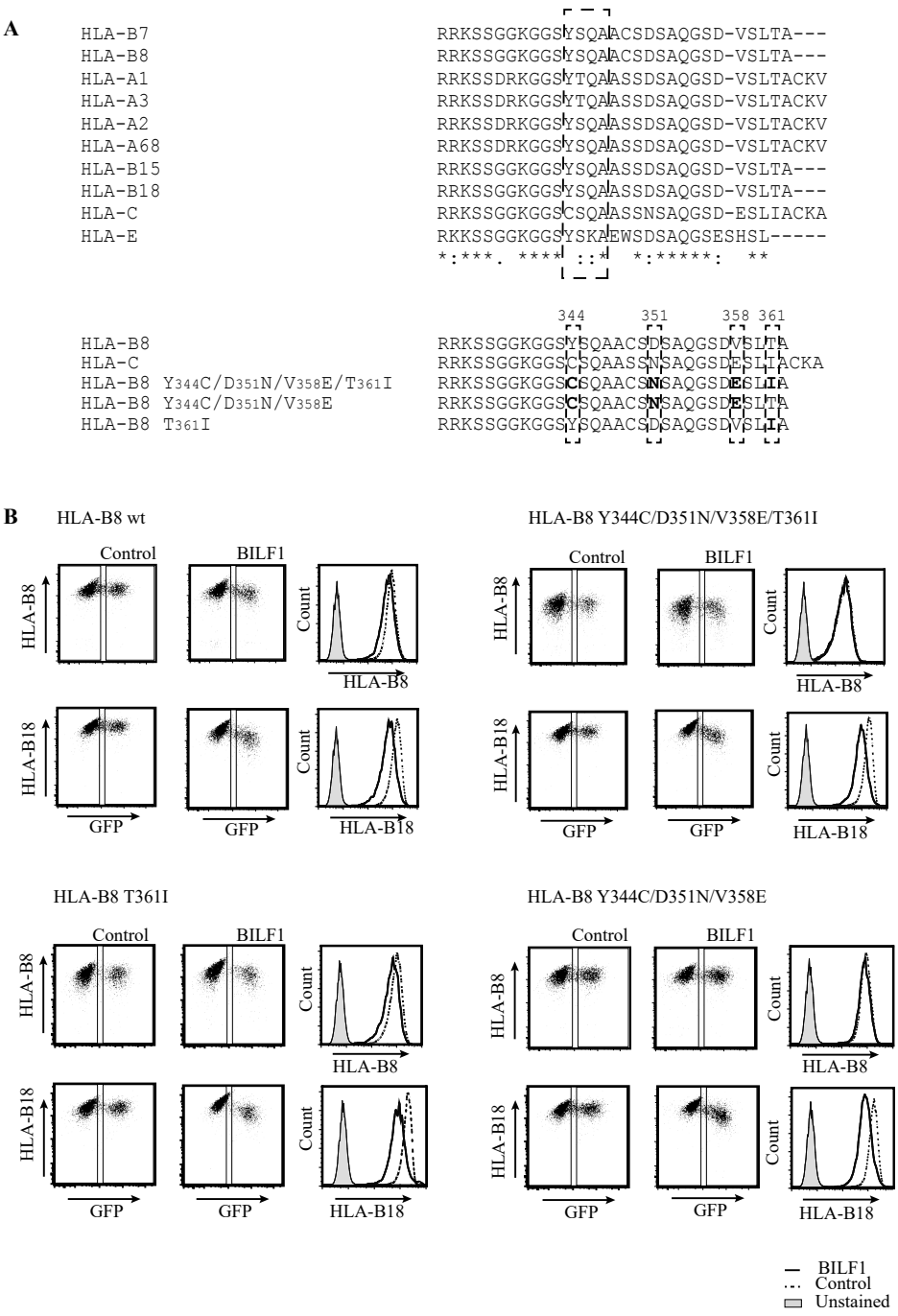
A
HLA-B7 RRKSSGGKGGSYSQAACSDSAQGSD-VSLTA---
HLA-B8 RRKSSGGKGGSYSQAACSDSAQGSD-VSLTA---
HLA-A1 RRKSSDRKGGSYTQAASSDSAQGSD-VSLTACKV
HLA-A3 RRKSSDRKGGSYTQAASSDSAQGSD-VSLTACKV
HLA-A2 RRKSSDRKGGSYSQAASSDSAQGSD-VSLTACKV
HLA-A68 RRKSSDRKGGSYSQAASSDSAQGSD-VSLTACKV
HLA-B15 RRKSSGGKGGSYSQAASSDSAQGSD-VSLTA---
HLA-B18 RRKSSGGKGGSYSQAASSDSAQGSD-VSLTA---
HLA-C RRKSSGGKGGSCSQAASSNSAQGSD-ESLIACKA
HLA-E RKKSSGGKGGSYSKAEWSDSAQGSESHSL-----
344 351 358 361
HLA-B8 RRKSSGGKGGSYSQAACSDSAQGSDVSLTA
HLA-C RRKSSGGKGGSCSQAASSNSAQGSDESLIACKA
HLA-B8 Y344C/D351N/V358E/T361I RRKSSGGKGGSCSQAACSNSAQGSDESLIA
HLA-B8 Y344C/D351N/V358E RRKSSGGKGGSCSQAACSNSAQGSDESLTA
HLA-B8 T361I RRKSSGGKGGSYSQAACSDSAQGSDVSLIA
B
HLA-B8 wt
HLA-B8 Y344C/D351N/V358E/T361I
HLA-B8 T361I
HLA-B8 Y344C/D351N/V358E
Control
BILF1
HLA-B8
HLA-B18
Count
GFP
BILF1
Control
Unstained

Figure 6 EBV BILF1-mediated HLA-B8 downregulation requires amino acid residues in the cytoplasmic region of the HLA-B8 molecule. *A*, *upper panel*, Amino acid sequence alignment of selected HLA class I heavy chain C-terminal tails. Alignments were generated using ClustalW2. An asterisk (*) indicates positions that have a single, fully conserved residue. A colon (:) indicates conservation between groups of strongly similar properties. A full stop (.) indicates conservation between groups of weakly similar properties. Sequences of the following subtypes were selected from the IMGT/HLA Database: A*01:01:01:01 (HLA-A1), A*02:01:01:01 (HLA-A2), A*03:01:01:01 (HLA-A3), A*68:01:01:01 (HLA-A68), B*07:02:01 (HLA-B7), B*08:01:01 (HLA-B8), B*15:03:01 (HLA-B15), B*18:01:01:01 (HLA-B18), C*01:02:01 (HLA-C), E*01:01:01:01 (HLA-E). The dashed box indicates a putative YXXA internalization motif present in HLA-A, -B and -E alleles but not HLA-C. *A, lower panel,* C-terminal tail amino acid sequences of HLA-B8 point mutants. Amino acids in the dashed boxes were subject to substitution. The numbers above the dashed boxes indicate the position of the residue in the HLA-B8 protein. *B,* U937 cells transduced to express HLA-B8 wild type, HLA-B8 $Y_{344}C/D_{351}N/V_{358}E/T_{361}I$, HLA-B8 $T_{361}I$, or HLA-B8 $Y_{344}C/D_{351}N/V_{358}E$ were transduced with GFP-encoding (control) or EBV BILF1/GFP-encoding (BILF1) replication-deficient retrovirus. Surface expression of HLA-B8 and HLA-B18 was determined by flow cytometry. Surface levels of the indicated proteins were compared between $BILF1^-/GFP^-$ cells and $BILF1^+/GFP^+$ cells (histograms).

All three BILF1 homologs contained N-terminal FLAG tags, allowing their surface expression to be assessed (**Figure 7B**). Marmoset LCV BILF1 was efficiently expressed at the cell surface, albeit to a lesser extent than its EBV and rhesus LCV counterparts. Further analysis showed HLA-B8 levels to be reduced in the population of MJS cells expressing EBV or rhesus LCV BILF1 at an equivalent level to that obtained with marmoset LCV BILF1 (**Figure 7C**).

Although marmoset LCV BILF1 failed to reduce surface expression of MHC class I in human cells, it remained possible that the viral protein could be functional in this respect in marmoset cells. We thus transiently transfected primary marmoset fibroblasts with a vector offering co-expression of FLAG-tagged marmoset LCV BILF1 and GFP. Marmoset MHC class I on these cells was detectable using the W6/32 mAb. At 48 hours post-transfection, transfected cells were identified by GFP expression. No decrease in surface MHC class I was evident in cells transfected with the marmoset LCV BILF1-IRES-GFP vector (**Figure 7D**; left, upper panel). As a population of GFP^+ cells failed to express detectable levels of marmoset LCV BILF1 at the surface (middle, upper panel), a double-staining procedure was performed to more precisely assess MHC class I levels in cells clearly expressing marmoset LCV BILF1. This confirmed that GFP^+ marmoset cells containing high surface levels of FLAG-tagged marmoset LCV BILF1 did not undergo a downregulation of MHC class I (right, upper panel). EBV BILF1 also failed to reduce surface expression of marmoset MHC class I (**Figure 7D**, lower panels). This indicates that a species restriction exists with regard to EBV BILF1 function, and suggests that a sufficient level of homology with the host protein(s) required for EBV BILF1-mediated MHC class I downregulation may not be present in marmoset cells.

Given the importance of the C-terminal domain of EBV BILF1 in mediating its effect towards HLA class I, we examined finally whether this domain was itself sufficient to confer the ability to downregulate HLA class I to a heterotypic protein. We therefore generated a chimeric BILF1 protein in which the 19 most C-terminal amino acids of marmoset LCV BILF1 were removed and replaced by those of EBV BILF1 (marmoset LCV-EBV C-tail swap BILF1).

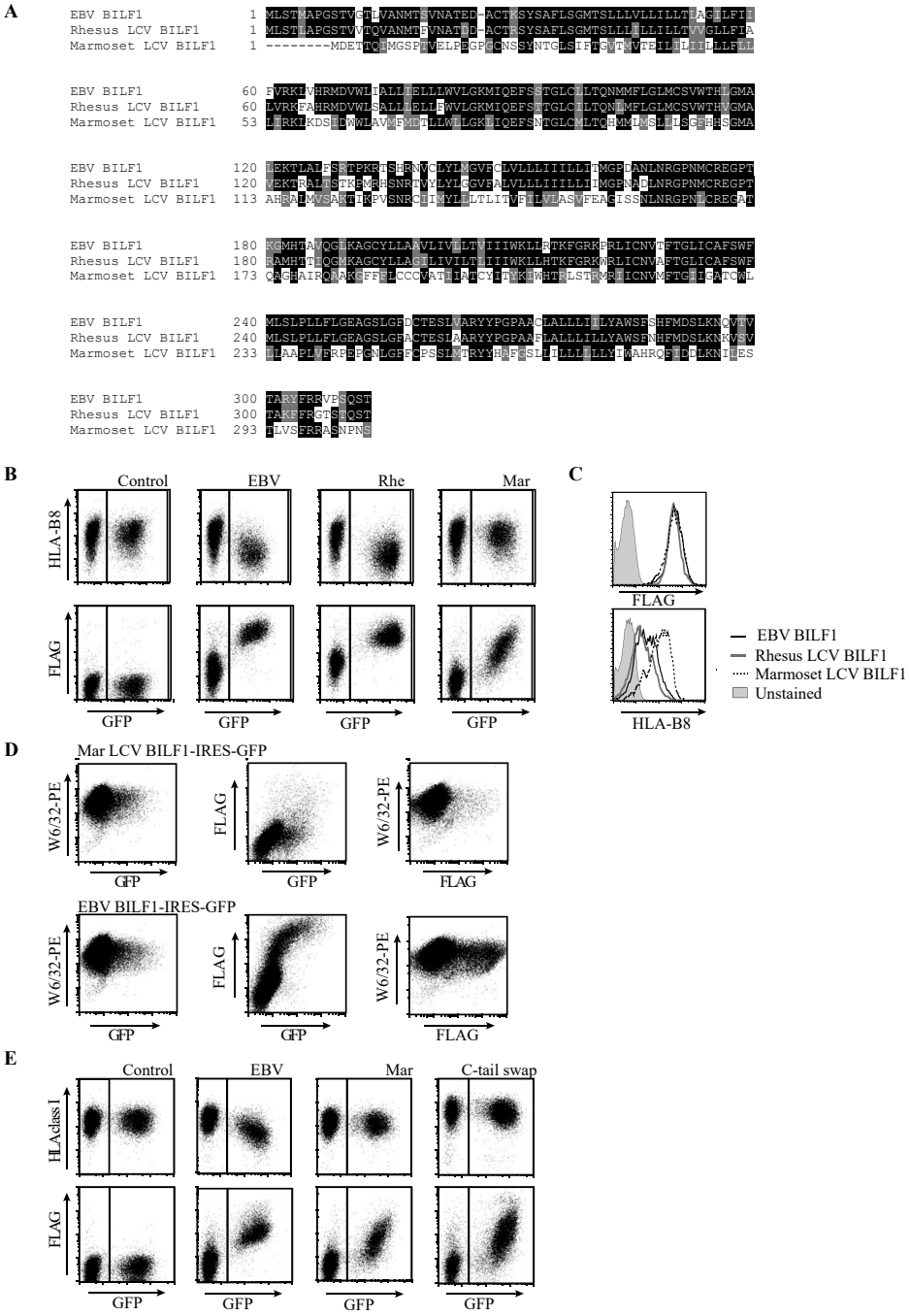
A
EBV BILF1
Rhesus LCV BILF1
Marmoset LCV BILF1
B
Control
EBV
Rhe
Mar
HLA-B8
FLAG
GFP
C
FLAG
HLA-B8
EBV BILF1
Rhesus LCV BILF1
Marmoset LCV BILF1
Unstained
D
Mar LCV BILF1-IRES-GFP
EBV BILF1-IRES-GFP
W6/32-PE
FLAG
GFP
E
Control
EBV
Mar
C-tail swap
HLAclass I
FLAG
GFP

Figure 7 MHC class I is not downregulated by the marmoset LCV BILF1 homolog. *A*, Amino acid sequence alignment of the BILF1 homologs encoded by EBV, rhesus LCV and marmoset LCV. Alignments were constructed using ClustalW2 and displayed using BOXSHADE version 3.21. *B*, MJS cells were transduced with GFP- (control), EBV BILF1/GFP- (EBV), rhesus LCV BILF1/GFP- (Rhe) or marmoset LCV BILF1 (Mar)-encoding replication-deficient lentivirus. After 7 days, surface expression of FLAG-tagged EBV BILF1 and HLA-B8 was determined by flow cytometry. Transduced and untransduced MJS cells were mixed before antibody staining to allow comparison in a single assay. *C*, Populations of transduced MJS cells expressing equivalent surface levels of FLAG-tagged EBV, rhesus LCV and marmoset LCV BILF1 were further analysed to examine their HLA-B8 surface expression levels. *D*, Marmoset primary fibroblasts were transfected with EBV or marmoset LCV FLAG-BILF1 genes in the pLV-IRES-GFP bicistronic vector. 48 h post-transfection, cells were stained with PE-conjugated W6/32 mAb, or with anti-FLAG and APC-conjugated anti-mouse antibody. Double staining of samples was performed to determine surface MHC class I expression on cells displaying cell surface FLAG-BILF1. *E*, MJS cells were transduced with GFP- (control), EBV BILF1wt/GFP- (EBV), marmoset LCV BILF1wt- (Mar), or marmoset LCV-EBV C-tail swap (C-tail swap)-encoding replication-deficient lentivirus. After 7 days, surface expression of FLAG-tagged EBV BILF1 and HLA class I was determined by flow cytometry. Transduced and untransduced MJS cells were mixed before antibody staining to allow comparison in a single assay.

This LCV BILF1 fusion gene was cloned into a lentiviral expression vector and human cells were transduced with control, EBV BILF1wt, marmoset LCV BILF1wt, or C-tail swap BILF1 lentivirus. C-tail swap BILF1 was expressed at the cell surface at a similar level to marmoset LCV BILF1wt in MJS cells but did not mediate downregulation of HLA class I (**Figure 7E**). This indicates that the intracellular C-terminal domain of EBV BILF1 is necessary but not alone sufficient for bringing about a reduction in surface HLA class I levels.

Discussion

Central to the execution of host anti-viral immunity is the action of $CD8^+$ T lymphocytes in detecting and eliminating virally infected cells. Herpesviruses have counter-evolved several strategies to thwart this system of immune surveillance by inhibiting the display of MHC class I:viral peptide complexes at the cell surface [7-9]. EBV inhibition of the HLA class I antigen presentation pathway during the viral lytic cycle is mediated through the concerted action of BGLF5 [10, 11], BNLF2a [12, 13], BILF1 [15, 16] and vIL-10 [14]. In understanding the dynamic process of multigenic immune evasion during the productive phase of the EBV life cycle, it is important to ascertain the temporal profile of individual gene expression. Previous studies have shown both BNLF2a and BGLF5 protein to be detectable approximately 3 h after lytic cycle induction [11, 41]. The vIL-10-encoding BCRF1 gene, in contrast, is expressed late in the lytic cycle [14]. Conflicting reports exist on the temporal expression of EBV BILF1 [24, 25, 35]. Here, we show BILF1 to be an early EBV lytic cycle gene, with transcripts first detectable by 4 h post induction. While this suggests that BILF1 protein is first expressed in conditions where BGLF5-mediated host-shutoff and BNLF2a-mediated TAP inhibition have

already been established, BILF1 may function to remove pre-existing HLA class I molecules from the cell surface. Furthermore, BNLF2a protein expression is transient, whereas the detection of BILF1 transcripts 16 h post-induction suggests that BILF1 is able to target HLA class I molecules presenting viral antigens late in the lytic cycle.

Although we have previously shown EBV BILF1 to downregulate HLA class I in human cells [15], the use of the pan-HLA class I-reactive mAb W6/32 could have masked selective targeting of specific HLA class I molecules. Other herpesvirus proteins targeting HLA class I for degradation have been found to demonstrate HLA class I type specificity. The Kaposi's sarcoma-associated herpesvirus (KSHV) E3 ligases K3 and K5 mediate ubiquitination of cell-surface HLA class I molecules, which are subsequently endocytosed and degraded by the lysosome [42-45]. While K3 is broadly reactive towards HLA-A, -B, -C and -E, K5 is selective for HLA-A and -B [45]. Meanwhile, HCMV US2 induces dislocation of newly synthesized HLA class I molecules from the ER to the cytosol for proteasomal degradation [46]. US2 targets HLA-A and certain HLA-B types, while surface expression of HLA-C, HLA-E and other HLA-B alleles is not affected [47].

Here, we employed a panel of alloantigen-specific mAbs to examine for the first time the effect of BILF1 on expression of individual HLA class I alleles in different cell types. Most cells express HLA-A, -B alleles, along with low levels of HLA-E, and in some cases HLA-C. HLA-A and -B molecules are most important for the presentation of viral peptides, a fact reflected in their high level of polymorphism. Surface expression of all HLA-A and -B alleles tested, was reduced by BILF1. Interestingly, the HLA-A2, -B7 and -B8 molecules shown here to undergo BILF1-mediated surface downregulation have been found to present epitopes derived from lytic cycle proteins, expressed by vaccinia virus infection of B cells, to EBV-specific $CD8^+$ T cells [48]. As some of the epitopes were from IE antigens, this provides further support to the idea that EBV BILF1 may target pre-existing HLA class I molecules for endocytosis upon its synthesis approximately 4 h after lytic cycle induction.

Additionally, it has been proposed that the repertoire of peptides bound by HLA-A2, -B7 and -B8 include those that do not require TAP for transport into the ER [49]. The EBV transmembrane protein LMP2 contains HLA-A2-restricted TAP-independent $CD8^+$ T cell epitopes [50], while a HLA-B7-restricted T cell clone specific for the EBV lytic cycle protein BMRF1 has been isolated from a TAP-deficient individual [51]. It is therefore possible that HLA class I molecules loaded with peptide in the presence of BNLF2a-mediated TAP inhibition during the lytic cycle may still be targeted by BILF1.

Different HLA class I molecules possess distinct peptide-binding specificity and will thus present a unique spectrum of viral peptides to $CD8^+$ T cells. A mechanism of immune escape confined to a narrow subset of HLA-A and -B alleles would not provide a selective advantage to the virus in transmission to a wide range of hosts. BILF1 likely mediates a broad-spectrum inhibition of HLA-A and -B surface expression that is particularly useful in a virus targeting a population with such high HLA-A and -B polymorphism.

HLA-C molecules are predominantly involved in regulating natural killer (NK) cell

function. HLA-C interacts with NK cell inhibitory receptors to prevent NK cell-mediated lysis. Interestingly, whereas a range of HLA-A and -B alleles were downregulated from the cell surface by EBV BILF1, HLA-C was only slightly affected. Through selective modulation of classical HLA class I molecules, BILF1 could target HLA-A and -B alleles presenting viral peptides to CD8+ T lymphocytes, while allowing the virally-infected cell to retain the inhibitory effect of HLA-C on NK cells. In this way, BILF1 could assist EBV evasion of both adaptive and innate immune mechanisms.

HLA-E is also best known for its role in regulating NK cell function. By presenting peptides derived from the HLA-A, -B and -C signal sequences and acting as a ligand for the CD94/NKG2A NK cell inhibitory receptor complex, HLA-E prevents killing by NK cells. EBV BILF1 did however decrease surface expression of HLA-E. Reducing surface HLA-E expression and removal of this inhibitory signal from the virally infected cell may therefore appear detrimental to virus survival. However, HLA-E has also been shown to bind viral antigens, including a BZLF1-derived peptide [52]. HLA-E:viral peptide complexes can then be recognized by $CD8^+$ T cells [53]. It may thus be advantageous for EBV to target HLA-E through BILF1, with the increased risk of NK cell attack being offset by other NK cell immune evasion tactics, such as the retention of HLA-C expression, and the action of miRNA BART-2 in reducing expression of the NK cell activating ligand MICB [54].

Previous studies have shown BILF1 to accelerate internalisation of HLA class I molecules, which are subsequently targeted for lysosomal degradation [15]. In an effort to gain further mechanistic insight into EBV BILF1-mediated HLA class I downregulation, we focussed on the role of BILF1 C-terminal tail, as this region of GPCRs is often involved in intracellular sorting and interaction with endocytic adaptor proteins [55]. While the C-tail is required for cell surface expression in the case of some GPCRs [56], our EBV BILF1 truncation mutant lacking the 19 most C-terminal residues was still detectable at the plasma membrane. The BILF1 deletion mutant was still functional with respect to modulation of intracellular signalling, as it retained the capacity to activate NFκB. However, BILF1-ΔC19 displayed a substantially abrogated ability to downregulate HLA class I surface expression relative to BILF1wt. Using a BILF1 deletion mutant lacking the 21 most C-terminal amino acid residues, a study by Zuo *et al.* indicated that the BILF1 C-tail is required for directing endocytosed HLA class I molecules to lysosomes [16]. The results of the present study are therefore in agreement with those of Zuo *et al.* in identifying a critical role for the BILF1 C-tail in reducing HLA class I levels.

As the C-tail of EBV BILF1 therefore likely contains motifs involved in the targeting of HLA class I to lysosomes, we scanned this domain for the short, linear amino acid sequences that typically mediate trafficking from the cell surface and sorting to lysosomes. Two candidate putative motifs related to protein endocytosis and intracellular sorting were identified. One, a type II PDZ ligand sequence, can be involved in regulating endocytosis, in addition to protein recycling [37]. The second, a non-classical tyrosine-based signal differs from the more common consensus motif by containing an extra residue between the tyrosine

and hydrophobic residue [37, 40]. Tyrosine-based motifs are recognized by clathrin adaptor proteins and can direct both protein endocytosis and lysosomal targeting [39]. In the case of both motifs, substitution of a key amino acid residue by alanine has been previously shown to functionally inactivate the signal [40, 57]. However, the failure of such point mutations to block the effect of BILF1 on HLA class I surface expression indicates that other structural determinants with the vGPCR C-tail are involved.

In addition to demonstrating the importance of the BILF1 C-tail, we found the intracellular portion of the HLA class I HC to be essential for BILF1-mediated downregulation. Other viral immunoevasins targeting the class I HC display a similar requirement. For instance, KSHV K3 catalyzes ubiquitination of lysine residues in the HC C-tail, thereby tagging the protein for internalisation and degradation [58]. However, the fact that BILF1 expression does not trigger ubiquitination of class I HC [15] points towards another basis for this dependence in the case of the EBV immunoevasin.

We identified three amino acid residues in the HLA-B8 intracellular tail that may be essential for BILF1-mediated downregulation. One of these, Y344, forms part of the tyrosine-based YXXA internalisation motif, which is conserved and required for constitutive endocytosis in the case of HLA-B27 [59]. This motif is present in all alleles tested in this study that were downregulated by BILF1, but absent from HLA-C (**Figure 6A**) and the HLA-B8 short mutant (**Figure 5A**). As it is reminiscent of the YXXΦ signal, the YXXA motif may also interact with clathrin adaptor proteins. However, the observation that the AP-2 is not required for BILF1-mediated HLA class I downregulation [16] would argue against the interaction of clathrin adaptor proteins with the HLA class I YXXA motif in facilitating the accelerated endocytosis or intracellular sorting directed by BILF1.

Thus, while the specific EBV BILF1 internalisation and sorting motifs and the intracellular adaptor protein(s) facilitating BILF1-mediated surface downregulation of HLA class I remain to be identified, our findings advance the molecular understanding of the process by identifying a critical domain in the viral effector protein and key amino acid residues in the host target proteins.

Besides EBV, other members of the LCV genus include *Callithricine herpesvirus 3* (marmoset LCV) and *Macacine herpesvirus 4* (rhesus LCV) [1]. These LCVs are prototypes for the New World and Old World primate LCVs, respectively, and display broadly similar biological properties to EBV. They can induce B cell growth transformation *in vitro*, possess inherent oncogenic potential *in vivo* and persistently infect their hosts. Rhesus LCV has a genetic repertoire that is identical to EBV, even though they are separated by an evolutionary distance of approximately 25 million years [60]. All EBV ORFs are accounted for in rhesus LCV and *vice versa*, with a similar relative genomic position. In contrast, marmoset LCV, which is estimated to have evolved 35 million years before EBV, has a similar, but less complete, genetic repertoire to EBV and rhesus LCV, suggesting a different evolutionary path [61, 62].

Notably, marmoset LCV lacks 14 genes encoded by the LCVs infecting higher-order primates [60-62]. None of these 14 genes are known to be essential for viral replication or B

cell immortalization, but they do include the immunomodulatory genes BNLF2a, vIL-10, the CSF receptor homolog BARF1 [63] and the EBERs [64]. Thus, the viral genes acquired during LCV evolution from New World hosts to Old World and human hosts may be less involved with intrinsic viral replication pathways during latent or lytic LCV infection, and may have evolved in order to survive in hosts with increasingly sophisticated host immunity, e.g. a more diverse MHC in humans and Old World versus New World hosts. While a BILF1 homolog is encoded by marmoset LCV, our results suggest the ability to downregulate cell surface MHC class I expression functionally evolved within the same related gene present in all LCV. Thus, LCV have made a concerted effort to target the host antigen presentation pathway and downregulate MHC by multiple evolutionary mechanisms, including (i) creation of novel immunomodulatory proteins (BNLF2a), (ii) acquisition of cellular homologs (vIL-10), and (iii) adaptation of novel functions into existing gene products (BILF1).

Materials and Methods

DNA Constructs

BILF1 wild type (wt) and mutant coding sequences were subcloned into the PstI/XhoI sites upstream of the internal ribosomal entry site (IRES) in the lentiviral expression vector pLV-IRES-GFP [28]. All were engineered to contain an N-terminal FLAG-tag. The EBV BILF1 wt sequence was subcloned from pcDNA5-FLAG-BILF1 and the EBV BILF1-K122A sequence from pcDNA3.1/TOPO-FLAG EBV BILF1 K122A (both gifts from H. Vischer, M. Smit, VU, Amsterdam, The Netherlands). The coding sequence of rhesus LCV BILF1 was amplified by PCR from pcDNA-Rhe LCV BILF1-IRES-GFP (a gift from M. Rowe, University of Birmingham, UK). The EBV BILF1 C-terminal truncation mutant ΔC19 was generated by PCR amplification of the wt sequence with the introduction of an early stop codon. To derive the EBV BILF1 point mutants, EBV BILF1-V_{299}A and EBV BILF1-Y_{303}A, the QuickChange site-directed mutagenesis kit (Stratagene) was used according to the manufacturer's instructions. The chimeric gene encoding a protein with amino acid residues 1-285 of marmoset LCV BILF1 fused to residues 293-312 of EBV BILF1 (BILF1 C-tail swap) was generated by PCR amplification using a reverse primer that encoded the 19 most C-terminal residues of the EBV BILF1 C-tail and annealed to nucleotides 830-855 of the marmoset LCV BILF1 coding sequence template.

The construct containing the N-terminally HA-tagged EBV BILF1 wt coding sequence in the retroviral expression vector pLZRS-IRES-GFP was a kind gift from J. Zuo and M. Rowe, University of Birmingham, UK.

The HLA-B8 wt sequence was PCR-amplified from pLZRS-HLA-B8-GFP (a gift from M. Heemskerk, LUMC, Leiden, the Netherlands) and subcloned into a lentiviral bidirectional vector, pCCLsin.PPT.pA.CTE.eGFP.mCMV.hPGK.NGFR.pre (kindly provided by L. Naldini, Milano, Italy), in which the human EF1A promoter replaced the minimal CMV-eGFP

cassette. The HLA-B8 short truncation mutant was generated by PCR amplification of the wt sequence with the introduction of an early stop codon.

To express HLA-B8 wt and C-tail mutants in U937 cells, cells were transduced with replication-deficient lentiviruses generated from the following lentiviral vector pSicoR-EF1a-Zeo-P2A (kindly provided by R.J. Lebbink, Utrecht, The Netherlands). The pSicoR-EGFP backbone vector (Addgene) was altered by removing the U6 promoter and replacing the CMV-EGFP cassette with the human EF1A promoter driving expression of the Zeocin resistance gene and the gene of interest. Zeocin and HLA-B8 genes were fused together by a self-cleaving 2A peptide derived from the porcine teschovirus-1 (P2A). The P2A peptide allows expression of both proteins from a single transcript. HLA-B8 wt was PCR-amplified from the bidirectional lentiviral vector mentioned above; the cytoplasmic tail mutants were generated by using reverse primers that encoded the mutations.

Restriction digests and sequence analysis verified the integrity of all gene sequences.

Cell Lines

The AKBM cell line is an EBV$^+$ Burkitt's lymphoma B cell line (Akata) stably transfected with the pHEBO-BMRF1p-rCD2-GFP reporter plasmid [29]. AKBM cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (PAA Laboratories, Pasching, Austria), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin and 0.3 mg/ml hygromycin B. The MJS (Mel JuSo; HLA typing A*01, B*08) melanoma-derived cell line [30], the monocytic U937 cell line (A*03, B*18, Cw*01), and primary fibroblasts obtained from a common marmoset (*Callithrix jacchus*) (kindly provided by G. Koopman, BPRC, Rijswijk) were both maintained in RPMI (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (European Union approved; Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. 293T (A*02, B*07), 293-kB-luc ([31] ; a gift from G. B. Lipford, Coley Pharmaceutical Group), U373 (A*0201, B*18) and HeLa (A*68, B*15) cells were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. 293-kB-luc cells were cultured in the presence of 0.7 mg/ml geneticin.

Replication-deficient lentiviruses and retroviruses

Replication-deficient recombinant lentiviruses were generated by calcium phosphate co-transfection of HEK 293T cells with a pLV-CMV-IRES-eGFP, pCCLsin.PPT.pA.CTE.EF1A.hPGK.NGFR.pre or pSicoR-EF1a-zeocin-P2A lentiviral vector encoding the gene of interest, and pCMV-VSVG, pMDLg-RRE and pRSV-REV (kindly provided by R. Hoeben, LUMC, Leiden, the Netherlands) [32]. Replication-deficient recombinant retroviruses were produced by using the Phoenix amphotropic packaging system as described previously [13]. After 48 to 96 h, culture supernatants were harvested and frozen or filtered through a 0.45 mm pore filter. MJS, HeLa, 293T, U373 and U937 cell lines were infected with 1 ml lentivirus-containing medium in tissue culture dishes coated with 12 mg/ml retronection. Transduction efficiency

was examined by measuring GFP or surface NGFR expression. In the case of U937 cells transduced with pSicoR-EF1a-zeocin-P2A-based vectors, cells were selected using zeocin (400 μg/ml) to obtain pure populations.

Induction of EBV lytic cycle in AKBM cells, RNA isolation and RT-PCR

The EBV lytic phase was induced in AKBM cells by cross-linking surface IgG with 50 mg/ml goat $F(ab')_2$ fragments to human IgG (Cappel; MP Biomedicals, Solon, OH). Discrimination between IE and late lytic phases was achieved by inhibition of viral DNA replication and late lytic phase gene expression using phosphonoacetic acid (PAA). PAA (pH 7.4 in 100 mM Hepes) at a final concentration of 300 mg/ml was added 1 h prior to EBV lytic phase induction. Total RNA was extracted using TRIzol reagent (Invitrogen) and treated with DNase (TURBO DNAsefree kit; Applied Biosystems), according to manufacturer's protocols. cDNA was synthesized using random hexamers and the Moloney murine leukemia virus reverse transcriptase (Finnzymes), and used for amplification with Taq DNA Polymerase. EBV BILF1 expression was measured using the primers 5'-GTATGGCGTTGGAGAAGACC-3' and 5'-TAATCAGCAGGAGTACCAGACA-3'; BZLF2/gp42 expression with the primers 5'- ATTCTACCTGTGGTAACTAGA-3' and 5'-TTAGCTATTTGATCTTTG-3'and 18S rRNA expression with the primers 5'-GTAACCCGTTGAACCCCATT-3' and 5'GATCCGAGGGCCTCACTAAAC-3'. DNA fragments of the expected length were visualized by 1% agarose gel electrophoresis and ethidium bromide (EtBr) staining.

Antibodies

The mouse monoclonal antibodies (mAbs) used in this study were: W6/32, which detects HLA class I molecules [33]; L243, which detects class II HLA-DR (American Type Culture Collection); anti-FLAG M2 (Sigma); and 3D12, detecting HLA-E (eBioscience). The rat mAb 3F10 (Roche) was used for detection of HA-tags. The human mAbs used were produced locally [34] and included VDK1D12, detecting HLA-A1; SN230G6 detecting HLA-A2; WIM8E5 detecting HLA-A68; VTM4D9 detecting HLA-B7; BVK 5B10 detecting HLA-B8; OUWF11 detecting HLA-B15, FVS4G4 detecting HLA-B18 and WK4C11 detecting HLA-C (Cw*01, Cw*03, Cw*08, Cw*12 and Cw*14). Additional antibodies used were Allophycocyanin (APC)-conjugated goat anti-mouse IgG (H+L) (Leinco Technologies); APC-conjugated goat anti-human IgG + IgM (H+L) (Jackson Immuno Research); APC-conjugated donkey anti-rat IgG $F(ab')_2$ (H+L) (Jackson Immuno Research); R-phycoerythrin (PE)-conjugated goat anti-mouse Ig $F(ab')_2$ (Dako); PE-conjugated goat anti-human IgM $F(ab')_2$ (Southern Biotech); biotinylated goat anti-mouse Ig (Dako) and APC-conjugated streptavidin (BD Pharmingen).

Flow Cytometry

Surface expression of specific molecules was determined using the indicated primary antibodies. Bound antibodies were detected using goat anti-mouse IgG-APC, or for staining of specific HLA class I alleles, goat anti-human IgG + IgM-APC. For HLA-E detection, a streptavidin-biotin-based three-step staining was performed. Double staining of marmoset fibroblasts, to examine co-expression of MHC class I and FLAG-BILF1 on single cells, was performed by consecutive incubation with anti-FLAG mAb, APC-conjugated anti-mouse Ab and PE-conjugated W6/32. Stained cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson) and using FlowJo software (TreeStar).

Transient transfections and luciferase assays

To examine activation of NF-κB by EBV BILF1wt, EBV BILF1-K122A and EBV BILF1-ΔC19, 293-κB-luc cells were seeded in a 96-well plate at a density of 2 x 10^5 cells/ml, 200 ml/well, 24 h before transfection with lipofectamine 2000 following the manufacturer's instructions. Cells were transfected with 70 ng of phRL-TK (constitutively expressing *Renilla* luciferase) and 160 ng of constructs encoding EBV BILF1 wt or mutants, or vector alone. NFκB-induced firefly luciferase and *Renilla* luciferase activity were assayed using the Luciferase Assay Reagent (Promega, Madison, WI) and Renilla Luciferase Assay System (both Promega), respectively, according to the instructions of the manufacturer. Luminescence was measured with the LB940 Mithras Research II microplate reader (Berthold Technologies). For transfection of primary marmoset fibroblasts, cells were seeded in a 6-well plate at a density of 2.5 x 10^5 per ml, 2 ml/well, 24 h before transfection with lipofectamine 2000 according to the manufacturer's instructions. Cells were transfected with 4 mg DNA and analyzed for expression of GFP and surface proteins after 48 h.

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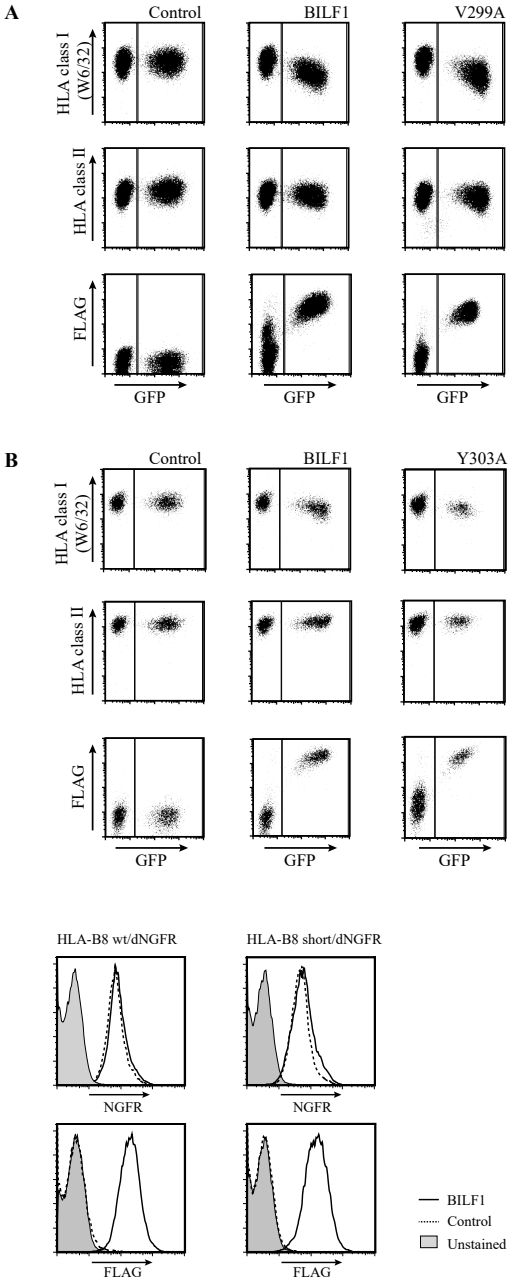
Supplementary Information



Supplementary Figure 1. C-tail point mutations fail to abrogate EBV BILF1-mediated downregulation of surface HLA class I. MJS cells were transduced with GFP- (control), EBV BILF1/GFP- (BILF1) and (*A*) EBV BILF1-V_{299}A/GFP- or (*B*) EBV BILF1-Y_{303}A/GFP-encoding lentivirus. After 7 days, surface expression of FLAG-BILF1, HLA class I and HLA class II was determined by flow cytometry. Transduced and untransduced MJS cells were mixed before antibody staining to allow comparison in a single assay.

Supplementary Figure 2. EBV BILF1 does not affect trNGFR levels in U373-HLA-B8 wt or U373 HLA-B8 short cells. U373-HLA-B8 wt and U373-HLA-B8 short cells were transduced with GFP-encoding (control) or EBV BILF1/GFP-encoding (BILF1) lentivirus. After 7 days, surface expression of trNGFR and FLAG-BILF1 was measured by flow cytometry.

References

1. Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC, *et al.* The order Herpesvirales. Arch Virol. 2009;154(1):171-7.
2. Kieff E, Rickinson AB. Epstein-Barr Virus and Its Replication. In: Knipe DM, Howley PM, editors. Field's Virology. 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 2603-54.
3. Rickinson AB, Kieff E. Epstein-Barr Virus. In: Knipe DM, Howley PM, editors. Field's Virology. 2. 5th ed. ed. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 2655-700.
4. Kutok JL, Wang F. Spectrum of Epstein-Barr virus-associated diseases. Annu Rev Pathol. 2006;1:375-404.
5. Hislop AD, Taylor GS, Sauce D, Rickinson AB. Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. Annu Rev Immunol. 2007;25:587-617.
6. Vossen MT, Westerhout EM, Soderberg-Naucler C, Wiertz EJ. Viral immune evasion: a masterpiece of evolution. Immunogenetics. 2002;54(8):527-42.
7. Griffin BD, Verweij MC, Wiertz EJ. Herpesviruses and immunity: the art of evasion. Vet Microbiol. 2010;143(1):89-100.
8. Hansen TH, Bouvier M. MHC class I antigen presentation: learning from viral evasion strategies. Nat Rev Immunol. 2009;9(7):503-13.
9. Ressing ME, Horst D, Griffin BD, Tellam J, Zuo J, Khanna R, *et al.* Epstein-Barr virus evasion of CD8(+) and CD4(+) T cell immunity via concerted actions of multiple gene products. Semin Cancer Biol. 2008;18(6):397-408.
10. Rowe M, Glaunsinger B, van LD, Zuo J, Sweetman D, Ganem D, *et al.* Host shutoff during productive Epstein-Barr virus infection is mediated by BGLF5 and may contribute to immune evasion. Proc Natl Acad Sci U S A. 2007;104(9):3366-71.
11. Zuo J, Thomas W, van LD, Middeldorp JM, Wiertz EJ, Ressing ME, *et al.* The DNase of gammaherpesviruses impairs recognition by virus-specific CD8+ T cells through an additional host shutoff function. J Virol. 2008;82(5):2385-93.
12. Hislop AD, Ressing ME, van LD, Pudney VA, Horst D, Koppers-Lalic D, *et al.* A CD8+ T cell immune evasion protein specific to Epstein-Barr virus and its close relatives in Old World primates. J Exp Med. 2007;204(8):1863-73.
13. Horst D, van LD, Croft NP, Garstka MA, Hislop AD, Kremmer E, *et al.* Specific targeting of the EBV lytic phase protein BNLF2a to the transporter associated with antigen processing results in impairment of HLA class I-restricted antigen presentation. J Immunol. 2009;182(4):2313-24.
14. Zeidler R, Eissner G, Meissner P, Uebel S, Tampe R, Lazis S, *et al.* Downregulation of TAP1 in B lymphocytes by cellular and Epstein-Barr virus-encoded interleukin-10. Blood. 1997;90(6):2390-7.
15. Zuo J, Currin A, Griffin BD, Shannon-Lowe C, Thomas WA, Ressing ME, *et al.* The Epstein-Barr virus G-protein-coupled receptor contributes to immune evasion by targeting MHC class I molecules for degradation. PLoS Pathog. 2009;5(1):e1000255.
16. Zuo J, Quinn LL, Tamblyn J, Thomas WA, Feederle R, Delecluse HJ, *et al.* The Epstein-Barr virus-encoded BILF1 protein modulates immune recognition of endogenously processed antigen by targeting major histocompatibility complex class I molecules trafficking on both the exocytic and endocytic pathways. J Virol. 2011;85(4):1604-14.
17. Alcami A. Viral mimicry of cytokines, chemokines and their receptors. Nat Rev Immunol. 2003;3(1):36-50.
18. Raftery M, Muller A, Schonrich G. Herpesvirus homologues of cellular genes. Virus Genes. 2000;21(1-2):65-75.
19. Brunovskis P, Kung HJ. Retrotransposition and herpesvirus evolution. Virus Genes. 1995;11(2-3):259-70.

20. Kuhn DE, Beall CJ, Kolattukudy PE. The cytomegalovirus US28 protein binds multiple CC chemokines with high affinity. Biochem Biophys Res Commun. 1995;211(1):325-30.
21. Beisser PS, Laurent L, Virelizier JL, Michelson S. Human cytomegalovirus chemokine receptor gene US28 is transcribed in latently infected THP-1 monocytes. J Virol. 2001;75(13):5949-57.
22. Vischer HF, Leurs R, Smit MJ. HCMV-encoded G-protein-coupled receptors as constitutively active modulators of cellular signaling networks. Trends Pharmacol Sci. 2006;27(1):56-63.
23. Davis-Poynter NJ, Farrell HE. Masters of deception: a review of herpesvirus immune evasion strategies. Immunol Cell Biol. 1996;74(6):513-22.
24. Beisser PS, Verzijl D, Gruijthuijsen YK, Beuken E, Smit MJ, Leurs R, *et al.* The Epstein-Barr virus BILF1 gene encodes a G protein-coupled receptor that inhibits phosphorylation of RNA-dependent protein kinase. J Virol. 2005;79(1):441-9.
25. Paulsen SJ, Rosenkilde MM, Eugen-Olsen J, Kledal TN. Epstein-Barr virus-encoded BILF1 is a constitutively active G protein-coupled receptor. J Virol. 2005;79(1):536-46.
26. Vischer HF, Nijmeijer S, Smit MJ, Leurs R. Viral hijacking of human receptors through heterodimerization. Biochem Biophys Res Commun. 2008;377(1):93-7.
27. Nijmeijer S, Leurs R, Smit MJ, Vischer HF. The Epstein-Barr virus-encoded G protein-coupled receptor BILF1 hetero-oligomerizes with human CXCR4, scavenges Galphai proteins, and constitutively impairs CXCR4 functioning. J Biol Chem. 2010;285(38):29632-41.
28. Vellinga J, Uil TG, de VJ, Rabelink MJ, Lindholm L, Hoeben RC. A system for efficient generation of adenovirus protein IX-producing helper cell lines. J Gene Med. 2006;8(2):147-54.
29. Ressing ME, Keating SE, van LD, Koppers-Lalic D, Pappworth IY, Wiertz EJ, *et al.* Impaired transporter associated with antigen processing-dependent peptide transport during productive EBV infection. J Immunol. 2005;174(11):6829-38.
30. Wubbolts R, Fernandez-Borja M, Oomen L, Verwoerd D, Janssen H, Calafat J, *et al.* Direct vesicular transport of MHC class II molecules from lysosomal structures to the cell surface. J Cell Biol. 1996;135(3):611-22.
31. Bauer S, Kirschning CJ, Hacker H, Redecke V, Hausmann S, Akira S, *et al.* Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci U S A. 2001;98(16):9237-42.
32. Carlotti F, Bazuine M, Kekarainen T, Seppen J, Pognonec P, Maassen JA, *et al.* Lentiviral vectors efficiently transduce quiescent mature 3T3-L1 adipocytes. Mol Ther. 2004;9(2):209-17.
33. Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, *et al.* Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. Cell. 1978;14(1):9-20.
34. Mulder A, Kardol MJ, Arn JS, Eijsink C, Franke ME, Schreuder GM, *et al.* Human monoclonal HLA antibodies reveal interspecies crossreactive swine MHC class I epitopes relevant for xenotransplantation. Mol Immunol. 2010;47(4):809-15. doi: 10.1016/j.molimm.2009.10.004. PubMed PMID: 19931911.
35. Rosenkilde MM, ned-Jensen T, Andersen H, Holst PJ, Kledal TN, Luttichau HR, *et al.* Molecular pharmacological phenotyping of EBI2. An orphan seven-transmembrane receptor with constitutive activity. J Biol Chem. 2006;281(19):13199-208.
36. Hiby SE, Apps R, Sharkey AM, Farrell LE, Gardner L, Mulder A, *et al.* Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. J Clin Invest. 2010;120(11):4102-10.
37. Marchese A, Paing MM, Temple BR, Trejo J. G protein-coupled receptor sorting to endosomes and lysosomes. Annu Rev Pharmacol Toxicol. 2008;48:601-29.
38. Hanyaloglu AC, von ZM. Regulation of GPCRs by endocytic membrane trafficking and its potential implications. Annu Rev Pharmacol Toxicol. 2008;48:537-68.
39. Bonifacino JS, Traub LM. Signals for sorting of transmembrane proteins to endosomes and

lysosomes. Annu Rev Biochem. 2003;72:395-447.
40. Parent JL, Labrecque P, Driss RM, Benovic JL. Role of the differentially spliced carboxyl terminus in thromboxane A2 receptor trafficking: identification of a distinct motif for tonic internalization. J Biol Chem. 2001;276(10):7079-85.
41. Croft NP, Shannon-Lowe C, Bell AI, Horst D, Kremmer E, Ressing ME, *et al.* Stage-specific inhibition of MHC class I presentation by the Epstein-Barr virus BNLF2a protein during virus lytic cycle. PLoS Pathog. 2009;5(6):e1000490.
42. Coscoy L, Ganem D. Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. Proc Natl Acad Sci U S A. 2000;97(14):8051-6.
43. Duncan LM, Piper S, Dodd RB, Saville MK, Sanderson CM, Luzio JP, *et al.* Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. EMBO J. 2006;25(8):1635-45.
44. Hewitt EW, Duncan L, Mufti D, Baker J, Stevenson PG, Lehner PJ. Ubiquitylation of MHC class I by the K3 viral protein signals internalization and TSG101-dependent degradation. EMBO J. 2002;21(10):2418-29.
45. Ishido S, Wang C, Lee BS, Cohen GB, Jung JU. Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. J Virol. 2000;74(11):5300-9.
46. Wiertz EJ, Tortorella D, Bogyo M, Yu J, Mothes W, Jones TR, *et al.* Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature. 1996;384(6608):432-8.
47. Barel MT, Ressing M, Pizzato N, van LD, Le BP, Lenfant F, *et al.* Human cytomegalovirus-encoded US2 differentially affects surface expression of MHC class I locus products and targets membrane-bound, but not soluble HLA-G1 for degradation. J Immunol. 2003;171(12):6757-65.
48. Pudney VA, Leese AM, Rickinson AB, Hislop AD. CD8+ immunodominance among Epstein-Barr virus lytic cycle antigens directly reflects the efficiency of antigen presentation in lytically infected cells. J Exp Med. 2005;201(3):349-60.
49. Petrovsky N, Brusic V. Virtual models of the HLA class I antigen processing pathway. Methods. 2004;34(4):429-35.
50. Lautscham G, Rickinson A, Blake N. TAP-independent antigen presentation on MHC class I molecules: lessons from Epstein-Barr virus. Microbes Infect. 2003;5(4):291-9.
51. de la SH, Saulquin X, Mansour I, Klayme S, Fricker D, Zimmer J, *et al.* Asymptomatic deficiency in the peptide transporter associated to antigen processing (TAP). Clin Exp Immunol. 2002;128(3):525-31.
52. Ulbrecht M, Modrow S, Srivastava R, Peterson PA, Weiss EH. Interaction of HLA-E with peptides and the peptide transporter in vitro: implications for its function in antigen presentation. J Immunol. 1998;160(9):4375-85.
53. Pietra G, Romagnani C, Manzini C, Moretta L, Mingari MC. The emerging role of HLA-E-restricted CD8+ T lymphocytes in the adaptive immune response to pathogens and tumors. J Biomed Biotechnol. 2010;2010:907092.
54. Nachmani D, Stern-Ginossar N, Sarid R, Mandelboim O. Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. Cell Host Microbe. 2009;5(4):376-85.
55. Rosenkilde MM, Smit MJ, Waldhoer M. Structure, function and physiological consequences of virally encoded chemokine seven transmembrane receptors. Br J Pharmacol. 2008;153 Suppl 1:S154-S66.
56. Blanpain C, Wittamer V, Vanderwinden JM, Boom A, Renneboog B, Lee B, *et al.* Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellular signaling pathways.

J Biol Chem. 2001;276(26):23795-804.
57. Dev KK, Nakajima Y, Kitano J, Braithwaite SP, Henley JM, Nakanishi S. PICK1 interacts with and regulates PKC phosphorylation of mGLUR7. J Neurosci. 2000;20(19):7252-7.
58. Wang X, Herr RA, Hansen T. Viral and cellular MARCH ubiquitin ligases and cancer. Semin Cancer Biol. 2008;18(6):441-50.
59. Santos SG, Antoniou AN, Sampaio P, Powis SJ, Arosa FA. Lack of tyrosine 320 impairs spontaneous endocytosis and enhances release of HLA-B27 molecules. J Immunol. 2006;176(5):2942-9.
60. Rivailler P, Jiang H, Cho YG, Quink C, Wang F. Complete nucleotide sequence of the rhesus lymphocryptovirus: genetic validation for an Epstein-Barr virus animal model. J Virol. 2002;76(1):421-6.
61. Cho Y, Ramer J, Rivailler P, Quink C, Garber RL, Beier DR, *et al.* An Epstein-Barr-related herpesvirus from marmoset lymphomas. Proc Natl Acad Sci U S A. 2001;98(3):1224-9.
62. Rivailler P, Cho YG, Wang F. Complete genomic sequence of an Epstein-Barr virus-related herpesvirus naturally infecting a new world primate: a defining point in the evolution of oncogenic lymphocryptoviruses. J Virol. 2002;76(23):12055-68.
63. Cohen JI, Lekstrom K. Epstein-Barr virus BARF1 protein is dispensable for B-cell transformation and inhibits alpha interferon secretion from mononuclear cells. J Virol. 1999;73(9):7627-32.
64. Sharp TV, Schwemmle M, Jeffrey I, Laing K, Mellor H, Proud CG, *et al.* Comparative analysis of the regulation of the interferon-inducible protein kinase PKR by Epstein-Barr virus RNAs EBER-1 and EBER-2 and adenovirus VAI RNA. Nucleic Acids Res. 1993;21(19):4483-90.

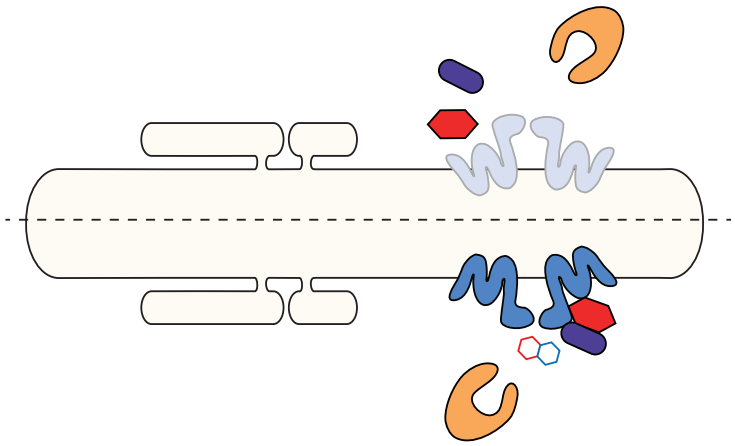
Chapter 5

Human B cells fail to secrete type I interferon upon cytoplasmic DNA exposure

Anna M. Gram[1], Chenglong Sun[2], Hester Koppejan[3], Rob C. Hoeben[1], Søren R. Paludan[2] and Maaike E. Ressing[1]

[1] Department of Molecular Cell Biology, Leiden University Medical Center, The Netherlands; [2]Department of Biomedicine, Aarhus University, Denmark, [3]Department of Rheumatology, Leiden University Medical Center, The Netherlands

Manuscript in preparation



Abstract

Cytoplasmic exposure to foreign DNA or mislocalized self-DNA can trigger secretion of type I interferon (IFN). The DNA sensors interferon-inducible protein 16 (IFI16) and cyclic GMP-AMP (cGAMP) synthetase (cGAS) recognize intracellular DNA and induce type I IFNs via the STING-TBK1-IRF3 axis. DNA sensing is protective as part of an anti-viral immune response, but may also be pathological by promoting auto-inflammatory and auto-immune diseases. B cells exert multiple functions such as antibody secretion, antigen presentation, and pathogen sensing. B cells sense CpG-containing DNA via the endosomal Toll-like receptor 9, thereby can contribute to anti-viral immune responses, but also to auto-immune diseases.

We report here that human B cells fail to secrete type I IFN upon cytoplasmic DNA exposure, although they possess the DNA sensors cGAS and IFI16 and the signalling components TBK1 and IRF3. Primary B lymphocytes and most B cell lines tested appear to lack the central adaptor protein STING that activates TBK1 and IRF3. B cells expressing STING also did not secrete type I IFNs upon dsDNA or cGAMP stimulation. Our data suggest that the cytoplasmic DNA sensing pathway may be dysfunctional in human B cells. Unresponsiveness of these cells to cytoplasmic DNA may render B cells attractive targets for infection by DNA viruses.

Introduction

Type I interferons (IFNs) play a central role in antiviral immunity as they induce the expression of interferon-stimulated genes (ISG) that create an anti-viral state [1]. Most cell types are believed to be able to produce the type I IFNs IFN-α and IFN-β [2]. They are encoded by 12 IFN-α genes and the IFN-β gene in humans. Type I IFNs are potently induced by viral nucleic acids (reviewed in [3,4]). Different RNA and DNA species trigger several pattern-recognition receptors (PRRs) resulting in activation of distinct downstream signalling cascades, which eventually converge and induce interferon regulatory factor 3 (IRF3)-induced transcription of type I IFN genes. The nucleic acid-sensing PRRs are located in endosomes, the cytoplasm, and even in the nucleus [3]. Viral RNA triggers Toll-like receptor 3 (TLR3) and the retinoic acid-inducible protein 1 (RIG-I)-like receptors, RIG-I and melanoma differentiation-associated protein 5 (MDA5), present in endosomes and the cytoplasm, respectively [5,6]. Foreign dsDNA is sensed by the endosomal TLR9 and several cytoplasmic and nuclear DNA sensors including interferon-inducible protein 16 (IFI16) and cyclic GMP-AMP (cGAMP) synthetase (cGAS) [7]. TLR9 senses unmethylated CpG DNA and induces the transcription of pro-inflammatory cytokines through NF-κB [8]. The cytoplasmic DNA sensor cGAS directly binds to the backbone of cytosolic dsDNA in a sequence-independent manner and produces the cyclic dinucleotide 2'3'-cGAMP [9,10]. cGAMP acts as a second messenger molecule and activates the central adaptor protein STING (stimulator of interferon genes; also known as MPYS, MITA, and ERIS) and in turn, TANK-binding kinase 1 (TBK1) and IRF3 are activated [11,12,13]. Nuclear and cytoplasmic IFI16-dependent DNA sensing has also been linked to type I IFN production via STING activation, but the underlying molecular details remain enigmatic at this point [14]. In certain cell types, such as fibroblasts and endothelial cells, IFI16 forms an inflammasome upon DNA sensing, which results in caspase-1-dependent IL-1β maturation [15,16].

Activation of nucleic-acid sensing PRRs must be tightly regulated in order to prevent recognition of cellular "self"-nucleic acids. To this end, the RNA sensor RIG-I recognizes 5'-triphosphates that are present on viral RNA, but absent from cellular mRNA [17]. In contrast, cGAS and IFI16 are triggered by dsDNA irrespective of any apparent sequence or modification. Therefore, they may be triggered by foreign or self-DNA when present in the cytoplasm. The cytoplasm is typically devoid of self-DNA. However, certain genetic defects allow cytoplasmic accumulation of self-DNA, thereby triggering an type I IFN response. Autoimmune diseases like systemic lupus erythematosus (SLE) exhibit an IFN signature, which has been suggested to involve activation of the cytoplasmic and endosomal DNA sensing pathways by accumulating and mislocated self-DNA (reviewed in [18,19]). Mouse models with genetic defects in DNase function are used to study auto-inflammatory diseases. The lethality observed in both DNase II$^{-/-}$ and Trex1$^{-/-}$ (DNase III) mice is abrogated by STING- or cGAS-deficiency [20,21,22,23]. Moreover, DNase II$^{-/-}$cGAS$^{-/-}$ and Trex1$^{-/-}$cGAS$^{-/-}$

did not develop polyarthritis and showed limited induction of auto-antibodies, respectively, suggesting that DNA-driven inflammation may cause auto-immunity [20]. B cells secreting anti-nuclear auto-antibodies contribute to the pathology of SLE and other auto-immune diseases. B cell-receptor-dependent uptake of immune complexes has been implicated in TLR9-dependent DNA sensing and subsequent expansion of autoreactive murine B cells [24,25]. DNA sensing by B cells may not only have pathological effects, but might also be protective in anti-viral immunity.

Herpesviruses are enveloped viruses with a DNA genome that establish latent infection in their hosts. Human B cells are target for infection with the human herpesvirus Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). EBV establishes latent infection in human B cells. Human B cells sense the genome of the herpesvirus EBV by TLR9, which initiates immune activation [26]. Interestingly, EBV infection has been postulated to be a trigger for SLE [27], but it remains mechanistically unclear how EBV infection could contribute to development of this auto-immune disease. IFI16 can be triggered by nuclear EBV or KSHV genomes resulting in inflammasome-mediated IL-1β maturation in B cells [28,29], but whether stimulation of IFI16 could also lead to type I IFN induction was not tested. It is currently unknown whether the dsDNA genome of EBV triggers the cytoplasmic DNA sensing pathway resulting in type I IFNs secretion. Human herpesviruses including herpes simplex virus-1 (HSV-1) and KSHV, but also the reverse-transcribed human immunodeficiency virus (HIV) genome and stem-loop structures of single-stranded HIV DNA are known to trigger the cytoplasmic DNA sensing pathway [30,31,32,33].

Given the central role of DNA recognition in immune responses to DNA viruses and auto-inflammatory diseases, we investigated whether human B cells produce type I IFNs upon exposure to cytoplasmic DNA.

Results

The B-LCL JY does not produce type I IFNs in response to cytoplasmic dsDNA

To investigate whether human B cells secrete type I IFNs upon cytoplasmic DNA exposure, we used both primary B lymphocytes and various B cell lines, as summarized in **Table 1** (s. Material and Methods). In all our experiments, PMA-activated THP1 cells served as a control, since these cells efficiently respond to cytoplasmic DNA exposure. To introduce DNA into the cytoplasm of cells we transfected cells with dsDNA. Fluorescein (FAM)-labeled dsDNA allowed monitoring of DNA-uptake efficiencies by flow cytometry (**Figure 1A**). The control cell line THP1 was efficiently transfected (>90%). Exposure to labeled DNA in the absence of transfection reagent also resulted in labeling of THP1 cells (26%) (**Figure 1A**, left panel). Transfection reagent-independent increase in fluorescence likely reflected cellular uptake or binding of labeled dsDNA to the cells. We examined the EBV-positive human B-lymphoblastoid cell line (B-LCL) JY, a commonly used B cell line first. In JY cells similar

uptake efficiencies were reached as in THP1 cells allowing assessment of type I IFNs secretion by cytoplasmic DNA (**Figure 1A**, right panel). Transfection reagent-independent uptake or binding of labeled dsDNA was low (8%) in JY cells.

Next, we transfected unconjugated dsDNA to trigger the cytosolic DNA sensing pathway in JY and THP1 cells and assessed type I IFNs secretion. In contrast to THP1 cells, JY cells did not secrete type I IFNs upon cytoplasmic DNA exposure (**Figure 1B**). As a control for type I IFN secretion, we transfected the RNA analogue poly(I:C). JY and THP1 cells secreted type I IFNs upon cytoplasmic RNA stimulation (**Figure 1B**). Exposure to dsDNA in the absence of transfection reagent did not trigger a type I IFN response in THP1 cells (data not shown) consistent with the DNA being unable to reach the cytoplasm, in contrast to transfected dsDNA. JY cells appeared able to produce and secrete type I IFNs upon cytoplasmic RNA exposure, but not after DNA stimulation.

To examine transcription of the IFN-β gene in JY cells, we determined levels of IFN-β1 transcripts at 6h and 24h after dsDNA or poly(I:C) transfection by quantitative real-time PCR (**Figure 1C**). There was no increase in IFN-β1 transcripts detected after dsDNA stimulation in JY cells. In response to poly(I:C), a low induction of IFN-β1 mRNA levels was detected in JY after 6h, but a robust increase at 24h post-transfection (**Figure 1C**). Upon dsDNA and poly(I:C) stimulation, THP1 cells exhibited very high levels of IFN-β1 mRNA at 6h and at lower levels at 24h. Following the pattern of IFN-β1 induction, transcription of IFN-stimulated gene (ISG)54 was observed to both stimuli in THP1 cells, but only in response to poly(I:C) in JY cells (**Figure 1D**). To test whether dsDNA stimulation resulted in gene expression of pro-inflammatory cytokines in JY and THP1 cells, we examined TNFα transcripts at 6h (**Figure 1E**) and 24h (data not shown). There was no increase detected in JY cells, and also THP1 showed only a very moderate and transient induction of TNFα mRNA to both nucleic acid stimuli at 6h (**Figure 1E**), which was absent at 24h (data not shown).

These data show that the human B-LCL JY did not produce and secrete type I IFN or the pro-inflammatory cytokine TNFα upon cytoplasmic DNA exposure. Poly(I:C) induced production of IFN-β1mRNA and secretion of type I IFNs in JY cells, which in turn stimulated induction of ISG54. This demonstrates that whereas the type I IFN pathway is functional in this B cell line, it is not activated upon cytoplasmic dsDNA stimulation.

EBV-negative B cells do not secrete type I IFN upon cytoplasmic DNA stimulation

To assess if EBV-negative B cells, in contrast to EBV-positive JY cells, produce type I IFNs in response to cytosolic dsDNA exposure, two non-EBV immortalized B cell clones were transfected with dsDNA. The determined transfection efficiency of about 50% may be an underestimation (**Figure 2A**, one clone depicted) due to the GFP marker present in these cells. The B cell clones did not respond to dsDNA or poly(I:C) by type I IFN secretion (**Figure 2B**, one clone depicted) suggesting that the B cell clones were unresponsive to intracellular nucleic acid exposure.

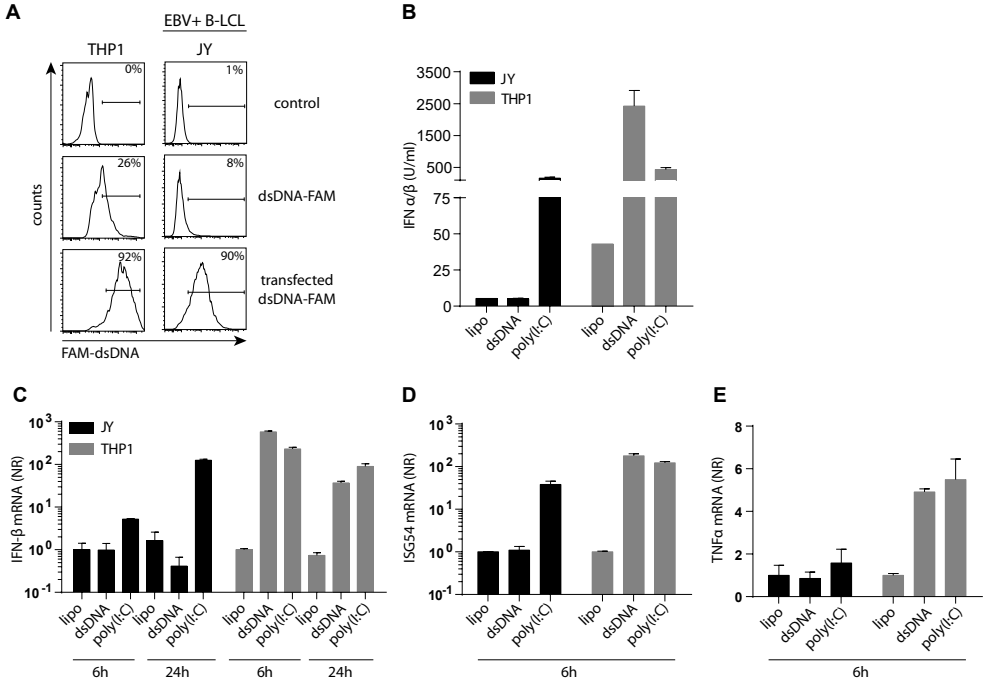
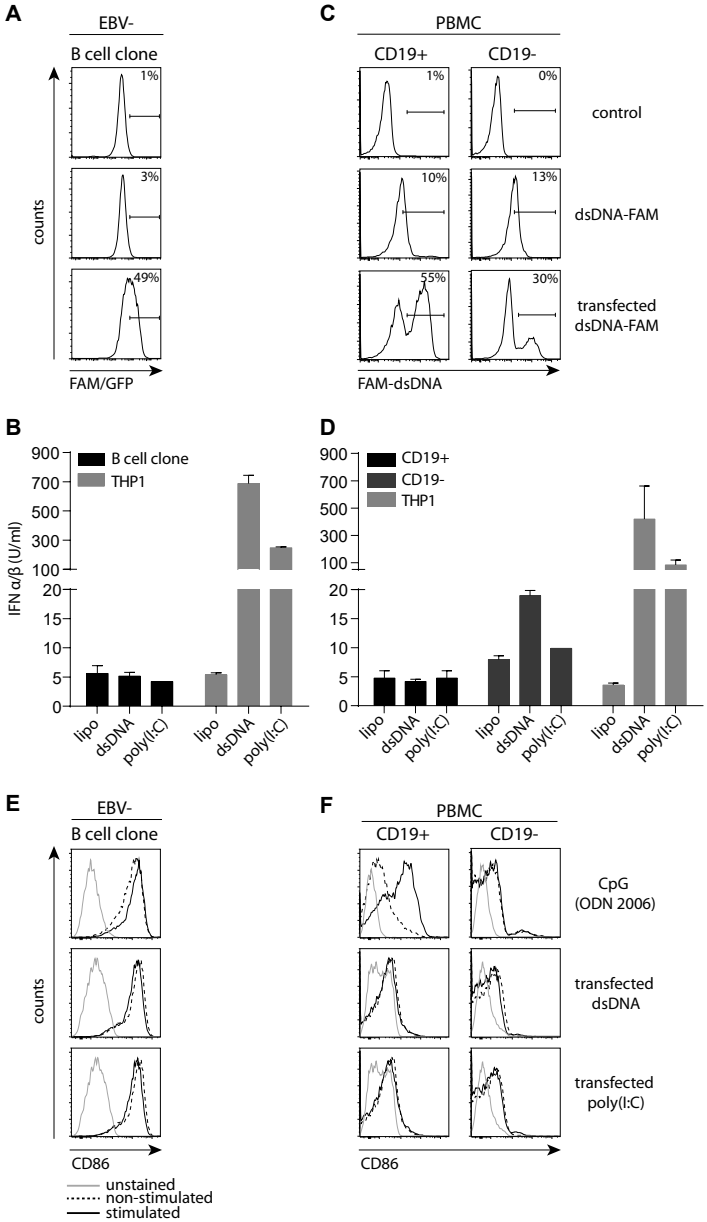


Figure 1 The human B-LCL JY fails to produce type I IFNs in response to dsDNA stimulation. A) Efficiencies of dsDNA transfection in the B-LCL JY and PMA-differentiated THP1 cells were determined by lipofectamine2000 transfection of FAM-labeled dsDNA. As controls, cells were either treated with the transfection reagent or FAM-labeled dsDNA alone. After overnight incubation, cells were analysed by flow cytometry. B) The B-LCL JY and PMA-differentiated THP1 cells were transfected with dsDNA or poly(I:C) using Lipofectamine2000. Cell-free supernatants were harvested 24h post-transfection and analysed for IFN-α/β in duplicates using a bioassay. One representative of four independent experiments is shown. Data are presented as mean ± SD. C-E) The B-LCL JY and PMA-differentiated THP1 cells were transfected as in B). RNA was isolated 6h or 24h post-transfection and analysed for mRNA levels of C) IFN-β, D) ISG54, E) TNFα by quantitative real-time PCR. Data are means (± SD) of normalized ratios relative to control sample, using β-actin as internal reference. One representative of two independent experiments is shown.

Figure 2 Human B cells do not secrete type I IFN upon exposure to cytoplasmic DNA. A) To determine transfection efficiencies, an immortalized B cell clone was transfected with FAM-labeled DNA or as a control treated with FAM-labeled dsDNA alone or left untreated. B) IFN-α/β secretion from tetanus- or influenza-specific immortalized B cell clones and PMA-differentiated THP1 cells transfected with dsDNA or poly(I:C) were determined by bioassay. Cell-free supernatants were harvested 24h post-transfection and analysed for IFN-α/β in duplicates. One representative of two independent experiments is shown for the influenza-specific clone. C) PBMC-derived $CD19^+$ or $CD19^-$ cells were treated as in A). Control cells were treated with lipofectamine2000 alone. D) PBMC-derived $CD19^+$, $CD19^-$ cells,



and PMA-differentiated THP1 cells were treated as in B). Supernatants were harvested 15 h post-transfection. One representative of two independent experiments is shown. E and F) An EBV-negative B cell clone (E) or CD19^{+} and CD19- PBMCs (F) were stimulated with CpG DNA or transfected with dsDNA or poly(I:C) (as in B) and cell surface levels of CD86 were determined by flow cytometry 24 h post-stimulation and compared to non-stimulated control cells, which were left untreated (CpG) or treated with lipofectamine alone (dsDNA and poly(I:C)). Data are depicted as histograms.

To test whether unresponsiveness to cytoplasmic DNA was a general feature of human B lymphocytes, we expanded our study to primary B cells. Transfection of dsDNA was successful in 55% of the primary $CD19^+$ B lymphocytes and in 30% of the $CD19^-$ PBMCs serving as control (**Figure 2C**). The $CD19^+$ B cells did not secrete type I IFN upon triggering with cytoplasmic DNA or RNA (**Figure 2D**). $CD19^-$ mononuclear cells showed only a slight response to dsDNA, but not to poly(I:C) stimulation (**Figure 2D**). The $CD19^-$ fraction contained about 50% $CD3^+$ cells, 9% $CD16^+$ cells, 4% $CD14^+$ cells and 35% of the cells were negative for these three surface markers as well as for CD19. Type I IFN secretion upon dsDNA stimulation was likely derived from monocytes, although the percentage of monocytes was low in the $CD19^-$ cell fraction. It is unclear why the cells did not secrete type I IFN after exposure to cytoplasmic poly(I:C). By speculation, dsDNA might induce higher levels of type I IFN upon cytoplasmic exposure in comparison to poly(I:C) in monocytes, as observed for THP1 cells. In these experiments the DNA-induced type I IFN levels were low, hence it could be that poly(I:C)-induced levels were not detectable.

We tested whether the primary B lymphocytes and the B cell clones were responsive to another nucleic acid stimulus indicating that the cells were viable at the time point of treatment. To this end, we stimulated the endosomal TLR9 present in B cells with the agonist CpG DNA (ODN 2006). CpG-mediated TLR9 stimulation of PBMC-derived B cells is known to result in upregulation of the activation marker CD86 [40]. The B cell clone tested expressed high cell surface levels CD86 prior to stimulation. A minute increase in the CD86 surface levels was observed upon stimulation with CpG DNA (MFI 15282 (unstimulated) vs 19915 (CpG)), but not with transfected dsDNA or poly(I:C) (**Figure 2E**). Stimulation with CpG DNA, but not transfection of the other two stimuli, resulted in upregulation of the activation marker CD86 in PBMC-derived $CD19^+$ cells (**Figure 2F**, left panel). The $CD19^-$ fraction showed no increase in surface levels of CD86 upon nucleic acid stimulation (**Figure 2F**, right panel). The primary $CD19^+$ and $CD19^-$ cells displayed sensitivity towards the cytotoxic transfection reagent, as a lower percentage of cells (around 25% for both $CD19^+$ and $CD19^-$) was present in the "live gate" in comparison to untreated or CpG-treated cells (about 80% for $CD19^+$ and 50% for $CD19^-$). This demonstrates that the primary B cells were responsive to endosomal DNA exposure suggesting that they were viable at the point of treatment.

In conclusion, primary human B lymphocytes and EBV-negative B cell clones did not secrete type I IFNs upon exposure to cytoplasmic DNA, while primary $CD19^-$ cells responded to transfected DNA.

Absence of STING protein in primary B cells and B cell lines, but not in B-LCL lines

To determine why human B cells did not respond to dsDNA stimulation, we examined the B cells for the following proteins of the cytoplasmic DNA sensing pathway: the DNA sensors cGAS and IFI16, the adaptor protein STING, and the downstream signalling molecules TBK1 and IRF3 (**Figure 3A**). PMA-differentiated THP1 cells having all these proteins served as a control (**Figure 3B-E**).

Although the transformed B-LCL JY did not respond to dsDNA stimulation, these cells produced cGAS, IFI16, STING, TBK1, and IRF3 (**Figure 3B**, lane 1). The B cell clones were grown in the presence of the CD40L-expressing murine feeder cell line. To exclude contamination of the B cell sample with feeder cells, this cell line was tested for cross-reactivity of the antibodies used. In feeder cells, we detected only a clear signal for TBK1, but not for the other proteins tested (lane 2). Therefore, we concluded that proteins detected in the B cell clones were not due to contamination with murine feeder cells. Both B cell clones had cGAS, IFI16, TBK1, and IRF3, but lacked the adaptor protein STING (lane 3 and 4). The EBV-negative cell line 2A8 (lane6) showed the same pattern as the B cell clones, whereas THP1 cells produced all proteins examined (lane 5).

To examine whether transformed B cells were representative for primary B cells, we probed for the proteins involved in the cytoplasmic DNA sensing pathway in primary PBMC-derived $CD19^+$ B cells (**Figure 3C**). In addition, we examined $CD19^-$ cells and total PBMCs. THP1 cells served as positive control. $CD19^+$ B cells from two donors possessed the DNA sensors cGAS and IFI16, and the signalling molecules TBK1 and IRF3, but lacked STING (lane 2 and 3). In the $CD19^-$ cells and total PBMCs cGAS, STING, TBK1, and IRF3 were detected, but IFI16 levels appeared to be low (lane 4, 5, and 6).

As an alternative source of primary human B cells, tonsillar $CD19^+$ B cells were examined for the proteins of the cytoplasmic DNA pathway and compared to tonsil-derived $CD19^-$ cells (**Figure 3D**). In $CD19^+$ cells, the DNA sensors, and the signalling intermediates TBK1 and IRF3 were detected, but STING protein was not (lane 2), while $CD19^-$ tonsillar cells had all proteins examined (lane 3). Thus, $CD19^+$ cells exhibited the same pattern as observed for blood-derived $CD19^+$ cells, respectively.

As JY cells were the only human B cells that appeared to have STING, we tested another B-LCL, MRJ, and compared it to JY and THP1 cells. MRJ displayed the same profile of proteins of the DNA sensing pathway as JY cells (**Figure 3E**, lane 3), and also these cells appeared to be unresponsive to dsDNA stimulation (data not shown).

In conclusion, human B lymphocytes appear to produce the cytoplasmic DNA sensors cGAS and IFI16 and the molecules TBK1 and IRF3. Whereas the EBV-positive B-LCLs JY and MRJ exhibited substantial levels of STING, this protein was undetectable in other human B cells tested. Absence of STING would provide an explanation why the EBV-negative blood-derived $CD19^+$ B cells and the B cell clones did not secrete type I IFN upon exposure to cytoplasmic dsDNA.

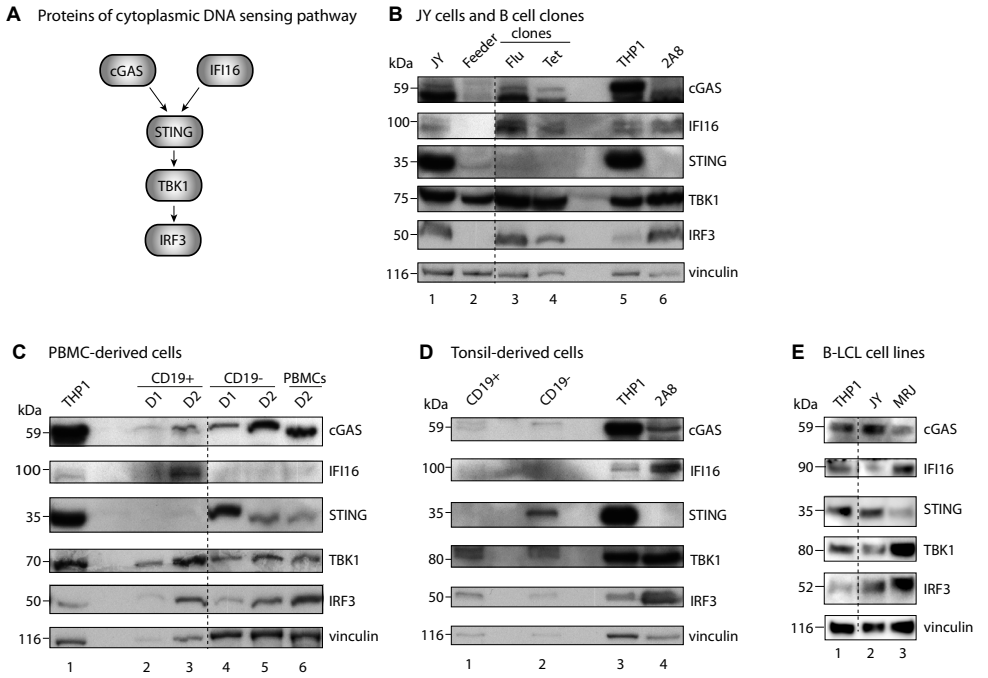


Figure 3 Most human B cells are deficient for STING protein. A) Schematic overview of proteins involved in the cytoplasmic DNA sensing pathway. B-E) Whole cell lysates were analysed for the presence of cGAS, IFI16, STING, TBK1, and IRF3 by Western blot analysis. Vinculin served as loading control. Dashed line indicate position of removed marker lanes. B) Tetanus (Tet)- and influenza (Flu)-specific immortalized B cell clones as well as the cell lines JY, 2A8, and PMA-differentiated THP1 cells were analysed. B cell clones were grown in the presence of murine feeder cells, but the feeder cells did not appear to account for proteins detected in B cell clone lysates. C) $CD19^+$ and $CD19^-$ PBMCs of two independent donors, total PBMCs of one donor and PMA-differentiated THP1 cells were analysed. D) Tonsillar $CD19^+$ and $CD19^-$ cells of one donor as well as the cell line 2A8, and PMA-differentiated THP1 cells were analysed for indicated proteins. E) The B-LCL cell lines JY and MRJ, and PMA-differentiated THP1 cells were analysed by Western blot analysis.

STING-expressing cells do not produce type I IFNs upon dsDNA or cGAMP stimulation

Despite presence of STING and the other proteins in the B-LCLs JY and MRJ, the cells were unresponsive to dsDNA stimulation. To get an indication whether the STING-TBK1-IRF3 pathway could be activated in B-LCLs, we bypassed the DNA sensing step by stimulating digitonin-permeabilized JY cells with the mammalian STING-activating molecule 2'3'-cGAMP. There was no type I IFN secreted from cGAMP-stimulated JY cells at 6h post-stimulation, while THP1 cells produced type I IFNs in the presence, but not in the absence of cGAMP (**Figure 4A**). This indicates that the STING pathway was not activated by cGAMP in JY cells. Sequence analysis revealed that JY cells had the same variant of STING as THP1 cells (data not shown), suggesting that JY cells did not carry a mutated form of STING.

To exclude potential inhibitory effects of EBV proteins, we turned to an EBV-negative human B cell line. As the EBV-negative B cell line 2A8 had the DNA sensors and the signalling component TBK1 and IRF3, but lacked STING, we assessed if reconstitution of STING induces a type I IFN response upon dsDNA stimulation. We stably introduced C-terminally HA-tagged murine STING in the EBV-negative cell line 2A8 that endogenously produced the sensors cGAS and IFI16, and the signalling molecules TBK1 and IRF3 (**Figure 3B**, lane 6 and **Figure 3D**, lane 4). Murine STING is able to reconstitute human cells lacking STING to respond to cyclic dinucleotides [41]. Two 2A8 cell lines were generated using different amounts of STING-coding lentivirus. The lentiviral vector also encoded GFP, which we used as a marker to FACS-sort transduced cells. Pure populations of GFP^+ 2A8-mSTING cells were used to examine presence of STING-HA by Western blot analysis (**Figure 4B**). The cell lines JY and THP1 displayed high levels of STING detected by a STING-specific antibody, whereas in the untransduced 2A8 cell line no signal was detected (**Figure 4B**). The 2A8-mSTING cells showed a weak signal using the STING-specific antibody, which binds human and murine STING. The 2A8 cell line transduced with a higher dose of lentivirus showed increased levels of STING in comparison to the other transduced cell line. Using an HA-reactive antibody allowed clear detection of HA-tagged STING in 2A8-mSTING lines (**Figure 4B**, lane 2 and 3). The bands detected with the HA-reactive antibody migrated at the same height as the weak STING antibody-reactive bands suggesting that these bands represented HA-tagged STING protein.

In a next step, we tested the transfection efficiency of 2A8 and 2A8-mSTING cells using the FAM-labeled dsDNA. Upon transfection, 40% of the 2A8 cells were labeled indicating successful uptake of the DNA (**Figure 4C**), while the GFP^+ 2A8-mSTING cells showed a transfection efficiency of around 20%, which may be an underestimation due to presence of GFP.

Upon transfection of poly(I:C) or dsDNA, neither the parental 2A8 cells, nor the 2A8-mSTING cells secreted type I IFNs, in contrast to control THP1 cells (**Figure 4D**). To assess whether 2A8 cells were responsive to another nucleic acid stimulus indicating that the cells were viable at the time point of treatment, we stimulated the 2A8-mSTING cells with CpG DNA. Prior to stimulation, the cells expressed high surface levels of CD86, but the levels slightly increased upon stimulation with CpG DNA suggesting that the cells were viable (**Figure 4E**).

To examine whether the STING-TBK1-IRF3 pathway could be activated in the EBV-negative 2A8-mSTING cell line, we stimulated these cells with cGAMP. Irrespective of STING expression, none of the 2A8 cell lines did secrete type I IFNs upon cGAMP stimulation, while THP1 cells produced type I IFNs (**Figure 4F**). Although direct evidence is missing at this point that mSTING introduced in 2A8 cells was functional and present at adequate levels, these data indicate that exogenous or endogenous STING expression was not sufficient for EBV-positive or -negative B cell lines to sense the second messenger molecule cGAMP. This may suggest that human B cells are unresponsive to dsDNA or cGAMP stimulation, irrespective of the presence or absence of EBV proteins.

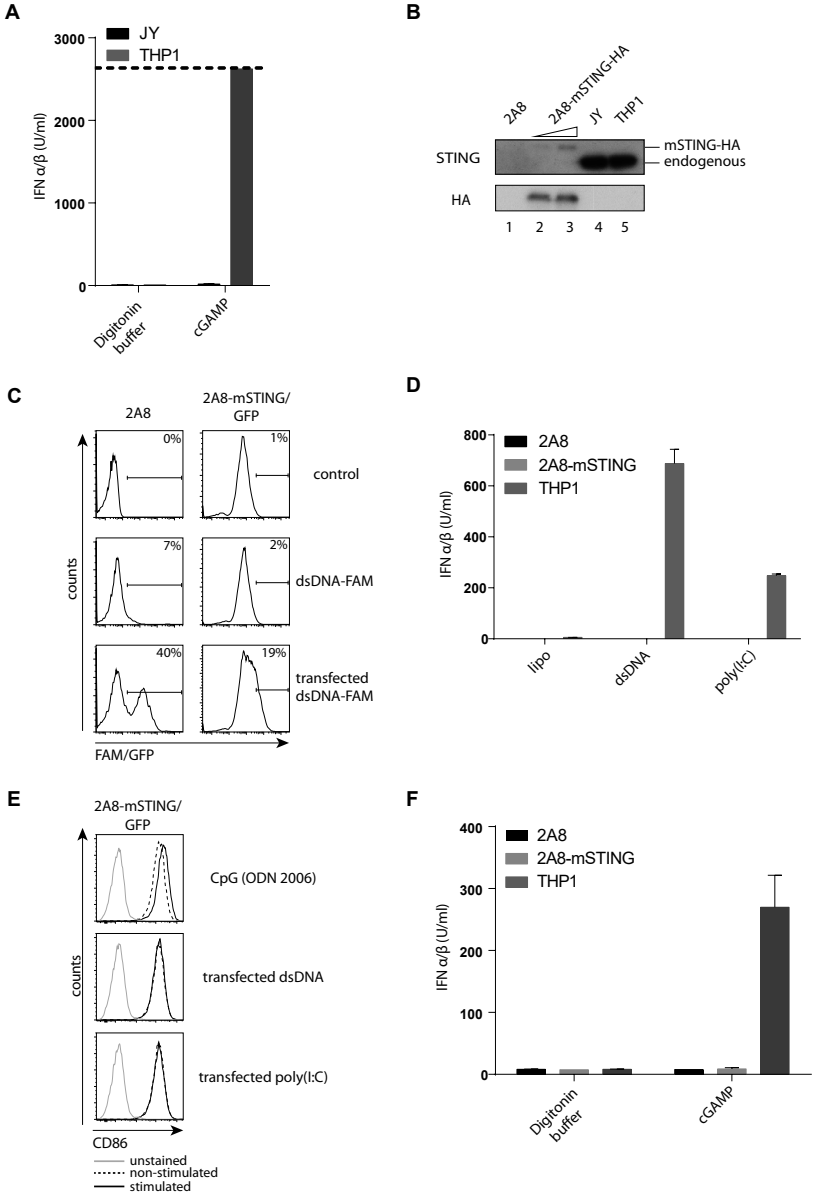


Figure 4 STING-expressing B cells fail to respond to cytoplasmic nucleic acids and cGAMP. A) JY and PMA-differentiated THP1 cells were stimulated with cGAMP using a buffer containing digitonin. Supernatants were harvested at 6 h post-stimulation. Data are mean ± SD of one representative out of three independent experiments. B) The cytoplasmic fraction of 2A8, mSTING-HA/GFP-transduced 2A8 (2A8-mSTING), JY, and PMA-differentiated THP1 cells was analysed for STING and HA-tag expression by Western blot analysis. C) Transfection efficiency of dsDNA into 2A8 and 2A8-mSTING cells was determined by lipofectamine2000 transfection of FAM-labeled dsDNA. As a control, cells were

left untreated or treated with dsDNA-FAM alone. Cells were analysed by flow cytometry after overnight incubation. D) 2A8, 2A8-mSTING, and PMA-differentiated THP1 cells were transfected with dsDNA or poly(I:C) using Lipofectamine2000. Cell-free supernatants were harvested at 24 h post-transfection and analysed for bioactive IFN-α/β in duplicates. E) 2A8-mSTING cells were stimulated with CpG DNA or transfected with dsDNA or poly(I:C) (as in D) and cell surface levels of CD86 were determined by flow cytometry 24 h post-stimulation and compared to non-stimulated control cells, which were left untreated (CpG) or treated with lipofectamine alone (dsDNA and poly(I:C)). Data are depicted as histograms. F) 2A8, 2A8-mSTING, and PMA-differentiated THP1 cells were stimulated with cGAMP using a buffer containing digitonin. Cell-free supernatants were harvested at 3,5 h post-stimulation and IFN-β bioactivity was determined by BioAssay.

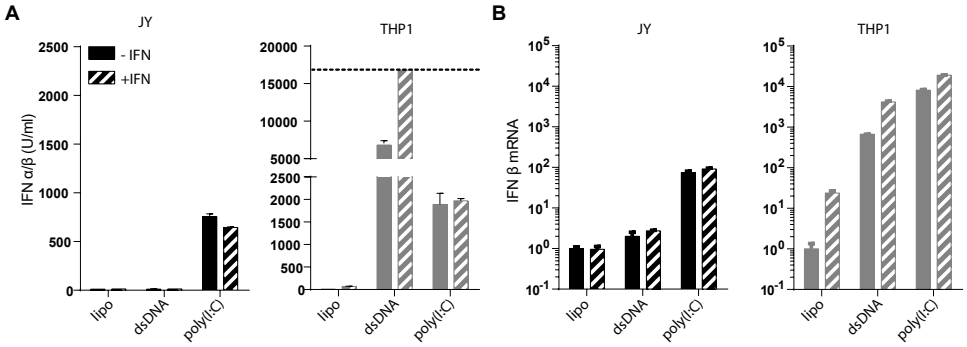


Figure 5 IFN-β priming does not rescue responsiveness to cytoplasmic DNA exposure of the B-LCL JY. A and B) JY (black bars) and PMA-activated THP1 cells (grey bars) were pre-stimulated with 1000 U/ml IFN-β for 24 h. Cells were then transfected with dsDNA or poly(I:C) using Lipofectamine2000. A) Cell-free supernatants were harvested at 24 h post-transfection and analysed for IFN-α/β in duplicates. Data are mean ± SD of one representative out of two independent experiments. A dashed line indicates maximum cut-off of assay. B) Cells were harvested, RNA isolated, and mRNA levels of IFN-β determined at 6 h post-transfection by quantitative real time-PCR. Data are means (± SD) of normalized ratios relative to control sample, using β-actin as internal reference. Representative data of three independent experiments are shown.

Type I IFN priming prior to dsDNA stimulation does not give rise to type I IFNs secretion in B-LCLs

Lastly, we examined whether the B-LCL JY respond to dsDNA when in a primed state. Type I IFNs induce expression of many ISGs via the JAK-STAT pathway, which acts downstream of the type I IFN receptor [1]. Upregulation of ISGs including PRRs such as RIG-I, MDA5, TLR3, cGAS, IFI16 and other signalling proteins limits viral replication [42,43]. Hence, type I IFN primes cells to detect and/or respond to PAMPs more vigorously. For example, human type I IFN-primed macrophages show enhanced responses to TLR stimuli in comparison to unprimed cells [44]. Therefore, we hypothesized that type I IFN-stimulated B cells might become responsive to dsDNA.

To this end, we pre-stimulated JY and THP1 cells with IFN-β for 24h prior to transfection with dsDNA or poly(I:C). Pre-stimulation of JY cells with IFN-β did not give rise to a dsDNA-

induced type I IFN response (**Figure 5A**, left panel). In contrast, enhanced IFN I levels were detected in IFN-β-primed THP1 cells upon dsDNA stimulation in comparison to unprimed cells (**Figure 5A**, right panel). Secretion of type I IFN following poly(I:C) stimulation was not altered by pre-stimulation with IFN-β in either of the cell lines (**Figure 5B**). In THP1 cells, IFN-β transcription appeared to be slightly increased in response to nucleic acids upon IFN pre-stimulation, but type I IFN treatment alone also elevated transcription (**Figure 5B**).

These data suggest that type I IFN-priming of the B-LCL JY did not enhance responsiveness to dsDNA. Overall, our data indicate that human B cells are unable to evoke type I IFN production in response to foreign dsDNA, and that this this is correlated with the absence of the adaptor protein STING in most of these cells. Curiously, despite the presence of STING, B cells appear to have a dysfunctional cytosolic DNA sensing pathway.

Discussion

In this study, we report that human B cells fail to secrete type I IFNs upon exposure to cytosolic dsDNA, although they produce the DNA sensors cGAS and IFI16. The EBV-negative B cell lines and primary B cells tested lacks the central adaptor protein STING that is required for signal transduction downstream of the cytosolic DNA sensors. B cell lines expressing STING did not secrete type I IFNs upon dsDNA or cGAMP stimulation.

Cytosolic DNA sensing plays a crucial role in host immune defense against invading DNA viruses as demonstrated by lethality in STING-knockout mice infected with HSV-1 [30]. The STING protein was originally identified in murine B cell lines as a molecule involved in apoptosis [45], but shortly thereafter, it was recognized as the central signalling hub for DNA sensing [11,12,13]. Murine B cell lines representing pre-B cells, immature, memory and plasma B cells were used to examine presence of STING [45]. Except for the plasma B cell line, STING was present in the different murine B cell lines, with the highest levels found in mature B cells. We examined human B lymphocytes, but only detected high levels of STING in B-LCLs, but not in other B cells. Thus, STING is present in murine, but not in most human B cells. This is interesting as murine and human STING exhibit a different activation profile in response to cyclic dinucleotides. Murine STING is potently triggered by bacterial 3'5'- and mammalian 2'3'-linked cyclic dinucleotides, while human STING is mainly activated by the mammalian 2'3'-cGAMP [41]. Hence, murine STING responds to a broader spectrum of ligands, which most likely dictates the role of this protein in innate immune activation. Therefore, one could speculate that a distinct expression profile in (immune) cells is evolutionarily favored due to differential sensitivity towards cyclic dinucleotide ligands.

Signaling via STING plays a central role in type I IFN induction following the recognition of the DNA virus, such as HSV-1 or KSHV [30,31]. Therefore, the absence of STING from

human B cells may render these cells more permissive for infection with the herpesviruses KSHV or EBV. Yet, human B cells are not devoid of functional DNA sensing. We have reported earlier that EBV genome activates the DNA sensor TLR9 in endosomes [26]. Moreover, IFI16 senses nuclear EBV and KSHV genomes resulting in subsequent formation of an IFI16 inflammasome and IL-1β maturation in B cells [28,29]. Thus, human B cells are likely to sense invading DNA viruses and initiate innate immune responses, yet they appear to fail to induce a type I IFN response upon cytoplasmic DNA exposure. It seems that STING may be expressed in human B cells under certain conditions suggested by high levels of the adaptor protein in the EBV-transformed B-LCLs. Since nine EBV latency proteins are present in B-LCLs, it remains to be determined whether they contribute to STING expression and/or type I IFN production.

Interestingly, B-LCLs or the 2A8-mSTING cell line were unable to mount a type I IFN response upon stimulation with dsDNA, despite presence of the proteins comprising the cytosolic DNA sensing pathway including STING. The B cell line JY has STING levels comparable to those observed in THP1 cells. JY cells carried the same STING variant as THP1 cells (data not shown), thus mutations interfering with its function could be excluded. Introduction of murine STING into the EBV-negative cell line 2A8 did not render these cells responsive to dsDNA or cGAMP stimulation, although murine STING can reconstitute human cells lacking STING [41]. Although we formally lack evidence that the construct coding for murine STING could functionally reconstitute STING in STING-deficient cells, our data imply that the cytoplasmic DNA sensing pathway is not functional in human B lymphocytes. A similar phenomenon has been reported for activated human T lymphocytes [38]. The T cells appeared to sense cytoplasmic DNA supporting formation of the STING signalosome including TBK1 recruitment, but still these cells failed to mount a type I IFN response [38]. In our study, we triggered STING-expressing B cells with the second messenger molecule cGAMP, which did not result in activation of the STING pathway and secretion of type I IFNs. This suggests that signal transduction was abrogated downstream of the STING protein, similar to the observations for T cells. We demonstrate that the B-LCLs responded to transfected poly(I:C), which is sensed by the cytosolic RNA sensors MDA5 and RIG-I. They signal via the adaptor protein MAVS that, in turn, recruits TBK1 and IRF3 suggesting that signalling involving the proteins TBK1 and IRF3 was supported, at least, in the B-LCLs. This implies, therefore, that STING failed to activate TBK1 and IRF3 in the B-LCLs.

It is unclear why poly(I:C) stimulation of the other B cells examined failed to induce production of type I IFNs. The signalling intermediates TBK1 and IRF3 are employed by the DNA and RNA sensing pathways. Western blot analysis (**Figure 3**) showed that primary B cells and the B cell clones had similar or even higher levels of the signalling molecules TBK1 and IRF3 in comparison to JY or THP1 cells. The latter two were responsive to poly(I:C). This suggests that all B lymphocytes examined were equipped with the molecules acting downstream of the adaptor proteins of the cytoplasmic nucleic acid sensors, but yet did not

respond. It remains to be determined whether human B cells have the cytoplasmic RNA sensors RIG-I and MDA5, as well as the adaptor protein MAVS.

Although B cells appear to have a dysfunctional cytosolic DNA sensing pathway, the DNA sensor cGAS might still contribute to induction of innate immune responses against B-lymphotropic viruses such as KSHV or EBV. Recently, two independent groups found that viral particles can transfer the second messenger molecule cGAMP to other cells [46,47]. cGAMP was found in virions of HIV, murine cytomegalovirus, and modified vaccinia virus Ankara suggesting that 1) these viruses trigger the DNA sensing pathway and 2) members of several viral families including herpesviruses, facilitate packaging of this cyclic dinucleotide into the virion. This viral particle-dependent cGAMP transfer triggered a type I IFN response in several cell types including the cell line THP1 and DCs derived from primary human monocytes [46,47]. Therefore, it would be interesting to examine whether virions of e.g. EBV or KSHV propagated in B lymphocytes contain cGAMP and whether this could stimulate other cell types infected by these viruses. Hence, triggering of cGAS in B cells could result in cGAMP production and packaging, and subsequent transfer to other cells by released virions. This may allow stimulation of STING prior to sensing of viral DNA released from the capsid.

Identification of several KSHV proteins, including the KSHV-specific K9/vIRF1 protein, interfering with the cGAS-STING pathway [31] argues that this pathway is involved in sensing of KSHV. The KSHV protein LANA inhibits cGAS and also the tegument protein ORF52 prevents cGAS-mediated cGAMP production *in vitro* and in infected cells [48,49]. The EBV homologue of ORF52, BLRF2, also prevented cGAMP formation in an *in vitro* assay, but whether the EBV protein EBNA1, the functional homologue of LANA, interferes with cGAS remains to be determined. Identification of viral proteins inhibiting cGAS function indicates that cGAS plays a role in detection of these viruses. As the cytoplasmic DNA sensing pathway appears to be dysfunctional in human B cells, it seems that viral inhibition of cGAS is not necessary in B cells, but required for infection of other cell types. For instance, KSHV infection of endothelial cells triggers activation of the cytosolic DNA sensing pathway [31].

Type I IFN stimulation of murine B cells was shown to augment B cell-receptor-dependent responses [50]. In human B cells, type I IFN enhances CpG-stimulated IgM production [51]. Hence, exposure of B cells to type I IFN appears to promote B cell functions such as antibody production that contribute to pathology of auto-immune diseases. Given the unresponsiveness of T and B cells to cytosolic DNA stimulation, it is tempting to speculate that lymphocytes might possess a safeguard mechanism that prevents them from triggering a type I IFN response upon exposure to intracellular DNA. Such a mechanism may present as a lymphocyte-specific inhibitor hampering activation or as an inducible, yet-unknown, factor facilitating signalling. In an attempt to unleash the cGAS-STING pathway in STING-expressing B cells, we pre-stimulated the cells with IFN-β prior to introduction of dsDNA. However, this did not result in induction or secretion of type I IFN in the B cells. Therefore,

further studies are needed to identify the underlying mechanism of frustrated type I IFN induction observed in T and B lymphocytes upon introduction of cytoplasmic DNA. Possibly, corroboration of such a mechanism may contribute to the development of autoimmunity or other inflammatory conditions upon DNA sensing of self-DNA.

Material and Methods

Isolation of primary human B cells

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation from buffy coats of healthy blood donors provided by Sanquin blood bank Amsterdam. Isolated PBMCs contained about 55% $CD3^+$, 15% $CD19^+$, 12% $CD16^+$, 7% $CD14^+$ cells and 11% of the isolated cells were negative for all these markers. The antibodies used for this analysis were the following: AlexaFluor700-conjugated anti-CD3 (BD Pharmingen, UCHT1), PE-conjugated anti-CD19 (BD Pharmingen, HIB19), FITC-conjugated anti-CD16 (BD Pharmingen, 3G8), and PE-Cy7-conjugated anti-CD14 (BD Pharmingen, M5E2).

Anonymized tonsil material was obtained from tonsillectomies. Tonsils were cut in small pieces and passed through a cell strainer. B cells from PBMCs and dispersed tonsils were positively isolated using anti-human CD19-coupled magnetic beads according to manufacturer's protocol (Invitrogen). The $CD19^-$ fraction left after B cell isolation was used as a control. Purity of isolated cells was determined by flow cytometry. For this, cells were stained using PE-conjugated anti-CD19 (BD Pharmingen, HIB19) and AlexaFluor700-conjugated anti-CD20 (Biolegends, 2H7) antibodies. In each experiment, purity of $CD19^+$ B cells was 95-99%, while $CD19^-$ cells contained less than 0,4% $CD19^+$ cells. Freshly isolated cells were used for experiments. Cells were resuspended in IMDM supplemented with 10% heat-inactivated FCS, 2 mM glutamax, 100 U/ml penicillin and 100 mg/ml streptomycin.

Cell lines

In this study, several transformed human B cell lines were included permitting long-term culture. The B cell lines were transformed either by EBV *in vitro* (i.e. B-lymphoblastoid cell lines (B-LCLs)) or *in vivo* (i.e. Burkitt's lymphoma-derived lines), or, alternatively, by B cell lymphoma protein 6 (BcL-6) and –xL transformation [34]. EBV-negative B cells originally isolated from BL-derived cell lines [35], were also included. An overview of all B lymphocytes used in this study is given **Table 1**.

The EBV-negative, antigen-specific B cell clones immortalized by introduction of Bcl-6 and Bcl-xL were cultured on murine CD40L-expressing feeder cells in the presence of recombinant mouse IL-21 as described elsewhere [34]. Prior to use in experiments, cells were carefully removed from the murine feeder cells and taken up in IMDM containing 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

The EBV-positive B-LCLs JY and MRJ, the BL-derived EBV-negative cell line 2A8, and

the monocytic THP1 cells were cultured in RPMI medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Prior to use in experiments, THP1 cells were differentiated into macrophage-like cells by treatment with 150 nM phorbol myristate acetate (PMA, Sigma-Aldrich) for 48h.

Table 1 B cells used in this study

Cells	Source	Immortalization	EBV status	References
Primary CD19⁺ B cells	Tonsil	no	unknown	
Primary CD19⁺ B cells	PBMC	no	unknown	
B cell clone (tet or flu)	PBMC	Yes, Bcl-6, Bcl-xL	-	[34]
B-LCL (JY or MRJ)	PBMC	Yes, EBV *(in vitro)*	+	[36,37]
2A8	BL	Yes, EBV *(in vivo)*	-	[35]

Replication-deficient lentivirus and transduction

Third generation SIN lentiviruses were generated in 293T cells transfected with the lentivirus plasmid encoding mSTING-HA-IRES-GFP driven by a PGK promoter and the vectors pCMV-VSV-G, pMDLg-RRE, and pRSV-REV to provide the helper functions. 2A8 cells were exposed to with lentivirus-containing supernatant to transfer the genes encoding murine STING and the GFP marker. To enhance transduction efficiency, cells were spin inoculated (1000x*g*, 33° C, 2h) in the presence of 4 ug/ml polybrene. GFP⁺ 2A8 cells were FACS-sorted. A pure GFP⁺ population of cells was propagated and used for experiments.

Reagents

For stimulation of the cytoplasmic DNA pathway, we used dsDNA (HSV60mer is derived from the HSV-1 genome (nucleotides 144,107-144,166 [38]), or 2',3'-cGAMP (Sigma-Aldrich). Unconjugated or fluorescein (FAM)-labeled DNA oligos were mixed and annealed in annealing buffer (10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA) by heating at 95° C for 5 min, followed by a slow cool-down. Annealed oligos were kept at 4° C until used. For stimulation of TLR9, 10 µg/ml CpG DNA (ODN 2006, Invivogen) was added to culture medium and incubated with cells for 24h. The cytoplasmic RNA pathway was triggered by polyinosinic-polycytidylic acid (poly(I:C), Invivogen). Where indicated, cells were treated with 1000 U/ml IFN-β (pbl assay science) added to culture medium 24h prior to transfection.

Transfections and transfection efficiencies

Cells were transfected with dsDNA or poly(I:C) using Lipofectamine2000 (Invitrogen). DNA or poly(I:C) were mixed at a ratio of 1 µg stimulant per 1ul lipofectamine2000. Complexes were allowed to form for 20 min before adding it to the cells. In general, $0.3x10^6$ cells seeded per well of a 24-well plate were transfected with 4 µg DNA or 2 µg poly(I:C) to introduce stimuli to the cytoplasm. Supernatants and/or cells were harvested for analysis at indicated time points, but typically 6h or 24h post-transfection. Transfection efficiencies were determined by exposing cells to cells with FAM-labeled dsDNA/lipofectamine, and assessing fluorescence by flow cytometry after overnight incubation.

Flow cytometry

To determine cell surface levels of indicated markers, cells were stained with antibodies of indicated specificity. Cell were washed in PBS supplemented with 0,5% BSA, and 0,02% sodium azide. Cells were antibody-stained with the following antibodies: mouse α-HLA-DR-PE (L243, BD Biosciences), and anti-CD86-PE (BD Pharmingen). Stained cells were washed, fixed, and subjected to a LSR II flow cytometer (BD Biosciences). Flow cytometry data were analysed using FlowJo (Treestar).

cGAMP stimulation

Digitonin-permeabilized cells were stimulated with cGAMP. For this, cells were taken up in digitonin buffer (5 µg/ml digitonin, 50 mM HEPES (pH 7.0), 100 mM KCl, 85 mM sucrose, 3 mM $MgCl_2$, 1 mM ATP, 0,1 mM GTP, 0,1 mM DTT, 0,2% BSA) containing 1 µM cGAMP. Cells were incubated for 10 min at 37° C, before buffer was replaced by culture medium.

Type I IFN Bioassay

IFN-α/β levels in cell-free culture supernatants were determined using the HEK Blue IFN-α/β reporter cell line (Invivogen), which in response to type I IFN stimulation produces the secreted embryonic alkaline phophatase (SEAP). Supernatants were plated with 5x104 reporter cells in 100 µl in a 96 well plate. Cells were incubated for Colorimetric analyses were performed at 620-655 nm using a plate reader.

Quantitative real-time PCR

RNA was isolated from stimulated cells at indicated time points by using the High Pure RNA Isolation Kit (Roche) according to manufacturer's protocol. Levels of mRNA were assessed in duplicates by real-time PCR using the TaqMan RNA-to-CT 1-step kit (Applied Biosystems). The following TaqMan probes were used: hIFN-β (Hs01077958_s1), hISG54 (IFIT2) (Hs01922738_s1), hTNF-α (Hs01113624_g1), and hβ-actin (Hs99999903_m1). Duplicates with CT values > 1 apart were excluded from analysis. Expression levels were

normalized to β-actin using the 2-ΔΔCT method, and data are presented as fold induction (±SD) over lipofectamine2000-treated control samples.

Western blot analysis

Total cell lysates were generated using RIPA lysis buffer (ThermoFisher) supplemented with 0,2% SDS, protease inhibitor mix (Roche), 50 U/ml Benzonase (Sigma-Aldrich), and 50 mM sodium fluoride (phosphatase inhibitor). NP40 lysis mix (0.5% NP40 (Igepal-CA630), 50 mM Tris HCl (pH 7.5), 150 mM NaCl) containing protease inhibitor mix (Roche) was used to generate post-nuclear lysates as described elsewhere [39]. Lysates were denatured using Laemmli sample buffer containing 20 mM DTT. Proteins were separate on Bio-Rad premade Criterion Tris-HCl gels (4-20% gradient) or on handcast gels (10%) and transferred on PVDF membranes. Membranes were blocked and probed with the following specific antibodies: anti-cGAS (HPA031700, Sigma-Aldrich), anti-IFI16 (C-18, sc-6050, Santa Cruz), anti-STING (D2P2F, Cell Signaling), anti-TBK1 (D1B4, Cell Signaling), anti-IRF3 (D6I4C, XP, Cell Signaling or sc9082X, Santa Cruz), anti-vinculin (hVIN-1, Sigma-Aldrich), and anti-HA (3F10, Roche). Membranes were incubated with secondary HRP-conjugated antibodies and ECL substrate subsequently to visualize bands using an ImageQuant LAS 4000 mini Luminescent Image Analyzer (GE Healthcare Life Sciences) or films.

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References

1. Schneider WM, Chevillotte MD, Rice CM (2014) Interferon-stimulated genes: a complex web of host defenses. Annu Rev Immunol 32: 513-545.
2. Theofilopoulos AN, Baccala R, Beutler B, Kono DH (2005) Type I interferons (alpha/beta) in immunity and autoimmunity. Annu Rev Immunol 23: 307-336.
3. Wu J, Chen ZJ (2014) Innate immune sensing and signaling of cytosolic nucleic acids. Annu Rev Immunol 32: 461-488.
4. Thompson MR, Kaminski JJ, Kurt-Jones EA, Fitzgerald KA (2011) Pattern recognition receptors and the innate immune response to viral infection. Viruses 3: 920-940.
5. Kawai T, Akira S (2011) Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 34: 637-650.
6. Loo YM, Gale M, Jr. (2011) Immune signaling by RIG-I-like receptors. Immunity 34: 680-692.
7. Unterholzner L (2013) The interferon response to intracellular DNA: why so many receptors? Immunobiology 218: 1312-1321.
8. Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, *et al.* (2004) TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat Immunol 5: 190-198.
9. Sun L, Wu J, Du F, Chen X, Chen ZJ (2013) Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science 339: 786-791.
10. Wu J, Sun L, Chen X, Du F, Shi H, *et al.* (2013) Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. Science 339: 826-830.
11. Sun W, Li Y, Chen L, Chen H, You F, *et al.* (2009) ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. Proc Natl Acad Sci U S A 106: 8653-8658.
12. Ishikawa H, Barber GN (2008) STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 455: 674-678.
13. Zhong B, Yang Y, Li S, Wang YY, Li Y, *et al.* (2008) The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. Immunity 29: 538-550.
14. Jakobsen MR, Paludan SR (2014) IFI16: At the interphase between innate DNA sensing and genome regulation. Cytokine Growth Factor Rev 25: 649-655.
15. Johnson KE, Chikoti L, Chandran B (2013) Herpes simplex virus 1 infection induces activation and subsequent inhibition of the IFI16 and NLRP3 inflammasomes. J Virol 87: 5005-5018.
16. Kerur N, Veettil MV, Sharma-Walia N, Bottero V, Sadagopan S, *et al.* (2011) IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi Sarcoma-associated herpesvirus infection. Cell Host Microbe 9: 363-375.
17. Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, *et al.* (2006) 5'-Triphosphate RNA is the ligand for RIG-I. Science 314: 994-997.
18. Ahn J, Barber GN (2014) Self-DNA, STING-dependent signaling and the origins of autoinflammatory disease. Curr Opin Immunol 31: 121-126.
19. Shrivastav M, Niewold TB (2013) Nucleic Acid sensors and type I interferon production in systemic lupus erythematosus. Front Immunol 4: 319.
20. Gao D, Li T, Li XD, Chen X, Li QZ, *et al.* (2015) Activation of cyclic GMP-AMP synthase by self-DNA causes autoimmune diseases. Proc Natl Acad Sci U S A 112: E5699-5705.
21. Ahn J, Gutman D, Saijo S, Barber GN (2012) STING manifests self DNA-dependent inflammatory disease. Proc Natl Acad Sci U S A 109: 19386-19391.
22. Ahn J, Ruiz P, Barber GN (2014) Intrinsic self-DNA triggers inflammatory disease dependent on STING. J Immunol 193: 4634-4642.
23. Gall A, Treuting P, Elkon KB, Loo YM, Gale M, Jr., *et al.* (2012) Autoimmunity initiates in nonhematopoietic cells and progresses via lymphocytes in an interferon-dependent autoimmune

disease. Immunity 36: 120-131.
24. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, *et al.* (2002) Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature 416: 603-607.
25. Uccellini MB, Busconi L, Green NM, Busto P, Christensen SR, *et al.* (2008) Autoreactive B cells discriminate CpG-rich and CpG-poor DNA and this response is modulated by IFN-alpha. J Immunol 181: 5875-5884.
26. van Gent M, Griffin BD, Berkhoff EG, van Leeuwen D, Boer IG, *et al.* (2011) EBV lytic-phase protein BGLF5 contributes to TLR9 downregulation during productive infection. J Immunol 186: 1694-1702.
27. James JA, Robertson JM (2012) Lupus and Epstein-Barr. Curr Opin Rheumatol 24: 383-388.
28. Ansari MA, Singh VV, Dutta S, Veettil MV, Dutta D, *et al.* (2013) Constitutive interferon-inducible protein 16-inflammasome activation during Epstein-Barr virus latency I, II, and III in B and epithelial cells. J Virol 87: 8606-8623.
29. Singh VV, Kerur N, Bottero V, Dutta S, Chakraborty S, *et al.* (2013) Kaposi's sarcoma-associated herpesvirus latency in endothelial and B cells activates gamma interferon-inducible protein 16-mediated inflammasomes. J Virol 87: 4417-4431.
30. Ishikawa H, Ma Z, Barber GN (2009) STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature 461: 788-792.
31. Ma Z, Jacobs SR, West JA, Stopford C, Zhang Z, *et al.* (2015) Modulation of the cGAS-STING DNA sensing pathway by gammaherpesviruses. Proc Natl Acad Sci U S A 112: E4306-4315.
32. Gao D, Wu J, Wu YT, Du F, Aroh C, *et al.* (2013) Cyclic GMP-AMP synthase is an innate immune sensor of HIV and other retroviruses. Science 341: 903-906.
33. Jakobsen MR, Bak RO, Andersen A, Berg RK, Jensen SB, *et al.* (2013) IFI16 senses DNA forms of the lentiviral replication cycle and controls HIV-1 replication. Proc Natl Acad Sci U S A 110: E4571-4580.
34. Kwakkenbos MJ, Diehl SA, Yasuda E, Bakker AQ, van Geelen CM, *et al.* (2010) Generation of stable monoclonal antibody-producing B cell receptor-positive human memory B cells by genetic programming. Nat Med 16: 123-128.
35. Chodosh J, Holder VP, Gan YJ, Belgaumi A, Sample J, *et al.* (1998) Eradication of latent Epstein-Barr virus by hydroxyurea alters the growth-transformed cell phenotype. J Infect Dis 177: 1194-1201.
36. Kremer AN, van der Meijden ED, Honders MW, Goeman JJ, Wiertz EJ, *et al.* (2012) Endogenous HLA class II epitopes that are immunogenic in vivo show distinct behavior toward HLA-DM and its natural inhibitor HLA-DO. Blood 120: 3246-3255.
37. Terhorst C, Parham P, Mann DL, Strominger JL (1976) Structure of HLA antigens: amino-acid and carbohydrate compositions and NH2-terminal sequences of four antigen preparations. Proc Natl Acad Sci U S A 73: 910-914.
38. Berg RK, Rahbek SH, Kofod-Olsen E, Holm CK, Melchjorsen J, *et al.* (2014) T cells detect intracellular DNA but fail to induce type I IFN responses: implications for restriction of HIV replication. PLoS One 9: e84513.
39. van Gent M, Braem SG, de Jong A, Delagic N, Peeters JG, *et al.* (2014) Epstein-Barr virus large tegument protein BPLF1 contributes to innate immune evasion through interference with toll-like receptor signaling. PLoS Pathog 10: e1003960.
40. Agrawal S, Gupta S (2011) TLR1/2, TLR7, and TLR9 signals directly activate human peripheral blood naive and memory B cell subsets to produce cytokines, chemokines, and hematopoietic growth factors. J Clin Immunol 31: 89-98.
41. Diner EJ, Burdette DL, Wilson SC, Monroe KM, Kellenberger CA, *et al.* (2013) The innate immune DNA sensor cGAS produces a noncanonical cyclic dinucleotide that activates human STING. Cell

Rep 3: 1355-1361.
42. Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, *et al.* (2011) A diverse range of gene products are effectors of the type I interferon antiviral response. Nature 472: 481-485.
43. Liu SY, Sanchez DJ, Aliyari R, Lu S, Cheng G (2012) Systematic identification of type I and type II interferon-induced antiviral factors. Proc Natl Acad Sci U S A 109: 4239-4244.
44. Siren J, Pirhonen J, Julkunen I, Matikainen S (2005) IFN-alpha regulates TLR-dependent gene expression of IFN-alpha, IFN-beta, IL-28, and IL-29. J Immunol 174: 1932-1937.
45. Jin L, Waterman PM, Jonscher KR, Short CM, Reisdorph NA, *et al.* (2008) MPYS, a novel membrane tetraspanner, is associated with major histocompatibility complex class II and mediates transduction of apoptotic signals. Mol Cell Biol 28: 5014-5026.
46. Bridgeman A, Maelfait J, Davenne T, Partridge T, Peng Y, *et al.* (2015) Viruses transfer the antiviral second messenger cGAMP between cells. Science 349: 1228-1232.
47. Gentili M, Kowal J, Tkach M, Satoh T, Lahaye X, *et al.* (2015) Transmission of innate immune signaling by packaging of cGAMP in viral particles. Science 349: 1232-1236.
48. Wu JJ, Li W, Shao Y, Avey D, Fu B, *et al.* (2015) Inhibition of cGAS DNA Sensing by a Herpesvirus Virion Protein. Cell Host Microbe 18: 333-344.
49. Zhang G, Chan B, Samarina N, Abere B, Weidner-Glunde M, *et al.* (2016) Cytoplasmic isoforms of Kaposi sarcoma herpesvirus LANA recruit and antagonize the innate immune DNA sensor cGAS. Proc Natl Acad Sci U S A.
50. Braun D, Caramalho I, Demengeot J (2002) IFN-alpha/beta enhances BCR-dependent B cell responses. Int Immunol 14: 411-419.
51. Giordani L, Sanchez M, Libri I, Quaranta MG, Mattioli B, *et al.* (2009) IFN-alpha amplifies human naive B cell TLR-9-mediated activation and Ig production. J Leukoc Biol 86: 261-271.

Chapter 6

Summarizing discussion

Summarizing discussion

Herpesviruses establish lifelong persistent infections in immunocompetent hosts. The herpesvirus Epstein-Barr virus (EBV) has evolved numerous immune evasion strategies that reduce immune activation and recognition of latently as well as productively infected cells. In this thesis, we defined new cellular targets of known and newly identified EBV evasion molecules acting during the lytic cycle and unraveled their underlying molecular mechanisms. In the first part, the findings of each chapter are summarized and discussed. In the second part, overarching points related to the research topic are addressed.

In **Chapter 2**, an shRNA-based approach was employed to reduce translation of the virus-encoded shutoff protein BGLF5 in productively EBV-infected B cells. The list of cellular molecules whose level is decreased by BGLF5-mediated RNA destabilization was extended by the innate immune molecules CD1d and TLR2. In lytically infected B cells, CD1d surface expression was reduced compared to latently infected cells. This phenotype was substantially rescued upon knock-down of the BGLF5 transcript. Expression of the *BGLF5* gene in EBV-negative cells decreased CD1d and TLR2 surface levels, thereby confirming the involvement of BGLF5. In addition to CD1d, a panel of cell surface markers was tested in lytically infected BGLF5-silenced B cells and compared to control cells. Some of these molecules displayed a limited downregulation, whereas certain molecules were strongly downregulated. Overall, the effect of BGLF5-mediated shutoff appeared to be rather limited, although the efficient shutoff by BGLF5 had been demonstrated using pulse-chase analysis in an earlier study [1]. Our findings on the limited effect of BGLF5 are supported by a study, in which the contribution of BGLF5 on the downregulation of HLA I in lytically infected B cells was found to be marginal [2]. Similar to our study, a shRNA approach was used to investigate the extent of BGLF5-mediated HLA I downregulation. In both studies BGLF5 transcript and protein levels were reduced up to 75%. The remaining levels of BGLF5 protein could contribute to downregulation of the molecules tested; thereby the effect of BGLF5 might be underestimated. Our preliminary pulse-chase analysis of BGLF5-silenced cells suggests that remaining BGLF5 protein was sufficient to induce efficient shutoff. Viral proteins are known to combine distinct functions. Also, BGLF5 has a dual function in EBV infection. The BGLF5 protein has RNase and DNase activity [3,4]. The RNase activity is responsible for shutoff, while the DNase function contributes to genome processing [1,5]. Therefore, silencing BGLF5 may also affect other processes of EBV replication. In BGLF5-silenced cells, the late phase of lytic replication was delayed. To exclude that this is due to insufficient genome processing, it might be interesting to selectively inhibit RNase function, without interfering with DNase activity. However, BGLF5 has one catalytic site for both activities making it challenging to generate a mutant that lacks only RNase activity. Two studies attempted to find such a mutant, but none of the BGLF5 mutants fulfilled these requirements entirely [6,7]. The best candidate obtained from these studies has a single mutation (K231M) in the so-called

"bridge", a structural feature of BGLF5 [4]. This mutant displayed severely impaired shutoff function as determined by rescued GFP reduction, HLA I cell surface expression and T cell recognition as well as cytosolic poly(A) binding protein relocalization compared to wild-type protein. Its DNase activity was reduced as well, but not absent. The maintained DNase activity determined differed significantly amongst the two studies, therefore, it remains unclear how suitable the BGLF5 mutant would be for complementation studies. Moreover, introduction of such a mutation into the BGLF5 gene in EBV-infected B cells is, however, laborious, if done in EBV-infected B cells (s. section on *Genetic modifications*). Still, this might be an elegant solution to yet another problem intrinsic to shRNA-based BGLF5 knockdown: the BGLF5-coding transcript also encodes BGLF4. Hence, shRNAs targeting the BGLF5 transcript simultaneously reduce BGLF4 levels. Knockdown of the BGLF4 transcript, coding for the EBV protein kinase, decreases expression of 31 late viral genes [8]. Indeed, we observed decreased levels of late protein expression on the cell surface of BGLF5-silenced cells during the lytic cycle. Therefore, the strong effect of the early gene product BGLF5 on CD1d observed in EBV-infected B cells may also be accounted to late viral proteins.

We describe late viral proteins to interfere with CD1d surface detection in **Chapter 3**. EBV gp150, the viral protein displaying the strongest effect on CD1d of all late viral glycoproteins, appeared to interfere with detection of several additional cell surface molecules including the antigen-presenting molecules HLA I and HLA II. Decreasing HLA I and II reduced the $CD8^+$ and $CD4^+$ T cell response to gp150-expressing cells by about 40% and 60%, respectively.. This has been confirmed by the Rowe lab [9] and is the first known function of EBV gp150. Importantly, comparing lytically infected B cells harboring gp150-deficient EBV to wild-type EBV revealed that in the absence of gp150 B cells displayed higher levels of antigen-presenting molecules. This suggests that gp150 contributes to immune evasion of several T cell subsets during the late phase of lytic replication. It has been postulated earlier that besides the early inhibitors BNLF2a, BGLF5, and BILF1, additional HLA I immune evasion molecules act during the late lytic phase [2]. It is currently unclear whether there are more late proteins targeting HLA I, but none of the late EBV glycoproteins showed substantial downregulation. We identified two additional glycoproteins, gp350 and BMRF2, interfering with CD1d surface expression, but the mechanisms underlying their action remains to be established. We focused on the elucidation of the mechanism of EBV gp150. Microscopy analyses of gp150-expressing cells led to the conclusion that gp150 acts on the cell surface, as antibody-based detection of GFP-tagged HLA molecules was impaired. In contrast, these HLA molecules were still present on the cell surface as indicated by GFP fluorescence. Supporting the hypothesis that the heavily glycosylated protein gp150 acts at the cell surface, inhibition or absence of sialoglycosidases or enzymatic removal of sialic acids from surface glycoproteins increased the antibody-mediated detection of surface molecules. This suggested that gp150 shields antigen-presenting molecules by means of its glycans. In sharp contrast, Quinn *et al.* claimed that EBV gp150 enhances ubiquitin-dependent internalization of HLA from the cell

surface, although a reduction in total HLA levels was not detected [9]. Furthermore, they report that proteasome inhibition completely rescued the gp150-mediated downregulation of HLA molecules, which is opposing to our findings. They observed only an effect of gp150 on HLA I molecules, but not on other molecules [9]. This discrepancy may be accounted to lower expression levels of gp150. We find that decreasing doses of gp150-coding lentivirus resulted in a reduced phenotype. Future studies should address the question whether gp150 acts in both suggested ways on antigen-presenting molecules and whether the expression levels of gp150 influence the mode of action. The Ebola glycoprotein is the only viral protein reported to act in a similar way [10]. It seems plausible that other heavily glycosylated (herpes)virus proteins shield cellular surface molecules. A homologue of the *BDLF3* gene is present in the genome of rhesus LCV, but displays the lowest degree of conservation among all glycoproteins [11]. As the glycans present on gp150 shield surface molecules, it may be assumed the exact amino acid sequence is less relevant to its function providing glycosylation is supported. Given that BDLF3 of rhesus LCV is predicted to be heavily glycosylated, it seems likely that it has immune evasive properties similar to EBV gp150. BDLF3 is absent from marmoset LCV [12]. This may suggest that *BDLF3* appeared rather late during evolution of the lymphocryptoviruses.

Another immune evasion molecule of EBV is the constitutively active orphan GPCR BILF1 [13,14]. BILF1 had been reported to interfere with HLA I surface expression by reducing the exocytic trafficking of HLA I to the cell surface and enhancing endocytosis resulting in degradation of this antigen-presenting molecule [14,15]. Reduction of HLA I impaired CD8+ T cell recognition of BILF1-expressing cells [14]. BILF1 appeared to interfere predominantly with presentation of late viral antigens [2]. In **Chapter 4**, we report that EBV BILF1 displays specificity towards certain HLA molecules as HLA-A, -B and, -E were targeted for downregulation, but HLA-C appeared virtually resistant to BILF1-mediated downregulation. To downregulate HLA I molecules, BILF1 required its C-terminal cytoplasmic tail. Yet, the cytoplasmic tail of EBV BILF1 appeared not to be sufficient to render marmoset LCV BILF1, which is unable to downregulate HLA I or LCV MHC I, capable of downregulating HLA I molecules. This suggests that an additional feature in BILF1 is required to allow it to act as immune evasion molecule. HLA I molecules lacking their cytoplasmic tail were not targeted for downregulation. Therefore, the cytoplasmic part of HLA I molecules was the determinant of BILF1 sensitivity. We identified three amino acid residues in the cytoplasmic tail of HLA-C that were not present in the cytoplasmic tail of BILF1-sensitive HLA I molecules. Changing the three residues into those of the HLA-C molecule rendered an otherwise sensitive HLA-B molecule resistant to downregulation. The molecular mechanism underlying BILF1-mediated downregulation is largely unclear, but BILF1 is reported to co-immunoprecipitate with HLA I [14]. To assess how the identified amino acids render HLA-C molecules resistant to BILF1-mediated downregulation, it may be interesting to determine whether BILF1 can interact with HLA-C, despite its inability to substantially downregulate this HLA molecule. This could

facilitate further elucidation of the mechanism of action of BILF1 and assist in identification of potential intracellular adaptor proteins involved in sorting or internalization of HLA I molecules.

Lastly, we aimed to learn more about evasion of innate immune pathways present in B cells by EBV. Herpesviruses have been shown to be recognized by the cytosolic DNA sensing pathway resulting in type I IFN production or inflammasome formation (reviewed in [16]). The genome of EBV and KSHV is sensed by the DNA sensor IFI16 resulting in inflammasome formation in B cells [17,18]. Several viral evasion molecules, including EBV BLRF2, interfering with DNA sensing pathway have been identified [19,20,21] supporting the notion that this immune pathway plays a role in the immune response to herpesviruses. The cytosolic DNA sensing pathway in human B cells was analysed in **Chapter 5,** as B lymphocytes are target cells for the DNA viruses EBV and KSHV. Neither primary B lymphocytes, nor the B cell lines examined, produced type I IFNs upon exposure to cytoplasmic DNA. A similar phenomenon is reported for T lymphocytes [22]. This raises the following question: Why do lymphocytes have the DNA sensors cGAS and IFI16, but are unresponsive to cytoplasmic DNA? Most B lymphocytes lacked the STING protein, an essential adaptor protein of the cytoplasmic DNA sensing pathway. Interestingly, B cells producing STING also did not mount a type I IFN response upon stimulation with DNA or cGAMP suggesting that STING expression was not sufficient to reconstitute the pathway in B cells. This resembles the situation in T lymphocytes: The DNA sensors, STING and the downstream molecules are present, but T lymphocytes do not respond to cytoplasmic DNA, although they sense the DNA [22]. Presence of the sensors in lymphocytes suggests that these cells are responsive under certain conditions that remain to be determined. It is tempting to speculate that lymphocytes possess a safeguard mechanism that regulates responsiveness to cytoplasmic DNA. Therefore, these cell types may serve as a study field to unravel regulatory pathways of cytoplasmic DNA sensing. Unresponsiveness to cytosolic DNA may make lymphocytes attractive target cells for DNA viruses such as herpesviruses. It remains to be determined whether other target cells of EBV sense the viral genome and initiate a type I IFN response.

Conclusions and future directions

It is well established that patients lacking functional T cells are prone to developing EBV-associated diseases or malignancies (**Chapter 1**). The EBV proteins studied in this thesis (**Chapter 2, 3, and 4**) interfere with generation and recognition of antigen-presenting molecules on the cell surface resulting in reduced T cell activation during the productive cycle of EBV infection. Investigation of EBV immune evasion molecules to understand their molecular mechanism of action and to assess their cellular targets significantly contributes to our understanding of EBV biology. Considering that EBV has even more T cell evasion molecules, this demonstrates that T cell activation is a major threat to EBV infection. To counteract efficient T cell recognition, this virus evolved several ways of interference acting at

different phases of the lytic cycle as well as during latency. Yet, we are far from comprehending how and when individual EBV proteins contribute to EBV infection and how this impacts pathogenesis on an organismic level. Studying the innate immune DNA sensing pathways of B cells (**Chapter 5**) expands our knowledge on immune pathways that may limit EBV infection. In conclusion, insights are gained on the cellular pathways that are involved in antiviral defense.

Animal models

Although attempts have been made to determine the contribution of individual EBV proteins to immune evasion during primary infection or lytic cycle *in vitro* [9,23,24], the role of distinct immune evasion strategies *in vivo* is largely unclear. One obstacle is the requirement for a permissive animal model that supports EBV infection. As EBV has a very narrow host range [25], the use of mice with reconstituted human immune system compartments (HIS mice) infected with EBV or infection of rhesus macaques with the closely related rhesus LCV is warranted [12,26,27]. Similar models have been informative for the investigation of the role of immune evasion *in vivo* for other human-specific viruses like HCMV. A cluster of rhesus CMV genes encoding immune evasion molecules (Rh182-189, which act homologous to the HCMV genes US2-US11) that interfere with MHC I-mediated antigen presentation to $CD8^+$ T cells are essential to establish a secondary persistent infection ('superinfection') in CMV-positive rhesus macaques [28]. The rhesus CMV-specific MHC I evasion molecule VIHCE was not required for superinfection. Depletion of $CD8^+$ T cells allowed the RhUS2-US11-deficient rhesus CMV to establish secondary persistent infection [28]. Interference with MHC I was not required for primary infection. This demonstrates that superinfection, but not primary infection relies on the presence of immune evasion molecules and that T cell control is effective, if not evaded. It is likely that the immune evasion genes are also not required for primary EBV infection in a naïve host when EBV-specific T cells are still absent. It remains to be investigated whether immune evasion plays a role during infection of uninfected cells in an EBV-experienced host or whether they rather contribute to prolonging the time span of virus production during reactivation. The EBV evasion genes *BCRF1* and *BNLF2a,* coding for vIL-10 and BNLF2a, respectively, are expressed upon primary infection of B cells *in vitro* [23]. Thereby, they may contribute to immune evasion during the pre-latent phase of infection in B cells.

To study infection with the EBV-related rhesus LCV, a specific pathogen-free colony of rhesus LCV-naïve rhesus macaques is required This animal model supports oral transmission, latent and lytic rhesus LCV infection and reproduces other key aspects of human EBV infection [12,29]. In a first study evaluating contribution of immune evasion, naïve rhesus macaques were infected with rhesus LCV lacking the innate immune evasion gene *BARF1* coding for the colony stimulating factor 1 (CSF-1) blocking protein. The viral load was reduced during lytic infection and frequency of latently infected cells was decreased [30]. This demonstrates that evasion of the innate immune system is important for efficient rhesus LCV infection.

HIS mice have hematopoietic cells of human origin and are used to study EBV infection *in vivo*. They improved our understanding of the role of NK cells early during EBV infection [31]. NK cells appear to control lytically, but not latently, infected B cells. In the absence of NK cells, CD8+ T cells expanded more dramatically and tumor incidence was higher [31]. However, the use of HIS mice is limited as not all steps of the EBV life cycle can be modeled, e.g. oral transmission, which requires infection of epithelial cells. Despite the human origin of the hematopoietic compartment, all non-hematopoietic cells including epithelial cells are of murine origin. Hence, life cycle steps involving non-hematopoietic cells cannot be investigated using this model.

These two examples show that the recent advances in establishing animal models to study EBV infection *in vivo* are promising. Further comprehension of the role of individual viral gene products, including immune evasion proteins, during infection may be expected within the coming decade. In addition, the contribution of specific immune pathways or cells to control viral infection should be studied in order to provide a complete picture of virus-host interaction.

Genetic manipulations

Genetic manipulation of the viral genome is a prerequisite to study the function and role of individual gene products of EBV or rhesus LCV *in vivo*. To investigate the contribution of gene products *in vitro*, transcript knock-down approaches can be employed, although often there is a preference for knock-out of viral genes. Once a gene-deficient viral genome (present in a cell line or a virus particle) is generated, many of the following steps in research become easier and clearer phenotypes can be obtained in comparison to knock-down approaches. The necessary genetic modification of the EBV genome can be achieved by the use of bacterial artificial chromosomes (BAC) or classical recombination [32]. For *in vitro* experiments, the major drawback of the BAC-based system is the necessity to infect B cells. Typically, *in vitro* infected B cells show a very low efficiency of entering the lytic cycle, thereby making it challenging to study genes expressed during the lytic cycle. Therefore, predominantly genes involved in primary infection and latency can be studied with viruses derived from the BAC system. In contrast, this system is indispensable for *in vivo* studies. The EBV BAC system was generated in the late 1990s [33], but a rhesus LCV BAC was only established recently [34] making it possible now to delete individual genes from rhesus LCV to test their function *in vitro* or *in vivo*.

As an alternative, and especially for lytic cycle-expressed genes, a classical recombination approach can be employed to delete, substitute, or introduce genes. The Akata cell line or the AKBM cell line, a derivative of the Akata cell line, allows efficient lytic replication, thereby being a suitable model to study the contribution of individual lytic phase gene products *in vitro*. As several copies of the EBV genome are present,, the classical recombination approaches to modify all genomes present are consequently very inefficient and laborious due to tedious screenings of clones, as described for the generation of the BDLF3-knock-

out Akata cell line [35]. The recently developed CRISPR/Cas9-based genome editing systems promise to facilitate fast and efficient editing of any kind of genome [36,37]. However, the genome editing systems do not prove to be a very effective tool to interrupt or introduce genes into the numerous EBV genomes present in a single cell as of yet [38]. Therefore, shRNA-based approaches as applied for BGLF5 (**Chapter 2**) or other gene products [2] may continue to be the tool of choice for studying essential, but also non-essential individual EBV proteins.

Coinfections

Endemic Burkitt's lymphoma occurring in equatorial Africa and Papua New Guinea is associated with coinfection of the malaria-causing parasite *Plasmodium falciparum* and EBV [39,40]. Although this link is known for half a century, it cannot be fully explained yet. Two recent studies provide important insights into the molecular mechanisms [41,42] (reviewed in [43]). In short, individuals chronically infected with *Plasmodium falciparum* had higher numbers of germinal center B cells that made high levels of activation-induced cytidine deaminase (AID), an enzyme involved in somatic hypermutation and class switching of immunoglobulin genes [42]. Consequently, EBV-positive germinal center B cells were more abundant in individuals infected with *Plasmodium falciparum* in comparison to uninfected ones [42]. In combination, the increased risk of AID-induced translocation and EBV-mediated survival of B cells, two factors favoring cancer cell development, may explain the higher incidence of BL in areas where *Plasmodium falciparum* –mediated malaria is holoendemic.

The example of *Plasmodium falciparum* and EBV coinfection resulting in lymphoma formation is extreme. There are more examples of microorganisms shaping their (micro)-environment, which may have deleterious or beneficial consequences. Interaction of the 'virome', defined as all viruses or virus-related sequences present in an individual, and the host influences phenotypes of health and disease (reviewed in [44]). Infection with persistent viruses such as herpesviruses, and especially with the highly disseminated EBV, might be a significant determinant. Continuous replication and shedding of EBV occurs in healthy carriers [45], hence there might be a low level of permanent immune stimulation by lytically replicating cells. Moreover, latently infected cells may release cytokines and type I IFNs as latent EBV gene products have been identified that activate and/or modulateinnate immune pathways **(Chapter 1)**. For example, LMP1 activates the several innate pathways, including the NF-κB pathway [46] and also the EBERs are sensed by different innate immune pathways [47,48]. Moreover, there are virally-encoded miRNAs present in EBV-infected cells that possibly induce transcriptional changes influencing immune-related genes ([49,50,51] and Hooykaas et al, personal communication). These different gene products are not only restricted to the virus-infected cell itself, since different RNA species including miRNA and EBERs might also be transferred by exosomes to other cells or even non-permissive cell types [52,53]. Thereby, EBV infection may shape our immune responses, but not *per se* in a negative fashion. One example of beneficial herpesvirus infection is illustrated in a report on knock-out mice lacking single immune factors such as HOIL-1, IL-6, or caspase-1, but being resistant

to lethal doses of *Listeria* due to latent infection with MHV68, a murine gamma-herpesvirus [54]. It is, therefore, exciting to consider our herpesviruses not only as pathogens, but also as "commensals" that might contribute to health and disease.

Glycosylation

Studying herpesviruses provides insights into its intriguing immune evasion mechanisms that enable the viruses to persistently infect their host, but also offer insights on fundamental molecular processes and modifications. EBV gp150 shields cell surface molecules by means of its abundant N and Olinked glycans (**Chapter 3**). A similar mechanism has been reported for Ebola glycoprotein [10]. Glycosylation of viral proteins also aids evasion of antibody-mediated neutralization (reviewed in [55]) including HCMV gN and BoHV-4 gp180 [56,57]. The glycosylation not only shielded epitopes within the glycosylated protein, but also prevented antibody-binding to other viral proteins [57]. A recent study suggests that viral glycoproteins can induce glycan-dependent immune responses [58]. The authors found that HSV-2 triggers a mucosal CXCL10 response preceding the early IFN response. The CXCL10 response appeared to be dependent on O-linked glycosylation of the viral glycoproteins. Cellular carbohydrate receptors like C-type lectins did not sense the glycosylation and activate signaling pathways. Instead, enzymatic or repeated flushing-induced disruption of the mucosal layer resulted in CXCL10 secretion. Therefore, the authors concluded that O-linked glycosylation of the virion was required to cross the mucus layer efficiently and to enter the underlying cells [58]. In summary, these studies suggest that glycosylation of viral proteins fine-tunes and adds functions to the glycoproteins. In addition, viral glycoproteins also interact with different cellular lectins (reviewed in [59]). Sialic acid–binding Ig-like lectins (Siglecs) are primarily present on immune cells and many of them trigger immune modulatory responses via intracellular signaling domains such as immunoreceptor tyrosine-based inhibitory motif (ITIM) Other Siglecs associate with a protein containing immunoreceptor tyrosine-based activating motif (ITAM). The signalling cascade initiated by sialic acid binding can dampen the outcome of other immune signalling pathways [60]. It is tempting to speculate that sialic acid-decorated glycoproteins of EBV such as gp150 may alter immune signalling events in *cis* or *trans* by activating Siglecs present on B cells or on cells interacting with B cells, respecitvely. This could be considered as immune evasion strategy. Altered glycosylation in cancer and auto-immunity is also increasingly recognized [61,62], but how and to what extent this impacts immune responses or other processes remains largely unclear. It is challenging to study the role of glycosylation of proteins without affecting the functionality of the protein. Yet, it is interesting to consider glycosylation as an additional layer of regulation similar to other post-translational modifications.

Concluding remarks

Investigation of the immune evasion strategies of the oncogenic γ-herpesvirus EBV provides essential insights in the underlying molecular working mechanisms and targets of the viral molecules. The knowledge on their different strategies might aid in designing anti-viral drugs or therapies to counteract these in order to cure EBV-associated diseases and malignancies. Currently, there is no drug available to treat infectious mononucleosis, which can be life-threatening in patients with certain immunodeficiencies. Targeting viral evasion molecules that interfere with innate or adaptive immune responses could improve immune recognition resulting in a better or faster control of the viral infection by the host. Alternatively, it is interesting to speculate whether viral evasion molecules might be exploited as tool to prevent or dampen unwanted immune responses [63,64]. Exploitation of molecules interfering with antigen presentation could be used to make transplanted or own organs such as the pancreas "invisible" to the immune system to prevent unwanted T cell attack. Viral molecules targeting innate immune responses might be exploited to reduce inflammation or auto-immunity. Of note, there are obvious obstacles such as delivery and safety issues, but also problems regarding preexisting immunity. In case of interference with antigen presentation, it needs to be considered that viral infection of the "immune-invisible" graft might not be properly controlled by the immune system. To identify the best viral target molecule to limit viral infection or the molecules with the greatest therapeutic potential, profound knowledge on the contribution and role of individual viral molecules during the replicative cycle and pathogenesis of the virus in vivo is required. Vaccination against EBV appears to be an attractive option to prevent associated diseases and malignancies. However, we should also consider that viruses that coevolved for millions of years together with their host may provide benefits, although certain individuals are at risk to develop disease or malignancies.

References

1. Rowe M, Glaunsinger B, van LD, Zuo J, Sweetman D, *et al.* (2007) Host shutoff during productive Epstein-Barr virus infection is mediated by BGLF5 and may contribute to immune evasion. Proc Natl Acad Sci U S A 104: 3366-3371.
2. Quinn LL, Zuo J, Abbott RJM, Shannon-Lowe C, Tierney RJ, *et al.* (2014) Cooperation between Epstein-Barr Virus Immune Evasion Proteins Spreads Protection from CD8+ T Cell Recognition across All Three Phases of the Lytic Cycle. PLoS Pathog 10: e1004322.
3. Baylis SA, Purifoy DJ, Littler E (1989) The characterization of the EBV alkaline deoxyribonuclease cloned and expressed in E. coli. Nucleic Acids Res 17: 7609-7622.
4. Buisson M, Geoui T, Flot D, Tarbouriech N, Ressing ME, *et al.* (2009) A bridge crosses the active-site canyon of the Epstein-Barr virus nuclease with DNase and RNase activities. J Mol Biol 391: 717-728.
5. Feederle R, Bannert H, Lips H, Muller-Lantzsch N, Delecluse HJ (2009) The Epstein-Barr virus alkaline exonuclease BGLF5 serves pleiotropic functions in virus replication. J Virol 83: 4952-4962.
6. Horst D, Burmeister WP, Boer IGJ, van Leeuwen D, Buisson M, *et al.* (2012) The Bridge in the Epstein-Barr Virus Alkaline Exonuclease Protein BGLF5 Contributes to Shutoff Activity during Productive Infection. Journal of Virology 86: 9175-9187.

7. Zuo J, Thomas W, van Leeuwen D, Middeldorp JM, Wiertz EJHJ, *et al.* (2008) The DNase of Gammaherpesviruses Impairs Recognition by Virus-Specific CD8+ T Cells through an Additional Host Shutoff Function. Journal of Virology 82: 2385-2393.
8. El-Guindy A, Lopez-Giraldez F, Delecluse HJ, McKenzie J, Miller G (2014) A locus encompassing the Epstein-Barr virus bglf4 kinase regulates expression of genes encoding viral structural proteins. PLoS Pathog 10: e1004307.
9. Quinn LL, Williams LR, White C, Forrest C, Zuo J, *et al.* (2015) The Missing Link in Epstein-Barr Virus Immune Evasion: the BDLF3 Gene Induces Ubiquitination and Downregulation of Major Histocompatibility Complex Class I (MHC-I) and MHC-II. J Virol 90: 356-367.
10. Francica JR, Varela-Rohena A, Medvec A, Plesa G, Riley JL, *et al.* (2010) Steric shielding of surface epitopes and impaired immune recognition induced by the ebola virus glycoprotein. PLoS Pathog 6: e1001098.
11. Rivailler P, Jiang H, Cho YG, Quink C, Wang F (2002) Complete nucleotide sequence of the rhesus lymphocryptovirus: genetic validation for an Epstein-Barr virus animal model. J Virol 76: 421-426.
12. Wang F (2013) Nonhuman primate models for Epstein-Barr virus infection. Curr Opin Virol 3: 233-237.
13. Paulsen SJ, Rosenkilde MM, Eugen-Olsen J, Kledal TN (2005) Epstein-Barr virus-encoded BILF1 is a constitutively active G protein-coupled receptor. J Virol 79: 536-546.
14. Zuo J, Currin A, Griffin BD, Shannon-Lowe C, Thomas WA, *et al.* (2009) The Epstein-Barr Virus G-Protein-Coupled Receptor Contributes to Immune Evasion by Targeting MHC Class I Molecules for Degradation. PLoS Pathog 5: e1000255.
15. Zuo J, Quinn LL, Tamblyn J, Thomas WA, Feederle R, *et al.* (2011) The Epstein-Barr virus-encoded BILF1 protein modulates immune recognition of endogenously processed antigen by targeting major histocompatibility complex class I molecules trafficking on both the exocytic and endocytic pathways. J Virol 85: 1604-1614.
16. Orzalli MH, Knipe DM (2014) Cellular sensing of viral DNA and viral evasion mechanisms. Annu Rev Microbiol 68: 477-492.
17. Ansari MA, Singh VV, Dutta S, Veettil MV, Dutta D, *et al.* (2013) Constitutive Interferon-Inducible Protein 16-Inflammasome Activation during Epstein-Barr Virus Latency I, II, and III in B and Epithelial Cells. Journal of Virology 87: 8606-8623.
18. Singh VV, Kerur N, Bottero V, Dutta S, Chakraborty S, *et al.* (2013) Kaposi's sarcoma-associated herpesvirus latency in endothelial and B cells activates gamma interferon-inducible protein 16-mediated inflammasomes. J Virol 87: 4417-4431.
19. Ma Z, Jacobs SR, West JA, Stopford C, Zhang Z, *et al.* (2015) Modulation of the cGAS-STING DNA sensing pathway by gammaherpesviruses. Proc Natl Acad Sci U S A 112: E4306-4315.
20. Wu JJ, Li W, Shao Y, Avey D, Fu B, *et al.* (2015) Inhibition of cGAS DNA Sensing by a Herpesvirus Virion Protein. Cell Host Microbe 18: 333-344.
21. Zhang G, Chan B, Samarina N, Abere B, Weidner-Glunde M, *et al.* (2016) Cytoplasmic isoforms of Kaposi sarcoma herpesvirus LANA recruit and antagonize the innate immune DNA sensor cGAS. Proc Natl Acad Sci U S A.
22. Berg RK, Rahbek SH, Kofod-Olsen E, Holm CK, Melchjorsen J, *et al.* (2014) T cells detect intracellular DNA but fail to induce type I IFN responses: implications for restriction of HIV replication. PLoS One 9: e84513.
23. Jochum S, Moosmann A, Lang S, Hammerschmidt W, Zeidler R (2012) The EBV Immunoevasins vIL-10 and BNLF2a Protect Newly Infected B Cells from Immune Recognition and Elimination. PLoS Pathog 8: e1002704.
24. Croft NP, Shannon-Lowe C, Bell AI, Horst Dl, Kremmer E, *et al.* (2009) Stage-Specific Inhibition of MHC Class I Presentation by the Epstein-Barr Virus BNLF2a Protein during Virus Lytic Cycle. PLoS Pathog 5: e1000490.

25. Muhe J, Wang F (2015) Host Range Restriction of Epstein-Barr Virus and Related Lymphocryptoviruses. J Virol 89: 9133-9136.
26. Dittmer DP, Damania B, Sin SH (2015) Animal models of tumorigenic herpesviruses--an update. Curr Opin Virol 14: 145-150.
27. Rickinson AB, Long HM, Palendira U, Munz C, Hislop AD (2014) Cellular immune controls over Epstein-Barr virus infection: new lessons from the clinic and the laboratory. Trends Immunol 35: 159-169.
28. Hansen SG, Powers CJ, Richards R, Ventura AB, Ford JC, *et al.* (2010) Evasion of CD8+ T cells is critical for superinfection by cytomegalovirus. Science 328: 102-106.
29. Moghaddam A, Rosenzweig M, Lee-Parritz D, Annis B, Johnson RP, *et al.* (1997) An animal model for acute and persistent Epstein-Barr virus infection. Science 276: 2030-2033.
30. Ohashi M, Fogg MH, Orlova N, Quink C, Wang F (2012) An Epstein-Barr Virus Encoded Inhibitor of Colony Stimulating Factor-1 Signaling Is an Important Determinant for Acute and Persistent EBV Infection. PLoS Pathog 8: e1003095.
31. Chijioke O, Muller A, Feederle R, Barros MH, Krieg C, *et al.* (2013) Human natural killer cells prevent infectious mononucleosis features by targeting lytic Epstein-Barr virus infection. Cell Rep 5: 1489-1498.
32. Feederle R, Bartlett EJ, Delecluse HJ (2010) Epstein-Barr virus genetics: talking about the BAC generation. Herpesviridae 1: 6.
33. Delecluse HJ, Hilsendegen T, Pich D, Zeidler R, Hammerschmidt W (1998) Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. Proc Natl Acad Sci U S A 95: 8245-8250.
34. Ohashi M, Orlova N, Quink C, Wang F (2011) Cloning of the Epstein-Barr virus-related rhesus lymphocryptovirus as a bacterial artificial chromosome: a loss-of-function mutation of the rhBARF1 immune evasion gene. J Virol 85: 1330-1339.
35. Borza CM, Hutt-Fletcher LM (1998) Epstein-Barr virus recombinant lacking expression of glycoprotein gp150 infects B cells normally but is enhanced for infection of epithelial cells. J Virol 72: 7577-7582.
36. Mali P, Yang L, Esvelt KM, Aach J, Guell M, *et al.* (2013) RNA-guided human genome engineering via Cas9. Science 339: 823-826.
37. Cong L, Ran FA, Cox D, Lin S, Barretto R, *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339: 819-823.
38. Yuen KS, Chan CP, Wong NH, Ho CH, Ho TH, *et al.* (2015) CRISPR/Cas9-mediated genome editing of Epstein-Barr virus in human cells. J Gen Virol 96: 626-636.
39. Burkitt D (1962) A children's cancer dependent on climatic factors. Nature 194: 232-234.
40. Burkitt DP (1983) The discovery of Burkitt's lymphoma. Cancer 51: 1777-1786.
41. Robbiani DF, Deroubaix S, Feldhahn N, Oliveira TY, Callen E, *et al.* (2015) Plasmodium Infection Promotes Genomic Instability and AID-Dependent B Cell Lymphoma. Cell 162: 727-737.
42. Torgbor C, Awuah P, Deitsch K, Kalantari P, Duca KA, *et al.* (2014) A multifactorial role for P. falciparum malaria in endemic Burkitt's lymphoma pathogenesis. PLoS Pathog 10: e1004170.
43. Thorley-Lawson D, Deitsch KW, Duca KA, Torgbor C (2016) The Link between Plasmodium falciparum Malaria and Endemic Burkitt's Lymphoma-New Insight into a 50-Year-Old Enigma. PLoS Pathog 12: e1005331.
44. Virgin HW (2014) The virome in mammalian physiology and disease. Cell 157: 142-150.
45. Hadinoto V, Shapiro M, Sun CC, Thorley-Lawson DA (2009) The dynamics of EBV shedding implicate a central role for epithelial cells in amplifying viral output. PLoS Pathog 5: e1000496.
46. Middeldorp JM, Pegtel DM (2008) Multiple roles of LMP1 in Epstein-Barr virus induced immune escape. Seminars in Cancer Biology
47. Manipulation of the immune response by Epstein-Barr virus and Kaposi's sarcoma-associated

herpes virus: Consequences for tumor development. pp. 388-396.
48. Iwakiri D, Zhou L, Samanta M, Matsumoto M, Ebihara T, *et al.* (2009) Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from Toll-like receptor 3. J Exp Med 206: 2091-2099.
49. Samanta M, Iwakiri D, Kanda T, Imaizumi T, Takada K (2006) EB virus-encoded RNAs are recognized by RIG-I and activate signaling to induce type I IFN. EMBO J 25: 4207-4214.
50. Xia T, O'Hara A, Araujo I, Barreto J, Carvalho E, *et al.* (2008) EBV MicroRNAs in Primary Lymphomas and Targeting of CXCL-11 by ebv-mir-BHRF1-3. Cancer Res 68: 1436-1442.
51. Nachmani D, Stern-Ginossar N, Sarid R, Mandelboim O (2009) Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. Cell Host Microbe 5: 376-385.
52. Haneklaus M, Gerlic M, Kurowska-Stolarska M, Rainey AA, Pich D, *et al.* (2012) Cutting edge: miR-223 and EBV miR-BART15 regulate the NLRP3 inflammasome and IL-1beta production. J Immunol 189: 3795-3799.
53. Baglio SR, van Eijndhoven MA, Koppers-Lalic D, Berenguer J, Lougheed SM, *et al.* (2016) Sensing of latent EBV infection through exosomal transfer of 5'pppRNA. Proc Natl Acad Sci U S A 113: E587-596.
54. Ahmed W, Philip PS, Tariq S, Khan G (2014) Epstein-Barr virus-encoded small RNAs (EBERs) are present in fractions related to exosomes released by EBV-transformed cells. PLoS One 9: e99163.
55. MacDuff DA, Reese TA, Kimmey JM, Weiss LA, Song C, *et al.* (2015) Phenotypic complementation of genetic immunodeficiency by chronic herpesvirus infection. Elife 4.
56. Cook JD, Lee JE (2013) The secret life of viral entry glycoproteins: moonlighting in immune evasion. PLoS Pathog 9: e1003258.
57. Machiels B, Lete C, Guillaume A, Mast J, Stevenson PG, *et al.* (2011) Antibody evasion by a gammaherpesvirus O-glycan shield. PLoS Pathog 7: e1002387.
58. Kropff B, Burkhardt C, Schott J, Nentwich J, Fisch T, *et al.* (2012) Glycoprotein N of human cytomegalovirus protects the virus from neutralizing antibodies. PLoS Pathog 8: e1002999.
59. Iversen MB, Reinert LS, Thomsen MK, Bagdonaite I, Nandakumar R, *et al.* (2016) An innate antiviral pathway acting before interferons at epithelial surfaces. Nat Immunol 17: 150-158.
60. Van Breedam W, Pohlmann S, Favoreel HW, de Groot RJ, Nauwynck HJ (2014) Bitter-sweet symphony: glycan-lectin interactions in virus biology. FEMS Microbiol Rev 38: 598-632.
61. Pillai S, Netravali IA, Cariappa A, Mattoo H (2012) Siglecs and immune regulation. Annu Rev Immunol 30: 357-392.
62. Bull C, Stoel MA, den Brok MH, Adema GJ (2014) Sialic acids sweeten a tumor's life. Cancer Res 74: 3199-3204.
63. van de Bovenkamp FS, Hafkenscheid L, Rispens T, Rombouts Y (2016) The Emerging Importance of IgG Fab Glycosylation in Immunity. J Immunol 196: 1435-1441.
64. Horst D, Ressing ME, Wiertz EJ (2011) Exploiting human herpesvirus immune evasion for therapeutic gain: potential and pitfalls. ImmunolCell Biol 89: 359-366.
65. Horst D, Verweij MC, Davison AJ, Ressing ME, Wiertz EJ (2011) Viral evasion of T cell immunity: ancient mechanisms offering new applications. CurrOpinImmunol 23: 96-103.

Addendum

Nederlandse samenvatting

Virussen hebben een gastheer nodig om zich te kunnen vermenigvuldigen (repliceren). Om de gastheer tegen virale en andere microbiële infecties te beschermen heeft het een immuunsysteem, waarmee het lichaamsvreemde moleculen kan herkennen en daarop kan reageren. Er wordt tussen het aangeboren en het verworven afweersysteem onderscheden. Een deel van de lichaamsvreemde moleculen, vooral virale nucleïnezuren, kan snel door gespecialiseerde sensoren van het aangeboren immuunsysteem herkend worden, bij voorbeeld door Toll-like receptoren. Een virus-specifieke immuunrespons wordt door het verworven immuunsysteem aangemaakt. De immuunrespons kan geïnitieerd worden als geïnfecteerde cellen stukjes (peptiden) van lichaamsvreemde virale eiwitten in de antigeen-presenterende moleculen HLA klasse I (HLA I, ook MHC I) en HLA klasse II (HLA II, ook MHC II) op het celoppervlak presenteren. De vreemde peptiden in de antigeen-presenterende moleculen kunnen door specifieke $CD8^+$ en $CD4^+$ T-cellen worden herkend. Dit leidt tot activatie van de T cel, die de virus-geïnfecteerde cel direct kan doden of andere cellen kan activeren, die bij de immuunrespons betrokken zijn. Deze immuunrespons verwijderd de meeste virusinfecties en geeft langdurige immuniteit tegen dit virus.

Herpesvirussen zijn DNA-virussen, die de bijzondere eigenschap hebben dat zij een latente infectie in een gastheer kunnen bewerkstelligen, ook al worden ze door het immuunsysteem herkend en wordt er immuniteit ontwikkeld. Dit is mogelijk doordat herpesvirussen verschillende indrukwekkende manieren hebben ontwikkeld om aan herkenning door het immuunsysteem te ontsnappen. De levenscyclus van herpesvirussen bestaat uit twee verschillende fases, de latente en de lytische infectie. Tijdens de latente infectie van cellen worden er geen nieuwe virusdeeltjes geproduceerd. In een latent geïnfecteerde cel worden er geen of nauwelijks nieuwe virale eiwitten gemaakt en ook het genoom wordt slechts op een laag niveau geamplificeerd, enkel om ervoor te zorgen dat de delende cellen het virale genoom behouden. Verder hebben sommige latente virale eiwitten immuun-modificerende eigenschappen. Zo zorgen deze mechanismen ervoor dat de latente infectie niet goed "zichtbaar" is voor het immuunsysteem. In tegenstelling tot de latente fase, worden tijdens de lytische fase veel virale eiwitten geproduceerd, het genoom wordt vermenigvuldigd en nieuwe virusdeeltjes worden gemaakt. Om te voorkomen dat de virus-producerende cellen snel door het immuunsysteem herkend en opgeruimd worden, hebben herpesvirussen verschillende manieren gevonden om herkenning van de geïnfecteerde cellen te beïnvloeden (immuun-evasie).

In dit proefschrift staan de resultaten beschreven van ons onderzoek naar hoe het herpesvirus Epstein-Barr virus (EBV) de antigeen-presentatie op het celoppervlak van EBV-geïnfecteerde cellen kan verhinderen. In **hoofdstuk 1** is de achtergrond van dit onderzoek gedetailleerd beschreven. EBV hordt bij de herpesvirussen, specifieker de lymfocryptovirussen. Voorbeelden van andere herpesvirussen, die mensen als gastheer hebben, zijn herpes simplex

virus, de veroorzaker van koortslip, of het waterpokkenvirus. EBV kan de ziekte van Pfeiffer veroorzaken als de gastheer voor het eerst tijdens adolescentie in aanraking met EBV komt. EBV infectie is ook geassocieerd met verschillende vormen van kanker, zoals het Burkitt's lymfoom en Hodgkin's lymfoom. In de gastheer infecteert EBV B-cellen en epitheelcellen. In B-cellen kan EBV een latente infectie veroorzaken, maar ook lytisch repliceren. Tijdens de lytische fase zorgen verschillende EBV eiwitten ervoor dat er minder antigeen-presenterende moleculen op het celoppervlak terechtkomen, zodat minder EBV-afkomstige peptiden gepresenteerd kunnen worden. Drie verschillende EBV eiwitten met deze eigenschap zijn in de hoofdstukken 2, 3, en 4 behandeld.

In **hoofdstuk 2** wordt beschreven hoe de aanmaak van het EBV 'shutoff' eiwit BGLF5 middels een shRNA approach verminderd wordt in B-cellen, waarin EBV lytisch in repliceert, en welke invloed dit heeft op diverse celoppervlakte-eiwitten. Op deze manier zijn twee tot dusver onbekende doelwitten van BGLF5 geïdentificeerd, namelijk de transcripten die coderen voor CD1d en Toll-like receptor 2. Doordat BGLF5 deze transcripten afbreekt wordt de expressie van deze eiwitten op het celoppervlak verlaagd. De twee eiwitten zijn onderdeel van het aangeboren immuun system. TLR2 draagt bij aan de herkenning van pathogenen en CD1d is een antigeen-presenterend molecuul, dat lipiden in plaats van peptiden aan T-cellen presenteert.

In **hoofdstuk 3** wordt immuun-evasie van HLA I, II, en CD1d door EBV tijdens de late lytische fase beschreven. Een EBV-eiwit, gp150, draagt eraan bij dat de detectie van alle drie de antigeen-presenterende moleculen op het celoppervlak van EBV-negatieve cellen verlaagd wordt. De functie van het EBV-eiwit gp150 was niet eerder bekend. Verder hebben we het moleculaire werkingsmechanisme van gp150 opgehelderd. EBV gp150 draagt niet bij aan een versnelde afbraak van de bestudeerde eiwitten, maar de moleculen blijven op het celoppervlak aanwezig. Het zwaar geglycosyleerde eiwit gp150 dekt middels zijn suikers (glycanen) moleculen op het celoppervlak af, wat de herkenning door $CD8^+$ en $CD4^+$ T-cellen vermindert. Voor herpesvirussen is dit een unieke manier van evasie, en er is een soortgelijke mechanisme slechts voor een ander viraal eiwit beschreven, namelijk het Ebola glycoeiwit. In afwezigheid van gp150 in lytisch replicerende EBV-geïnfecteerde B-cellen komt een groter aantal HLA en CD1d moleculen op het celoppervlak terecht dan wanneer gp150 ook tot expressie komt. Dit geeft aan dat tijdens de productieve EBV-infectie van B-cellen gp150 ook een bijdrage levert aan de verminderde herkenning van antigeen-presenterende moleculen op het celoppervlak en dus ontsnapping aan T-celherkenning.

In **hoofdstuk 4** zijn de moleculaire details beschreven van BILF1-afhankelijke verlaging van HLA I moleculen op het celoppervlak. Voor interferentie met HLA I zijn de cytoplasmatische staart van zowel BILF1 als het HLA I molecuul noodzakelijk. Verder wordt aangetoond dat niet alle HLA allelen gevoelig zijn voor BILF1. De celoppervlakte-niveaus

van HLA-C moleculen worden namelijk niet of nauwelijks door BILF1 verlaagd. We hebben de verantwoordelijke aminozuren in de cytoplasmatische staart van HLA-C moleculen geïdentificeerd en introductie van deze aminozuren in een BILF1-gevoelig HLA molecuul maakt het resistent. BILF1 wordt door meerdere relateerde lymfocryptovirussen (LCV), waaronder EBV, rhesus LCV, en marmoset LCV gecodeerd. Of de BILF1 eiwitten van deze drie LCVs allemaal de immuunevasie-eigenschap hebben was onbekend. BILF1 van EBV en van rhesus LCV kunnen de celoppervlakte-expressie van humaan MHC I verlagen, maar marmoset BILF1 kan dit niet. Ook kan marmoset BILF1 niet de oppervlakte expressie van marmoset MHC I beïnvloeden. Dit zou kunnen betekenen dat BILF1 evolutionair gezien pas recent zijn evasie-eigenschap heeft ontwikkeld.

Over de herkenning van EBV door het aangeboren immuunsysteem is minder bekend. B-cellen hebben verschillende sensoren zoals Toll-like receptoren, maar over de aanwezigheid van cytosolische en nucleaire DNA-sensoren in B-cellen was vrijwel weinig bekend. Het DNA-genoom van EBV zou door dit soort sensoren herkend kunnen worden. In **hoofdstuk 5** is beschreven dat hoewel B-cellen wel cytoplasmatische DNA-sensoren tot expressie brengen, het essentiële adaptereiwit STING afwezig was in de meerderheid van de bestudeerde primaire B-cellen en cellijnen. B-cellen, die wel de eiwitten van de volledige signaleringscascade leken te hebben, reageerden ook niet op geïntroduceerd DNA , waaruit we concluderen dat de cascade in B-cellen niet functioneel is. Het is interessant om te speculeren of dit B-cellen tot attractieve gastheercellen voor EBV en andere DNA-virussen maakt.

Het laatste hoofdstuk, **hoofdstuk 6**, geeft een samenvatting van de bevindingen uit alle eerdere hoofdstukken van dit proefschrift alsmede een discussie in de context van de gerelateerde vak literatuur. Verder worden de ontwikkelingen en mogelijkheden van het EBV- en herpesvirusonderzoek en implicaties daarvan voor de kliniek besproken. Uit de eerste drie hoofdstukken blijkt dat evasie van HLA II, CD1d, en vooral HLA I een belangrijke rol in de levenscyclus van EBV moet spelen, omdat EBV hier zoveel verschillende manieren voor ontwikkeld heeft, die in verschillende fasen van de lytische replicatie actief zijn. De hier besproken eiwitten BGLF5, gp150, en BILF1 kunnen samen met andere EBV-eiwitten, zoals BNLF2a en gp42, de herkenning door $CD8^+$ en $CD4^+$ T-cellen verstoren. Het is belangrijk om de werkwijze van deze evasieeiwitten te begrijpen, want dit zou de grondlaag voor de ontwikkeling van anti-virale therapien, bij voorbeeld van de ziekte van Pfeiffer, kunnen zijn. Verder is het interessant om er over na te denken, of (gemodificeerde) virale evasieeitwitten, die de antigen-presentatie verminderen, kunnen gebruikt worden om getransplanteerde of eigen organen (bi voorbeeld de pancreas, het insuline producerend orgaan) tegen ongewilde aanval door T cellen te voorkomen zo als in een orgaanafstoting-reactie of type I diabetes.

Deutsche Zusammenfassung

Viren sind kleine infektiöse Partikel, die aus einem viralen Genom, das die Erbinformation beinhaltet, und Eiweißen (Proteinen) bestehen. Im Gegensatz zu Bakterien können Viren nicht eigenständig replizieren (sich vermehren). Viren benötigen eine Wirtszelle, die sie infizieren, „kapern" und dazu benutzen können, um zu replizieren. Das Immunsystem des Menschen sorgt dafür, dass virale und andere Infektionen erkannt und beseitigt werden. Man unterscheidet zwischen dem angeborenen und dem erworbenen Immunsystem. Das angeborene Immunsystem erkennt körperfremde Moleküle (z.B. das virale Genom) durch zelluläre Sensoren in und auf Körper- und Immunzellen. Ein Beispiel für solche Sensoren sind die Toll-like-Rezeptoren. Die Erkennung von körperfremden Molekülen führt zur Aktivierung der Sensoren, die andere zelluläre Eiweiße hinzuziehen und damit eine Signalkaskade auslösen, um so eine schnelle, aber unspezifische Immunantwort auszulösen. Diese angeborene Immunantwort stimuliert die Entwicklung der Erreger-spezifischen (erworbenen) Immunantwort. Die erworbene Immunantwort besteht einerseits aus der Antikörper-basierten humoralen Immunität, und andererseits aus der zellulären Immunität. Hauptbestandteil der zellulären Immunität sind bestimmte Blutzellen, die sogenannten T-Zellen, die kleine Stückchen (Peptide) von körperfremden Eiweißen (Antigenen) erkennen können. Die Peptide werden auf der Zelloberfläche durch antigen-präsentierende Moleküle, die sogenannten MHC-Moleküle (die im Menschen auch HLA-Moleküle genannt werden) präsentiert. Es gibt HLA-Moleküle der Klasse I und II. T-Zellen erkennen diese HLA-Moleküle zusammen mit den präsentierten Peptiden und werden dadurch aktiviert. Aktivierte T-Zellen können entweder (Virus-infizierte) Zellen, die die fremden Peptide auf der Zelloberfläche präsentieren, direkt töten oder anderen Zellen, die an einer Immunantwort beteiligt sind, helfen, aktiv zu werden. Die erworbene Immunantwort sorgt normalerweise dafür, dass Infektionsherde entfernt werden und eine langanhaltende Immunität gegen den spezifischen Erreger etabliert wird.

Die Familie der Herpesviren umfasst behüllte Viren mit einem DNA-Genom. Es gibt mehrere humane Herpesviren, die Erkrankungen wie Lippenherpes (Herpes simplex Virus 1), Genitalherpes (Herpes simplex 2), Windpocken (und bei Reaktivierung Gürtelrose) (Varicella-Zoster Virus) oder Pfeiffersches Drüsenfieber (Epstein-Barr-Virus) verursachen können. Herpesviren haben die Besonderheit, dass sie eine latente Infektion verursachen, d.h. das Virengenom verbleibt nach einer Infektion im Körper, obwohl das Immunsystem die Viren erkannt und Immunität entwickelt hat. Herpesviren haben verschiedene beeindruckende Strategien entwickelt, um die Immunantwort zu umgehen oder ins Leere laufen zu lassen. Der Lebenszyklus von Herpesviren besteht aus zwei Phasen, der latenten und der lytischen Infektion. Während der latenten Phase werden keine neuen Viruspartikel produziert. Auch werden keine oder kaum virale Proteine generiert, und das virale Genom wird nur auf einem niedrigen Niveau vervielfältigt, was nur dazu dient, dass das virale Genom auch in den Tochterzellen der sich teilenden und infizierten Zelle vorhanden bleibt.

Durch die wenigen viralen Proteine (und somit präsentierten Peptide) und die niedrige Genomvervielfältigungsrate sind die latent infizierten Zellen für das Immunsystem fast gänzlich „unsichtbar". Zusätzlich haben einige der viralen Proteine, die in der latenten Phase produziert werden, immunmodifizierende Eigenschaften, die dafür sorgen, dass die Immunerkennung und –aktivierung reduziert wird. Im Gegensatz zur latenten Phase werden in der lytischen Phase neue virale Eiweiße produziert, das virale Genom stark vervielfältigt und neue Viruspartikel generiert. Um zu verhindern, dass infizierte Zellen, die sich in der lytischen Phase befinden, vom Immunsystem - aus viraler Sicht - zu schnell entdeckt und zerstört werden, haben Herpesviren eine Reihe von Strategien entwickelt, um die Erkennung der infizierten Zelle zu verspäten und somit mehr Zeit zur Produktion von noch mehr Viruspartikeln zu haben.

In dieser Arbeit werden die Ergebnisse unserer Forschung zu der Frage, wie das Epstein-Barr-Virus (EBV) die Antigenpräsentation auf der Zelloberfläche von EBV-infizierten Zellen verhindern kann, vorgestellt. In **Kapitel 1** sind die Hintergründe unserer Forschungsarbeit detailliert beschrieben. EBV gehört zur Familie der Herpesviren und zur Unterfamilie der Lymphocryptoviren. Mehr als 90% der erwachsenen Weltbevölkerung sind mit EBV infiziert. EBV kann das Pfeiffersche Drüsenfieber verursachen, welches bei erstmaliger EBV-Infektion vor allem, aber nicht ausschließlich, im Jugend- oder Erwachsenenalter auftritt. Die Infektion mit EBV spielt auch bei verschiedenen Formen von Krebs, zum Beispiel dem Burkittslymphom oder dem Hodgekinslymphom, eine Rolle. EBV infiziert B-Zellen (die Antikörper-produzierenden Zellen des Immunsystems) und Epithelzellen. EBV kann B-Zellen latent infizieren, aber auch in ihnen lytisch replizieren. Während der lytischen Phase sorgen verschiedene EBV-Proteine dafür, dass weniger Antigen-präsentierende Moleküle auf die Zelloberfläche kommen und somit weniger Peptide von EBV-Eiweißen präsentiert werden. Drei verschiedene EBV-Eiweiße mit dieser Eigenschaft sind Gegenstand der Kapitel 2, 3 und 4.

Das EBV-Protein BGLF5 ist Gegenstand von **Kapitel 2**. BGLF5 sorgt während der lytischen Phase von EBV dafür, dass die Produktion von Wirtsproteinen, unter anderem HLA-Moleküle, zu Gunsten von viralen Eiweißen massiv abnimmt. In Kapitel 2 ist beschrieben wie die Menge an BGLF5 in lytisch infizierten B-Zellen unter Zuhilfenahme von shRNAs reduziert werden kann. In den Zellen mit verringerter Anzahl BGLF5-Molekülen wurde die Menge von verschiedenen Zelloberflächenmolekülen bestimmt und mit Zellen, die die normale BGLF5 Menge haben, verglichen. Auf diese Art und Weise konnten wir das Eiweiß CD1d als Ziele identifizieren, dessen kodierende mRNA von BGLF5 abgebaut wird. Dies resultiert in einer geringeren Produktion von CD1d und einer reduzierten Menge dieser Eiweiße auf der Zelloberfläche. In Zellen, in denen das BGLF5-Gen zur Expression gebracht wurde, konnte auch TLR2 als Ziel festgestellt werden. Die zwei Proteine CD1d und TLR2 sind Teil des angeborenen Immunsystems. TLR2 trägt zur Erkennung von Erregern bei. CD1d ist

ein nicht-klassisches Antigen-präsentierendes Molekül, das Lipide statt Peptide an T-Zellen präsentiert. Die beschriebene Methode erlaubt somit, neue Zieleiweiße zu identifizieren.

In **Kapitel 3** wird dargestellt, wie EBV die Antigenpräsentation durch HLA I, II und CD1d während der späten lytischen Phase verringert. Das EBV-Eiweiß gp150, das die Zelloberfläche erreicht, trägt dazu bei, dass die Erkennung aller drei Antigen-präsentierender Moleküle durch T- Zellen abnimmt. Die Funktion von EBV gp150 war zuvor unbekannt. Des Weiteren haben wir den molekularen Wirkmechanismus studiert. EBV gp150 sorgt nicht dafür, dass die Zieleiweiße schneller als gewöhnlich abgebaut werden, sondern kann die Eiweiße auf der Zelloberfläche maskieren, sodass sie nicht mehr für T-Zellen erkennbar sind. Dies wird dem relativ kleinen EBV-Protein gp150 durch seine ungewöhnlich vielen Zuckermodifikationen ermöglicht. Diese Vermeidungsstrategie ist bisher einzigartig für Herpesviren, und ein vergleichbarer Mechanismus ist nur für ein anderes virales zucker-modifiziertes Eiweiß, das des Ebolaviruses, bekannt. In der Abwesenheit von gp150 in der lytischen Infektionsphase sind mehr Zelloberflächen-HLA und –CD1d-Moleküle in B-Zellen detektierbar als in Abwesenheit von gp150. Das heißt, es ist denkbar, dass auch in EBV-infizierten und lytisch-replizierenden B-Zellen das Eiweiß gp150 zur Abnahme von T -Zellerkennung beiträgt.

In **Kapitel 4** werden die Ergebnisse unserer weiterführenden Studie über EBV BILF1 dargelegt. BILF1 ist ein EBV-Protein, das während der frühen lytischen Phase produziert wird und dafür sorgt, dass weniger HLA I-Moleküle auf der Zelloberfläche anwesend sind. Wir beschreiben die Erkenntnisse über die molekularen Details der BILF1-abhängige Abnahme von HLA I-Eiweißen auf der Zelloberfläche. Dazu wurden verschiedene BILF1 und HLA I-Mutanten von uns generiert und studiert. BILF1 benötigt seinen cytoplasmatischen Teil um die Menge an Zelloberflächen-HLA I zu verringern. Des Weiteren kann BILF1 das Erreichen der Zelloberfläche des HLA I-Eiweißes nur verhindern, wenn HLA I seinen cytoplasmatischen Teil besitzt. Überdies können wir zeigen, dass nicht alle HLA I-Proteine, die durch die verschiedenen HLA-Allele kodiert werden, durch BILF1 beeinflusst werden. Die Menge der HLA-C-Zelloberflächenmoleküle wird nämlich kaum oder gar nicht verringert. Die dafür verantwortlichen Aminosäuren konnten im cytoplasmatischen Teil von HLA-C ausfindig gemacht werden. Wenn diese Aminosäuren an den entsprechenden Positionen in einem BILF1-sensitiven HLA I-Protein eingefügt werden, wird das HLA I-Eiweiß resistent gegen BILF1. Mehrere verwandte Lymphocryptoviren (LCV), unter anderem EBV, Rhesus-LCV und Marmosetten-LCV kodieren BILF1. Es war unbekannt, ob BILF1 von diesen drei LCVs über die Eigenschaft verfügt MHC-Moleküle am Erreichen der Zelloberfläche zu hindern. BILF1 von EBV und Rhesus-LCV können humanes MHC I auf der Zelloberfläche verringern, während Marmosetten-LCV BILF1 dies nicht kann. Auch Marmosetten-MHC I-Eiweiße kann Marmosetten-LCV BILF1 nicht vermindern, d.h. es verfügt nicht über die Eigenschaft, die Menge an Zelloberflächen-MHC I zu verringern. Dies könnte bedeuten, dass evolutionär gesehen BILF1 über die Eigenschaft der Verminderungsstrategie erst relativ kurz

verfügt.

Im Gegensatz zum erworbenen Immunsystem ist über die Erkennung von EBV durch das angeborene Immunsystem weniger bekannt. B-Zellen verfügen über verschiedene Sensoren wie Toll-like-Rezeptoren, aber ob B-Zellen cytoplasmatische und Zellkern-ständige DNA-Sensoren haben, war weitestgehend unbekannt. Das DNA-Genom von EBV könnte von dieser Sorte Sensoren erkannt werden. In **Kapitel 5** ist beschrieben, dass B-Zellen cytoplasmatische DNA-Sensoren haben, aber das Adaptereiweiß STING in den meisten primären B-Zellen und B-Zelllinien abwesend ist. Das Protein STING ist für den cytoplasmatischen DNA-Erkennungssignalweg essentiell und ohne dieses Eiweiß kann das Erkennungssignal nicht weitergegen werden. Somit verfügen die meisten der getesteten B-Zellen nicht über einen vollständigen Signalkaskadenweg für die Erkennung von DNA. Interessanterweise reagieren B-Zellen, die anscheinend alle Eiweiße der DNA-Erkennungs- und Signalkaskade haben, nicht auf cytoplasmatische DNA. Daraus ziehen wir die Schlussfolgerung, dass die Signalkaskade in B-Zellen nicht funktionstüchtig ist. Es ist interessant zu spekulieren, ob die Abwesenheit von dem DNA-Erkennungsweg B-Zellen zu attraktiven Wirtszellen für EBV und andere DNA-Viren macht.

Das letzte Kapitel, **Kapitel 6**, fasst die Ergebnisse aus den anderen Kapiteln dieser Arbeit zusammen und beinhaltet eine Diskussion dieser im Kontext relevanten Fachliteratur. Des Weiteren werden die möglichen zukünftigen Entwicklungen in der EBV- und Herpesvirenforschung und deren klinische Anwendbarkeit diskutiert. Die in den ersten vier Kapiteln dargestellten Ergebnisse zeigen, dass die Umgehung der Antigenpräsentation der HLA II-, CD1d- und vor allem HLA I-Eiweiße eine wichtige Rolle spielen muss, weil EBV so viele verschiedene Strategien dafür entwickelt hat, die in verschiedenen Phasen der lytischen Replikationsphase aktiv sind. Die hier besprochenen EBV-Proteine BGLF5, BILF1 und gp150 können gemeinsam mit anderen EBV-Eiweißen, wie zum Beispiel BNLF2a und gp42, die Erkennung durch T-Zellen stören. Es ist wichtig, die molekularen Arbeitsweisen dieser Eiweiße zu verstehen, weil dies die Grundlage für die Entwicklung von anti-viralen Therapien, zum Beispiel gegen das Pfeiffersche Drüsenfieber, bilden kann. Außerdem ist es interessant darüber nachzudenken, inwiefern (modifizierte) virale Eiweiße, die die Antigenpräsentation verringern, therapeutisch genutzt werden können, um zum Beispiel transplantierte oder eigene Organe vor ungewollten T-Zell-Attacken zu bewahren, wie dies der Fall in Organabstoßungsreaktionen nach Transplantationen oder Diabetes Typ I der Fall ist.

List of publications

Gram AM, Oosenbrug T, Marthe F.S. Lindenbergh, Christian Büll, Anouskha Comvalius, Kathryn J.I. Dickson, Joop Wiegant, Hans Vrolijk, Robert Jan Lebbink, Gosse J. Adema, Marieke Griffioen, Mirjam H.M. Heemskerk, David C. Tscharke, Lindsey M. Hutt-Fletcher, Emmanuel J.H.J. Wiertz, Rob C. Hoeben, Maaike E. Ressing; *The Epstein-Barr Virus Glycoprotein gp150 Forms an Immune-evasive Glycan Shield at the Surface of Infected Cells;* PLoS Pathog. 2016 Apr 14;12(4):e1005550.

Ressing ME, van Gent M, **Gram AM**, Hooykaas MJ, Piersma SJ, Wiertz EJ; *Immune Evasion by Epstein-Barr Virus*. Curr Top Microbiol Immunol. 2015;391:355-81.

van Gent M, **Gram AM**, Boer IG, Geerdink RJ, Lindenbergh MF, Lebbink RJ, Wiertz EJ, Ressing ME. *Silencing the shutoff protein of Epstein-Barr virus in productively infected B cells points to (innate) targets for immune evasion;* J Gen Virol. 2015 Apr;96(Pt 4):858-65.

Griffin BD*, **Gram AM***, Mulder A, Van Leeuwen D, Claas FH, Wang F, Ressing ME, Wiertz E; *EBV BILF1 evolved to downregulate cell surface display of a wide range of HLA class I molecules through their cytoplasmic tail;* J Immunol. 2013 Feb 15;190(4):1672-84. * Equal first authorships

Horst D, Geerdink RJ, **Gram AM**, Stoppelenburg AJ, Ressing ME; *Hiding lipid presentation: viral interference with CD1d-restricted invariant natural killer T (iNKT) cell activation;* Viruses. 2012 Oct 23;4(10):2379-99.

Gram AM, Frenkel J, Ressing ME.; *Inflammasomes and viruses: cellular defence versus viral offence*; J Gen Virol. 2012 Oct;93(Pt 10):2063-75.

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Curriculum Vitae

Anna Gram was born on May 30, 1987 in Basel, Switzerland. She grew up in Germany and finished her school education in 2006. The same year, she started her Biology study at the Ruprecht-Karls-Universität in Heidelberg, Germany and obtained her Bachelor diploma in 2009. Thereafter, she moved to Utrecht, The Netherlands to study 'Biomedical Sciences' with a focus on 'Infection & Immunity'. Anna spent her research internships at the UMC Utrecht in the groups of Prof. Leo Koenderman (supervised by Dr. Laurien Ulfman) and Prof. Emmanuel Wiertz/Dr. Maaike Ressing (supervised by Dr. Sytse Piersma) working on cell adhesion mechanisms (9 month) and evasion of the complement system by Epstein-Barr virus (6 month), respectively. She wrote her Master thesis about inflammasomes under supervision of Dr. Joost Frenkel.

After obtaining her Master diploma in 2011, Anna started her PhD training at the UMC Utrecht in the group of Prof. Emmanuel Wiertz under supervision of Dr. Maaike Ressing. In 2013, she moved with Dr. Maaike Ressing to the LUMC in Leiden to join the group of Prof. Rob Hoeben, where she continued her PhD studies. In 2015, Anna spent three month as visiting scientist in the group of Prof. Søren Paludan at Aarhus University, Denmark. The stay was supported by two fellowships. The results of her PhD studies are described in this thesis.

Currently, Anna is working as a postdoc on a multi-disciplinary joint project between the department of Molecular Cell Biology and Hematology (both LUMC) in close collaboration with Genmab.