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

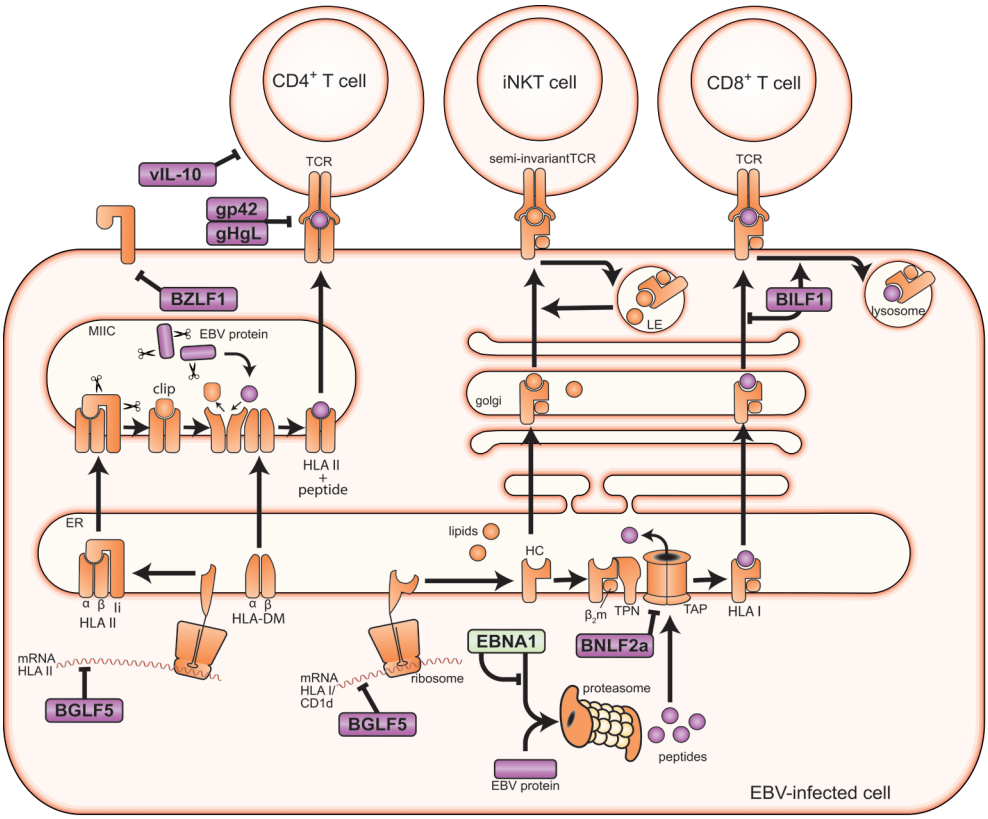

Figure 1 Antigen presentation pathways targeted by EBV. Multiple EBV-derived peptides are presented in the context of HLA I and II molecules to be scrutinized by specific T cells. Endogenous lipids are presented by CD1d to iNKT cells. Presentation to CD4⁺ T cells occurs by HLA II⁺ antigen-presenting 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.

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