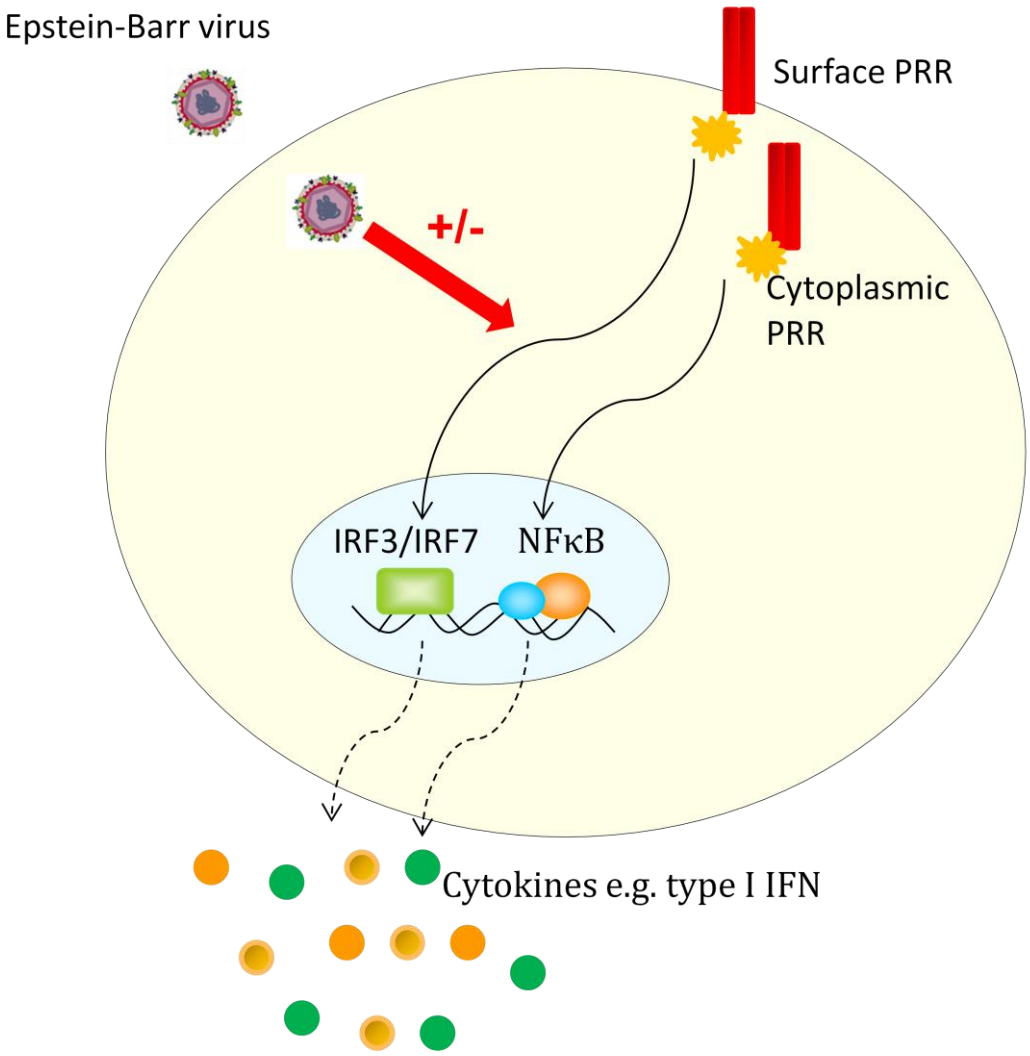
Clever tricks EBV employed to modulate innate immunity during latency and lytic infection



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About the cover: Upon recognition of PAMPs, activated PRRs initiate innate immune signaling routes via IRF3/IRF7 and NFκB to elicit antiviral immune responses[1, 2]. Epstein-Barr virus developed multiple strategies to interfere with this signaling, by both enhancing and inhibiting these cascades.

*Picture of virion is acquired from www.712designs.com/illustrations/kshv.

Title: Clever tricks EBV employed to modulate innate immunity during latency and lytic infection

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Outline

Abstract

Epstein-Barr virus, a human γ-herpesvirus, persists in humans for life without being cleared by their immune system. Both innate and adaptive immunity contribute to the control of viral infections. However, antiviral immunity initiates with innate immune responses, for which recognition of conserved viral patterns by pattern recognition receptors (PRRs) is essential. Upon triggering, PRRs induce signaling routes through activation of transcription factors IRF3/IRF7 and NFκB. Since EBV is able to establish and maintain latency, and to complete virion synthesis without being eliminated, EBV is likely to modulate PPR-induced innate immune signaling. Multiple EBV-encoded and cellular gene products are involved in modulating innate immunity during both latent and lytic phase of infection by targeting PRR-induced signaling. Remarkably, EBV gene products have dual roles during infection, as innate immune signaling pathways are both induced and inhibited. This review first discusses briefly the herpesviridae and EBV, then innate immunity in host cells, focusing on Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and cytoplasmic DNA sensors in particular. Furthermore, this review provides a detailed overview of EBV recognition by these PRRs, and EBV-encoded gene products involved in modulating PRR-induced immune signaling during both latent and lytic infection. In addition, I propose a model for EBV modulation in the course of innate immunity depending on the time of infection. Knowledge of EBV evasion strategies should broaden our understanding of EBV infection and may reveal potential candidates for future prevention of EBV-associated diseases, including malignancies.

1. Introduction

Herpesviridae and EBV

The herpesviridae family comprises a large group of viruses, which share some typical features[3]. Herpesviruses are characterized by their long linear double-stranded DNA genome, which is enclosed by an icosahedral capsid. The icosahedral capsid is surrounded by a tegument layer and an envelope[3]. The envelope contains various glycoproteins, which determine cell tropism and are essential for cell entry. Another, more important, feature shared by all herpesviruses is their ability to establish persistent latent infections during which the viral genome is present, but no viral genes are expressed, without being eliminated by the host immune system[3]. Aside from the shared key features, the herpesviridae family is classified into three subgroups, α-herpesviruses, β-herpesviruses, and γ-herpesviruses, based on genome similarities and host cell tropism[2, 3]. Examples of classical α-herpesvirus infections are chickenpox caused by Varicella zoster virus (VZV, HHV3) and typical Herpes simplex virus (HSV-1, HHV1 and HSV-2, HHV2) orofacial and genital infections. Infections caused by β-herpesviruses include congenital defects caused by human Cytomegalovirus (HCMV, HHV5) and rashes typical for HHV6 (variant A and B) and HHV7 infection. The γ-herpesviruses Epstein-Barr virus (EBV, HHV4) and Kaposi's sarcoma-associated herpesvirus (KSHV, HHV8) are associated with various lymphomas and are the only human herpesviruses able to transform B cells[2, 3]. EBV was the first human tumor virus discovered, which was already described in 1958[3]. This review focuses on γ-herpesvirus Epstein-Barr virus (EBV).

Worldwide, more than 90% of the adult human population is infected with EBV. In the majority of carriers, EBV infection acquired during childhood is asymptomatic [3]. However, primary EBV infection during adolescence causes infectious mononucleosis (IM) in 25% of the cases[3]. Furthermore, EBV infection is associated with a variety of malignancies, such as Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL), nasopharyngeal carcinoma (NPC), and post-transplant lymphoproliferative disorder (PTLD)[3]. Each of these malignancies is linked to one of the four EBV latency programmes (Figure 1)[3-6]. PTLD is the prime example of disease caused by EBV and appears in solid organ transplant recipients due to immune suppression. An adoptive transfer of autologous EBV-specific cytotoxic T cells in transplant recipients eliminates EBV-infected cells and prevents development of PTLD[78]. In addition, in transplant recipients who already had developed PTLD, an adoptive transfer of EBV-specific CTLs dramatically reduced PTLD severity{{117 Khanna,R. 1999}}. Thus, it is clear that EBV causes PTLD, because upon elimination of the virus, the host does not develop PTLD.

EBV infection and replication

EBV infects humans through the oral route and spreads via the saliva[3, 6]. The prime target cells of EBV are naïve B cells and epithelial cells, but EBV can also infect or can be taken up by other cell types, including monocytes and plasmacytoid dendritic cells (pDCs)[9]. EBV tethers to B cells by binding of the viral glycoprotein gp350 to complement receptor CR2 (CD21), a receptor expressed by B cells[9]. This interaction is followed by binding of gp42 to surface HLA class II molecules, thereby triggering fusion for which gH-gL and gB are required. For infection of epithelial cells, gp42 is not required and it has been shown that soluble gp42 inhibits fusion of EBV lacking gp42 with epithelial cells[9]. The requirement of gp42 to infect B cells and the absence of gp42 to infect epithelial cells suggests a role for gp42 in determining EBV cell tropism[9]. After infection of a host cell by EBV, there are two options: EBV induces the lytic infection characterized by *de novo* production of virus particles in epithelial cells and occasionally in B cells, or EBV establishes latency in B cells, which permits the virus to persist for the life-time of the host[9].

Latent phase. Latency is characterized by the presence of the EBV genome, but the lack of *de novo* production of viral particles[3]. To establish latent infection, EBV induces the sequential expression of latency proteins in four phases (Figure 1), which involve Epstein-Barr nuclear antigens (EBNA1, EBNA2A/B, EBNA3A/B/C) and latent membrane proteins (LMP1 and LMP2) [3, 6, 10]. EBV-encoded small RNAs (EBERs) are present in all latency programmes. To induce expression of these genes, the EBV genome enters the host cell nucleus where it remains as extrachromosal DNA (episome) and causes expression of latency gene EBNA2. EBNA2 is involved in establishment of the growth transcription program (latency type III) by inducing expression of the latency genes [3, 6, 10]. The genes expressed during the growth transcription program induce B-cell activation and proliferation, and stimulate the infected naive B cell to enter the germinal centre (GC) of a follicle in the lymph nodes. Inside the follicle, the EBV growth transcription program switches to the default

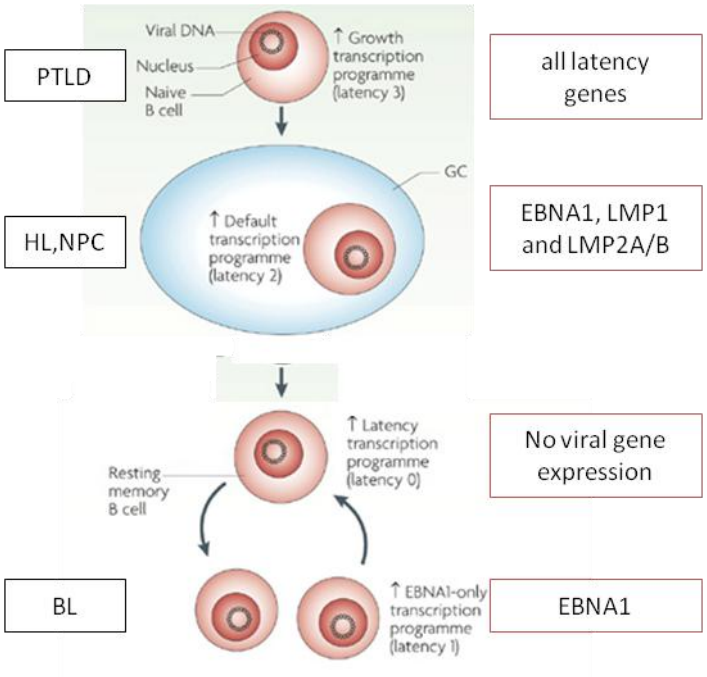

Figure 1 adapted from Thorley-Lawson *et al.* and adjusted: Establishment of latency by EBV using 4 latency programmes. Each programma is characterized by expression of a specific subset of latency genes. EBV-associated tumors are linked to one of these programmes.

program (latency II) which induces differentiation of the naïve B cell into a memory B cell[3, 6]. During latency type 0 all viral protein expression is shut off, allowing the virus to persist in resting memory B cells for the lifetime of the host. Only during division latency protein EBNA1 is expressed, required for the maintenance of the viral genome (Latency I; EBNA1-only program)[3, 6]. Interestingly, EBNA1 contains Gly-Ala repeats which prevent EBNA1 breakdown by the cellular proteasome and thus prevent recognition of EBNA1 by cytotoxic T cells [11]. The EBV proteins and RNAs expressed during the different latency phases and their functions are listed in table 1.

Lytic protein/RNA	Function
EBNA1	Maintenance of EBV episome
EBNA2	Induces expression of latent genes to create latency III state
EBNA3a, EBNA3b, EBNA3c	Inhibit EBNA2 expression and induce transition from latency III to latency II
LMP1	Acts as an active tumor necrosis factor (TNF) receptor by mimicking host CD40, and induces B-cell activation
LMP2a	Mimics B-cell receptor and its signaling, and thereby induces B-cell survival
LMP2b	Exact function remains to be elucidated, but suggested to play a role in the modulation of LMP2a activity which makes B-cells more susceptible for EBV reactivation
EBERs	Function in latency unknown, but are implications for EBERs in evading the immune system

Table 1: Overview of all latent proteins and RNAs expressed with their role during EBV latency[3, 6, 10, 12,13].

Lytic phase. Lytic infection is established in epithelial cells directly after initial infection or in latently infected B cells upon reactivation from latency[3, 6, 10]. During lytic infection, the complete viral genome, which are more than 80 viral genes, is expressed with the ultimate goal to produce new virions. The lytic phase is a tightly regulated process by the consecutive expression of immediate-early, early, and late lytic genes[3, 10]. Immediate early proteins BZLF1 and BRLF1 are essential to switch from latent to lytic infection as they induce expression of early genes (like viral polymerases), which subsequently induce expression of late lytic genes (structural proteins, including viral glycoproteins)[3, 10]. Late lytic genes code for structural and non-structural proteins, and factors required for viral DNA replication. Lytic genes are not expressed during lytic infection solely, as few of them appear at the start of infection[10]. Currently, research focuses on the recently discovered herpesvirus encoded micro RNAs (miRNAs), which are abundantly expressed by all herpesviruses[14, 15]. EBV expresses micro RNAs (BARTs) during both latent and lytic cycles, but their exact function and their role in EBV pathogenesis need to be revealed[15].

Innate immunity in virus-infected host cells

In order to replicate, a virus requires a host cell. Therefore, it seems likely that innate immunity of the infected host cell plays an important role in the control of viral infection, for which recognition of conserved viral patterns by pattern recognition receptors (PRRs) is essential[1].

Pattern Recognition receptors. Innate recognition of viruses by host cells is accomplished by pattern recognition receptors (PRRs)[1, 2]. PRRs are subdivided into several families, including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and a family of cytoplasmic DNA sensors[13]. PRRs detect pathogen associated molecular patterns (PAMPs), which are distinct and conserved pathogen structures[1, 2]. This review focuses on three PRR families, namely TLRs, RLRs, and cytoplasmic DNA sensors. In addition, although there are several signaling pathways induced by these PRRs, this review focuses only on the signaling routes resulting in activation of transcription factors IRF3/IRF7, IRF3 induces expression of IRF7, and NFκB, which contribute to the initiation of antiviral responses (Figure 2).

Sensing of PAMPs by these PRRs results in recruitment of adaptor proteins, which subsequently activate downstream signaling proteins[1, 2, 16]. Upon TLR triggering, adaptor protein MyD88 (all TLRs, except TLR3) or TRIF (only TLR3 and TLR4) is activated. Triggering of cytosolic RNA sensors induces activation of adaptor protein IFNB-promoter stimulator 1 (IPS1, also known as MAVS, CARDIF or VISA), which is bound to the outer mitochondrial membrane[1]. The adaptor protein required for signaling induced by DNA sensors is unknown. Upon PRR triggering, adaptor protein TRIF activates the protein complex (consisting of IKKε, TBK1, Tank and NEMO) required for activation of transcription factor IRF3 via phosphorylation, which allows IRF3 to translocate to the nucleus and induce expression of type I IFN[1, 2]. Adaptor protein MyD88 causes the consecutive activation or IRAK4, IRAK1 and TRAF6, which subsequently activates IKKα causing activation of IRF7 that

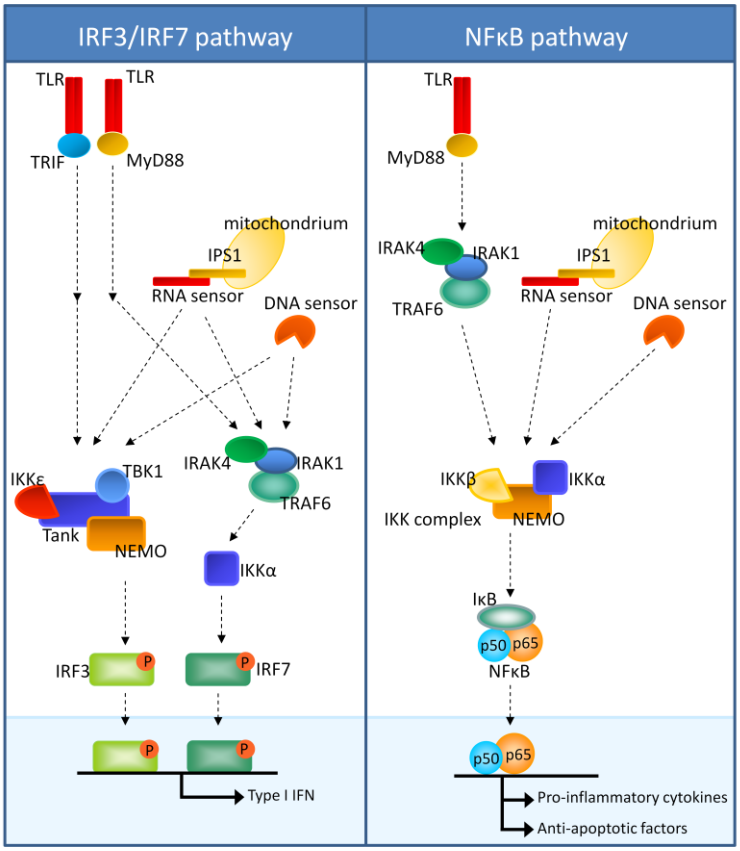

Figure 2: Signaling pathways induced upon recognition of viruses by PRRs signal through IRF3/IRF7 and NFκB.

translocates to the nucleus where it induces type I IFN expression. In the other signaling route resulting in activation of transcription factor NFκB, TRAF6 activates the IKK complex (consisting of IKKα, IKKβ, and NEMO), which phosphorylates inhibitor IκB and thereby induces its degradation via ubiquitination[1, 2, 16]. This allows for NFκB to translocate to the nucleus where it induces expression of pro-inflammatory cytokines and anti-apoptotic factors. In addition, adaptor protein TRIF causes delayed activation of NFκB[1, 2, 16]. These signaling routes and the proteins involved in these cascades are depicted in figure 2.

Effects of IRF3/IRF7 activation. An important arm of innate immunity is the production of type I IFN, including IFNα and IFNβ, which is expressed upon activation of IRF3 and IRF7. Production of type I IFN induces activation of several types of innate immune cells, including NK cells, macrophages and DCs, but also of the adaptive immune system resulting in antigen-specific responses[1, 17]. In addition, in host cells type I IFN induces expression of antiviral genes, which interfere with viral replication and increase recognition of viruses by enhancing PRR expression. The induction of an antiviral state is accomplished by binding of type I IFN to its receptor IFNAR1/2 on both infected and neighboring cells, which results in the activation of two associated kinases, JAK1 and Tyk2[1, 18]. The active kinases activate STAT1 and STAT2, which form a complex with IRF9. This complex translocates to the nucleus and induces expression of the IFN-stimulated genes (ISGs), see figure 3. These ISGs comprise genes that augment the IFN response and genes that interfere with viral replication, creating an antiviral state in both infected and neighboring cells[1]. For example, ISGs are factors involved in initiation of the host immune response, inhibition of viral transcription, and factors involved in pathogen recognition with the goal to eliminate the virus.

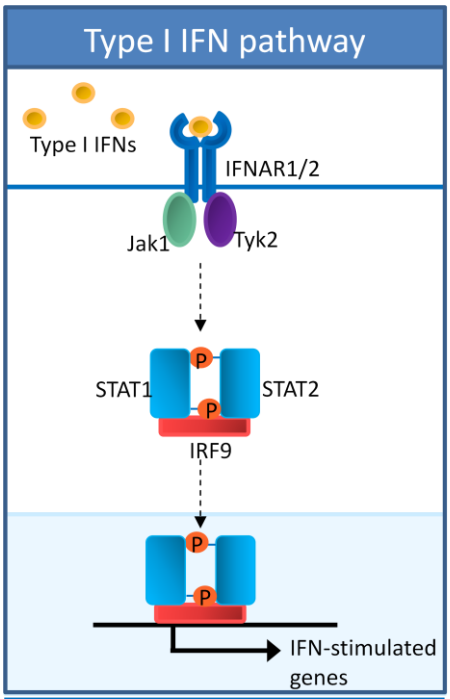

Figure 3: Type I IFN signaling

Effects of NFκB activation. Activated NFκB causes expression of a wide variety of genes involved in different processes[19]. Firstly, NFκB causes expression of pro-inflammatory cytokines and adhesion molecules, which result in the recruitment of immune cells to the site of infection. Secondly, expression of anti-apoptotic genes is enhanced by NFκB, including proteins IAP-1/2, Bcl-2 and BclX. Thirdly, NFκB is involved in B-cell differentiation and survival, but also in T-cell activation. This observation together with the fact that NFκB is activated upon PRR triggering, implies a role for NFκB in linking innate and adaptive immunity. Another effect of NFκB activation is the expression of

proteins with microbicidal activities and proteins involved in ROS production. These are only few of the many versatile effects induced by NFκB, underlining that NFκB signaling is a complex signaling pathway[19].

Toll-like receptors. The Toll-like receptor family consists of 10 members in humans and each member recognizes distinct conserved pathogen structures (Table 2)[1, 2, 13]. The majority of TLRs are located on the cell surface, namely TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10. The other members, TLR3, TLR7, TLR8, and TLR9, are located in endosomes, where they sense nucleic acids derived from pathogens[1, 2, 13]. Recognition of herpesviral PAMPs is mediated by TLR2, TLR3, TLR7 and TLR9[2]. The importance of TLRs in controlling viral infections is highlighted by the fact that both humans and mice deficient for certain TLRs show abrogated immune responses and increased susceptibility to viruses[2, 20, 21]. In addition, patients which bear a mutation in UNC93B, an ER-associated protein required for TLR3, TLR7, and TLR9 signaling, show impaired type I IFN responses[22].

Toll-like receptor	Cellular localization	PAMP
TLR1/2	Cell surface	Triacyl lipopeptides
TLR2	Cell surface	Peptidoglycan, hemagglutinin, viral surface glycoproteins
TLR3	Endosome	ssRNA
TLR4	Cell surface	Lipopolysaccharide, mannan
TLR5	Cell surface	Flagellin
TLR6/2	Cell surface	Diacyl lipopeptides, lipoteichoic acid
TLR7	Endosome	ssRNA
TLR8	Endosome	ssRNA
TLR9	Endosome	dsRNA
TLR10	Cell surface	Unknown

Table 2: Overview of different Toll-like receptors with their localization and PAMP[13].

Cytoplasmic RNA and DNA sensors. Upon infection of host cells, viruses often enter the cytoplasm. It would therefore be beneficial for the host when also the cytoplasm contains PRRs to detect intracellular pathogens. Indeed, PRRs reside in the cytoplasm and can recognize in particular viral RNA and DNA[1, 2]. Until now, various RNA and DNA sensors have been identified. Currently, there are two cytoplasmic PRRs known to sense viral RNA, namely RIG-I and MDA5, which induce signaling cascades via IPS1 as depicted in figure 2[1, 2]. RIG-I senses 5' triphosphate ssRNA and short dsRNA fragments, and MDA5 recognizes higher order RNA structures. Both cytoplasmic RNA sensors are involved in recognition of herpesviruses. So far, six cytosolic DNA sensors have been discovered, DAI, DHX9, DHX36, AIM2, IFI16, and LRRFIP1[2]. Only for the latter two there is evidence available for a role

in the recognition of herpesviruses[2, 23]. Little is known about signaling induced by DNA sensors. However, it has been shown recently that DNA sensor LRRFIP1 activates both IRF3/IRF7 and NFκB upon triggering, which suggests that DNA sensors may induce similar signaling cascades as depicted in figure 2[23].

To maintain latency, and to permit replication and new virus production, EBV has to actively evade the immune system. Indeed, there is evidence for EBV evading the adaptive immune system. For instance, the EBV lytic early protein BNLF2a prevents recognition of viral proteins loaded in HLA class I, by T cells via inhibition of Transporter associated with Antigen Processing (TAP)[24,25]. Another EBV protein, BGLF5 induces host shut off, meaning that protein synthesis in the host is suppressed through mRNA degradation. This results in reduced expression of HLA class I and II on the cell surface and thereby diminished recognition by cytotoxic T cells[26]. EBNA1, the only protein present during latency type I, contains Gly-Ala repeats, which prevent breakdown by the proteasome and thereby evade recognition by cytotoxic T cells[11]. These are only three examples of the many strategies EBV has evolved to escape from the adaptive immune system. Recently, more is published about modulation of the innate immune system by EBV. For instance, infectious mononucleosis is characterized by over exaggerated immune responses, which potentially involve innate immune responses as well[3]. This is supported by the recent observation that patients with IM contained EBERs in their serum and that these EBERs were able to trigger TLR3, resulting in production of pro-inflammatory cytokines that cause IM symptoms[27]. As PRRs are important in the recognition of viruses to establish an antiviral state and EBV already has been found to evade adaptive immunity, it is likely that EBV has various manners to evade the human innate immune system as well. The hypothesis that EBV interferes with innate immunity is even more strengthened by the slow mutation rate and long co-evolution with the immune system, even before humans existed, suggesting that EBV is well adapted to the human immune system. This review summarizes the tricks of EBV known to modulate PRR-induced signaling during different stages of the EBV replication cycle, and speculates about its role in evasion and about other plausible tricks for EBV to evade innate immunity.

2. Recognition of EBV by the innate immune system

The prime target cells of EBV are naïve B cells. As B cells express a wide repertoire of TLRs, namely TLR1, TLR2, TLR6, and TLR10 on their cell surface, and TLR7 and TLR9 in their endosomes, they may recognize EBV in different ways[28]. However, B cells are not the only cells which recognize EBV. EBV can also be taken up by pDCs or can infect monocytes, which upon TLR triggering mount a robust response against the virus[9, 29]. Moreover, as various EBV proteins and EBV mRNAs may reside in the cytoplasm, recognition might also occur by cytoplasmic sensors.

Recognition by Toll-like receptors.

TLR2. During viral infection, TLR2 is involved in the recognition of lipoproteins on the surface of virions. Also EBV is able to trigger TLR2 signaling. In TLR2 transfected HEK293 cells, both infectious and UV-inactivated EBV virions induce activation of transcription factor NFκB in a TLR2 dependent manner, presumably via gp350 recognition, as antibodies directed against TLR2 or gp350 inhibited the production of NFκB[30]. Given that TLR2 binds to hydrophobic PAMPs, it seems logical that TLR2 may sense hydrophobic fusion proteins on the EBV virion surface, which may appear only during fusion, presumably after binding of gp350 to CR2 on the host cell[2]. It has been shown for the β-herpesvirus HCMV that antibodies directed against fusion proteins gB and gH of the HCMV virion inhibit TLR2 activation[2, 31]. As EBV also contains gB and gH, fusion proteins, which are conserved among herpesviruses, it is thought that these proteins are implicated in EBV triggering TLR2 as well[9]. Thus, it may be that EBV virions trigger TLR2 activation in host cells by interacting with tethering protein gp350 or with fusion proteins gB and gH. In addition, proteins gp42 and gB may be potential targets for TLR2, as these proteins are also involved in fusion of EBV with host cells.

In general, TLR2 is thought to be involved in the recognition of proteins present on the virion surface. Interestingly, EBV lytic protein dUTPase, essential for viral replication, is able to induce TLR2 activation as well[32, 33]. In macrophages and HEK293 cells expressing TLR2, EBV dUTPase induces NFκB activation via adaptor protein MyD88, resulting in the expression of pro-inflammatory cytokines, like IL-6 and IL-10. This activation depends solely on TLR2, as HEK293 cells expressing other TLRs were not able to enhance NFκB activity[32]. However, dUTPase and TLR2 are spatially separated and may not interact during EBV infection, as dUTPase is present in the cytoplasm of EBV-infected cells and TLR2 is located on the cell surface[13, 33]. Ariza and coworkers suggest that dUTPase might be released from infected cells and thus can interact with TLR2 on the same and other host cells. However, evidence is needed to determine whether TLR2 and dUTPase interaction occurs at all during EBV infection.

TLR3. TLR3 senses dsRNA and it is therefore not surprising that TLR3 recognizes dsRNA viruses, like Rhinovirus [34, 35]. EBV is a DNA virus, but produces RNA structures during infection as well, which may be recognized by TLR3. Latently EBV-infected cells produce large numbers of nonpolyadenylated, noncoding RNA structures (also known as EBERs) which, due to intermolecular base-pairing, form dsRNA structures[3]. Although EBERs reside in the nucleus and TLR3 in endosomes, these noncoding RNA structures may function as TLR3 agonists[3]. EBERs are present in the supernatant of EBV-infected cells, suggesting that EBV-infected cells may secrete EBERs into the extracellular environment, where it could be taken up by cells via the endosomal route and activate TLR3[27]. In addition, Iwakiri and colleagues found that of the two EBERs expressed in EBV-infected cells, only EBER1 triggers TLR3, resulting in activation of IRF3 and NFκB, and eventually in the production of type I IFN[27]. EBER1 binds to cellular protein La, which is involved in the correct folding and maturation of RNA polymerase III transcripts and is present in the nucleus. This suggests that EBER1 and La might be secreted as a complex by EBV-infected cells, which has been shown to activate TLR3 signaling, resulting in activation of NFκB and IRF3[2727, 36]. These findings show a role for extracellularly provided EBERs triggering TLR3 only *in vitro* and thus, it should be kept in mind that during EBV infection interaction between EBERs and TLR3 may not occur. In addition, it is not clear whether EBV actively regulates EBER release. The presence of EBERs in the supernatant of cells infected with EBV may also be simply a consequence of release by dying EBV-infected cells.

TLR7. There is evidence for a role of TLR7, sensor of ssRNA, in EBV induced type I IFN production. Although EBERs form dsRNA structures, these mRNAs still contain ssRNA motifs, which may interact with TLR7 causing its activation. Indeed, EBERs induce type I IFN production in pDCs, an effect abrogated by the use of inhibitory TLR7 agonists, which supports a role for EBERs in triggering TLR7[37]. In addition, for the β-herpesvirus HCMV, a sufficient type I IFN response is dependent on TLR7 triggering[38]. The same may be true for EBV, presumably via EBERs interacting with TLR7[27]. Again, it should be kept in mind that TLR7 and EBERs may not interact during EBV infection due to spatial separation.

TLR9. The genomic dsDNA structures of EBV are recognized by TLR9[29, 37]. Upon EBV uptake by a pDC, TLR9 senses EBV dsDNA resulting in type I IFN production, a process for which endosomal acidification is required[29, 37]. Moreover, TLR9 inhibitor treatment of EBV-infected pDCs and monocytes causes a marked decrease in production of cytokines type I IFN, IL-8, IL-10 and MCP-1. Given that EBV infects or is taken up by host cells expressing high levels of TLR9, recognition of dsDNA of EBV by TLR9 may be important in the control of EBV infection by the host[28].

Recognition by cytoplasmic sensors

RNA sensors. In RIG-I expressing Daudi cells, both EBER1 and EBER2 induce expression of type I IFN, indicating that cytoplasmic RNA sensor senses EBERs [39]. Moreover, triggering of RIG-I by EBERs induces activation of NFκB and IRF3. Given that EBERs are already present quite early in infection, they are suggested to be involved in type I IFN production during initial EBV infection[39]. In addition, these findings imply a role for RIG-I in the recognition of EBERs, but whether this indeed occurs *in vivo* requires further investigation, as EBERs (nucleus or extracellular environment) and RIG-I (cytoplasm) are spatially separated[2, 27, 36]. MDA5 may also be implicated in recognition of EBV, as α-herpesvirus HSV-1 induces signaling via MDA5 and IPS1, resulting in production of type I IFN[2, 40].

DNA sensors. Nowadays, proof of recognition of EBV by cytoplasmic DNA sensors is lacking, but there is evidence for other human herpes viruses. CMV is sensed by DAI (also known as ZBP1), which activates IRF3, resulting in production of type I IFN [2, 41-43]. Other DNA sensors DHX9 and DHX36 are associated with the production of pro-inflammatory cytokines and type I IFN by pDCs upon HSV-1 infection, through activation of NFκB and IRF7[2, 44]. Although there is no evidence yet for EBV recognition by DNA sensors, these findings obtained for other human herpesviruses suggest a role for DNA sensors in the recognition of EBV as well.

Taken together, TLR2 and TLR9 are actually involved in the recognition of EBV. Whether EBERs trigger TLR3, TLR7, and RIG-1 during EBV infection is questionable, as their interaction was only shown *in vitro* where EBERs were provided to cells in the extracellular milieu. MDA5 and various DNA sensors may sense EBV as well, given the role these PRRs have in recognition of other human herpes viruses. Triggering of PRRs is thought to be detrimental for EBV infection as it induces signaling via IRF3/IRF7 and NFκB, resulting in the induction of antiviral responses and the establishment of an antiviral state in both infected and neighboring cells. Therefore, EBV may aim to suppress these responses in order to prevent elimination and thus to remain in humans for decades. PRRs recognizing EBV are potential candidates for the virus to interfere with and thus to suppress PRR-induced signaling.

3. Modulation of innate immunity during EBV latency phase

Upon infection of B cells EBV immediately goes into latent phase to establish persistence in humans for life. Latency is characterized by the presence of EBV viral genome, but the absence of viral replication[3]. EBV latency is divided into three phases, which are characterized by the expression of a specific subset of latency genes, important for the establishment and maintenance of EBV latent infection[3, 6, 10]. The ability of EBV to induce persistence for life without being noticed by the immune system points towards a role for EBV latency genes in evasion of innate immunity. Indeed, EBV latency gene products interfere with innate immunity at the level of recognition as well as at the level of signaling to create an environment beneficial for its own survival.

EBV interferes with innate immune signaling at the level of pattern recognition

The induction of an immune response against EBV starts with the recognition of the virus by pattern recognition receptors, of which TLR2, TLR3, TLR7, TLR9 and RIG-I have so far been shown to be involved. Recognition of EBV by PRRs would be detrimental for the virus and therefore, it would be likely that EBV developed various strategies to counteract recognition by PRRs. Currently, there is evidence for EBV modulating expression and signaling of TLR7 and TLR9, and cytoplasmic RNA sensor RIG-I (Table 3).

Toll-like receptors. Treatment of uninfected B cells with TLR7/8 and TLR9 agonists induces B-cell proliferation. However, in EBV-infected B cells proliferation was significantly lower upon TLR7/8 and TLR9 triggering, which suggests that EBV suppresses TLR-induced proliferation[45]. Inhibition of proliferation was not due to reduced expression of TLRs, which implies that EBV probably dampens the TLR-induced effects via interference with TLR signaling. However, simultaneous treatment of B cells with TLR9 agonist and EBV particles enhances B-cell proliferation, while B-cell proliferation was lower in B cells treated with EBV particles only[46]. The differential effect of TLR triggering on B-cell proliferation may be dependent on the time of EBV infection. In the setting where proliferation is decreased in B cells, EBV infection may already have been established allowing for expression of EBV gene products, which interfere with TLR-induced signaling and thus proliferation. On the other hand, B-cell proliferation was enhanced in B cells treated with EBV particles and TLR agonists simultaneously. Due to addition of EBV particles and TLR agonists to B cells at the same time, it may be that EBV infection was not fully established yet and thus no viral genes were expressed to interfere with effects induced by TLR ligands. This implies that in this setting B-cell proliferation may be a direct effect of TLR stimulation rather than modulation by EBV. This is supported by the fact that in response to TLR9 treatment uninfected B cells start to proliferate[47]. Overall, these findings imply

that EBV infection has to be established in B cells in order for EBV to express gene products which may counteract TLR-induced signaling and thus TLR-induced effects like B-cell proliferation.

TLR7. There are indications that EBV EBERs could activate TLR7 resulting in the production of type I IFN and initiation of antiviral responses, which makes TLR7 a potential target for EBV to interfere with[37]. In naive B cells infected with UV-inactivated EBV virions (no viral gene expression), EBV causes increased expression of TLR7 already after 5 and 11 hours, which reaches a peak after 72 hours of infection, suggesting that EBV infection enhances TLR7 expression[48]. Simultaneously, expression of a splice variant of cellular protein IRF5, involved in production of type I IFN downstream of TLR7, is enhanced in B cells 72 hours post-infection [48, 49]. This splice variant called V12 bears only the DNA binding domain and thereby functions as a repressor of IRF5 activity[48]. Besides TLR7 and IRF5, expression of IRF4, involved in B-cell transformation, was enhanced in B cells treated with UV-inactivated EBV virions[48, 50]. IRF4 represses IRF5 expression, which is supported by the observation that reduced levels of IRF4 result in increased expression of IRF5[50]. Thus, EBV has developed two strategies to suppress IRF5 activity; by increasing expression of IRF5 splice variant V12 and negative regulator IRF4.

TLR9. EBV dsDNA activates TLR9 in pDCs and monocytes, inducing production of type I IFN and other pro-inflammatory cytokines[51, 52]. In addition, TLR9 expression increases upon EBV infection, which may contribute to an even more robust antiviral response[52]. There is evidence that EBV interferes with TLR9 by actively dampening signaling[48, 53]. In naive B cells treated with EBV virions, EBV causes downregulation of TLR9 expression after 24 hours of infection[48]. In addition, both TLR9 mRNA and protein levels are reduced in EBV-infected B cells[53], which is mediated by LMP1, as this protein reduces TLR9 promoter activity through activation of NFκB. These results suggest that EBV actively hampers TLR9-induced signaling by suppressing TLR9 expression.

It seems that EBV has dual roles in modulating TLR7 and TLR9 expression levels. Although it is evident that EBV inhibits TLR-induced signaling by inducing expression of both EBV latency and cellular genes to suppress this signaling, it is less clear whether the enhanced TLR expression is due to EBV actively modulating innate immunity or that another mechanism is involved. In host cells expression of TLR7 and TLR9 quickly increases upon EBV infection and TLR activation is thought to be detrimental for EBV. Therefore, it may be that the enhanced TLR7 and TLR9 expression is a response of the host cell towards EBV infection, rather than active modulation by the virus self. This idea is supported by the observation that activation of B cells, for instance due to viral infection, is accompanied by an increase in TLR expression[54]. In addition, transcription factor EBNA2 is expressed not before 11 hours of infection, which implies that EBV can not affect host signaling pathways before EBNA2 is present, as other latency genes are not yet expressed[48]. Thus, the increase in TLR7 expression observed 5 hours post-infection might not be due to modulation of innate immunity by

EBV, but may be a host response towards EBV infection. Another possibility is that gene products present in the EBV virion, such as tegument proteins, may interfere with TLR expression and TLR signaling immediately after infection. This is shown for HSV-1 tegument protein UL37, which induces NFκB activation via TLR2 during initial infection[55].

Cytoplasmic sensors. As certain EBV latency proteins and mRNAs reside in the cytoplasm, where the virus may be recognized by cytoplasmic sensors, it would be likely that the virus interferes with signaling induced upon cytoplasmic PRR triggering. Currently, there are examples found only for cytoplasmic RNA sensor RIG-I[56].

RIG-I. EBERs are able to activate RIG-I resulting in the production of type I IFN[56]. In uninfected B cells treated with EBERs only, EBERs contributed to B-cell growth. This suggests that during EBV infection, EBERs may be of additive value to induce B-cell transformation and proliferation, processes essential in establishing latency[56]. In addition to type I IFN production as a result of RIG-I activation by EBERs, activation of signaling protein IRF3 induces the production of anti-inflammatory cytokine IL-10, which implies a role for EBERs in dampening immune responses[57]. The fact that EBERs are abundantly produced by the virus and are thought to be actively secreted by EBV-infected cells, suggests that EBERs may play an important role in EBV infection. Therefore, EBERs may be implicated in modulating innate immunity for the benefit of EBV, presumably via production of IL-10, rather than solely inducing production of disadvantageous type I IFN[56, 58]. Another possibility is that EBERs serve a function in EBV infection outside immunity.

Pattern Recognition Receptor	PRR signaling enhanced + or inhibited -	Role EBV in modulating PRR signaling
Toll-like receptor 7	+	TLR7 expression enhanced
	-	Splice variant IRF5 V12 inhibits IRF5 activity
	-	IRF4 inhibits IRF5 activity
Toll-like receptor 9	+	TLR9 expression increased
	-	TLR9 expression decreased via activation of NFκB by LMP1
RIG-I	+	EBERs stimulate via RIG-I production of anti-inflammatory cytokine IL-10

Table 3: Overview of EBV modulation with PRR signaling. Infection of EBV causes increased TLR7 and TLR9 expression in host cells. In addition, EBV induces expression of both cellular and viral proteins to suppress TLR expression and to counteract TLR-induced signaling. Evidently, EBV has dual roles in modulating innate immunity.

EBV latency proteins modulate and IRF3/7 pathway and type I IFN signaling

EBV recognition by PRRs in host cells induces the production of type I IFN[1, 51, 52]. As type I IFN is critical in the first line of defence against pathogens, EBV has developed several strategies to counteract the production and downstream effects of type I IFN during latency. EBV gene products EBNA2, LMP1, and LMP2A/B are known to interfere with IRF3/IRF7 and type I IFN-induced signaling by targeting signaling at various levels.

EBNA2. Transcription factor EBNA2, expressed 11 hours post-infection, is essential for the initiation of latency phase III by inducing expression of all latency genes[3, 6, 10, 48]. Latency protein EBNA2 is associated with the resistance against antiviral and anti-proliferative effects of type I IFN in various tumors, which implies that EBNA2 might inhibit type I IFN signaling[59, 60]. Unexpectedly, in Burkitt's lymphoma cell lines, EBNA2 promotes production of type I IFN resulting in the expression of ISGs, measured 24 hours after EBNA2 expression was induced (Figure 4)[61]. A plausible explanation for this observation is that EBNA2 exploits cellular IRF3/IRF7 signaling by activating transcription factor IRF7 to induce expression of LMP1, a protein critical in establishment of latency, presumably by binding to the interferon-stimulated response element (ISRE) in the LMP1 promoter[62]. More recent findings imply a role for EBNA2 in inhibition of type I IFN-induced signaling by promoting cellular STAT3 expression and enhancing its activity via cooperative interaction with LMP1, as shown after 48 hours of transfection[63]. Expression of STAT3 may result in inhibition of type I IFN-induced effects as it binds STAT1[64]. Sequestering of STAT1 prevents heterodimerization of STAT1 with STAT2 and thereby suppresses expression of ISGs (Figure 4)[1, 18, 64]. Taken together, EBNA2 may promote IRF7 activation to express LMP1, but EBNA2 suppresses the establishment of an antiviral state by interfering with type I IFN-induced signaling.

LMP1. Latency transmembrane protein LMP1 is expressed 24h post-infection and is critical in establishing latency by inducing B-cell activation[3, 53]. In addition, LMP1 has implications for type I IFN signaling[3, 6, 10, 53]. LMP1 contains a long C-terminal cytoplasmic tail which interacts with signaling proteins TRAF1, -2, -3, -5, JAK3, and NFκB, all components of the two main signaling pathways in innate immunity (Figure 2)[65, 66, 66, 67, 67, 68]. LMP1 may interact with these proteins to hamper type I IFN production. However, there are conflicting findings that show a role for LMP1 in affecting type I IFN signaling (Figure 4)[65, 66]. Firstly, in transfected cells after 48 hours of culture, LMP1 enhances type I IFN signaling by inducing expression and activation of transcription factor IRF7[65]. In addition, LMP1 induces activation of transcription factor NFκB and signaling protein JAK3, via TRAF1, -2, -3, and -5. JAK3 activates STAT1 involved in type I IFN-induced signaling causing expression of ISGs[65, 67]. It has been recently discovered that LMP1 also interacts with TRAF6, a protein involved in both IRF3/IRF7 and NFκB signaling (Figure 4)[69]. As TRAF6 may activate IRF7 and thus LMP1, it may be that LMP1

activates TRAF6 as a positive feedback mechanism to promotes its expression required for establishment of latency.

LMP1 also plays a role in suppressing type I IFN signaling. In EBV positive LCLs 48 hours post-transfection, LMP1 interacts with Tyk2, one of the two kinases associated with IFNAR and responsible for activation of STAT1 and STAT2, and inhibits Tyk2 activation by preventing its phosphorylation (Figure 4)[66]. Inhibition of Tyk2 is accompanied by reduced STAT activation and less expression of ISGs, showing that LMP1 inhibits type I IFN-induced signaling. Remarkably, LMP1 works as both an enhancer as well as an inhibitor of type I IFN signaling[65, 66]. However, the role of LMP1 as suppressor of type I IFN signaling is more compatible with the observations that EBV-infected cells and EBV-positive tumor cells are resistant to type I IFN stimulation[59, 60].

LMP2A/B. Besides their role in establishing latency, LMP2A and LMP2B play a role in evading innate immunity by modulating type I IFN signaling (Figure 4)[70]. In epithelial cells, LMP2A and LMP2B stimulate the uptake of IFNAR and prevent expression of ISGs, thereby inhibit the initiation of an antiviral state. Thus, both LMP2A and LMP2B inhibit downstream effects of type I IFN by increasing IFNAR recycling and may contribute to the resistance to type I IFN in EBV-infected cells[70].

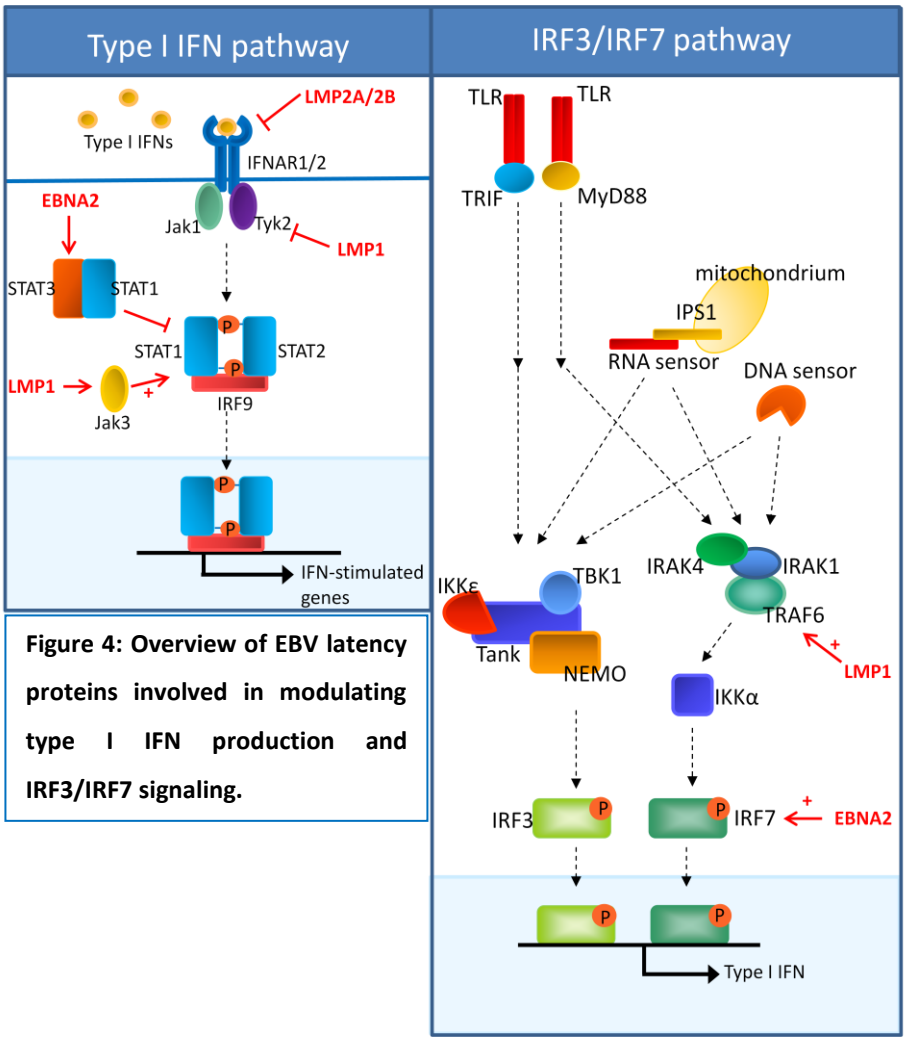

Figure 4: Overview of EBV latency proteins involved in modulating type I IFN production and IRF3/IRF7 signaling.

EBV has contrasting roles in modulating IRF3/IRF7 and type I IFN-induced signaling, but the exact cause of this is unknown. Given that type I IFN production results in the expression of ISGs, genes which are thought to be detrimental for EBV as an antiviral state will be created, it would not be likely that EBV enhances signaling pathways with the aim to induce production of type I IFN[1]. Presumably, EBV EBNA2 induces activation of IRF3/IRF7 with the goal to induce LMP1 expression required for establishing latency and that the additional production of type I IFN may be simply a disadvantageous side-effect. Furthermore, once expressed, LMP1 may activate IRF7 via TRAF6 as a positive feedback mechanism to enhance its expression. Another possibility is that the augmented production of type I IFN may be induced upon recognition of latency proteins EBNA2 and LMP1 by PRRs. Overall, more latency proteins are implicated in inhibition of type I IFN-induced signaling, indicating that inhibition of type I IFN-induced effects may be of particular importance in EBV infection. This is in agreement with the fact that EBV is associated with resistance to type I IFN, to prevent induction of antiviral responses and to persist in humans for decades[61, 65, 71, 72].

EBV latency gene products modulate NFκB pathway

There is quite some evidence for EBV interference with signaling routes via IRF3/IRF7 and type I IFN-induced signaling, but less is known about EBV modulating the NFκB pathway. Recent findings point towards a role for latency proteins EBNA1, LMP2A and LMP1 in modulating innate immunity by interfering with the NFκB signaling pathway.

EBNA1 contributes to innate immune modulation by suppressing NFκB signaling via inhibition of IKKα and IKKβ, components of the IKK complex (Figure 5)[73]. In carcinoma cell lines, reduced IKKα and IKKβ activity results in reduced degradation of IκB and thus in decreased nuclear dislocation of NFκB. Similarly, EBV latent protein LMP2A is able to inhibit NFκB activity resulting in reduced production of pro-inflammatory cytokines[74]. However, the underlying mechanism of this inhibition is not discovered yet. An opposite effect on NFκB activity is observed for latency protein LMP1, as LMP1 constitutively activates the NFκB signaling pathway[75, 76]. In LMP1 tranfected B cells, LMP1 is loaded in vesicles lacking HLA Class II at the Golgi, which are subsequently secreted in the extracellular environment[76]. This mechanism may prevent LMP1 degradation by the host proteasome and thus recognition by cytotoxic T cells, but also inhibits NFκB signaling by suppressing LMP1 activity. So, LMP1 may enhance initially NFκB signaling in order to establish EBV latency and that secretion of this protein in exosomes is a mechanism to regulate its activity. Suppression of antiviral responses seems to be important for EBV, as latency proteins EBNA1 and LMP2A inhibit NFκB signaling.

Regulation of NFκB signaling by cellular and EBV-encoded miRNAs

Herpesviruses abundantly express miRNAs during both latent and lytic infection[14, 15]. MiRNAs exert their suppressing function on protein activity by complementary binding to specific target mRNAs[14]. Although little is known about the precise function of these miRNAs in EBV infection, findings reveal a role in modulating NFκB signaling. Currently, some miRNAs induced by EBV, both cellular and viral, are known to influence NFκB activity by targeting NFκB signaling components and by targeting EBV latency proteins.

Cellular miR-155 and miR-146a. Upon infection of B cells, cellular miR-155 expression is induced by latency protein LMP1 and is involved in inhibition of NFκB signaling by targeting cellular protein IKKε, which is part of a complex required for activation of NFκB (Figure 5)[77]. Expression of cellular miR-155 may be a negative feedback mechanism for LMP1 to modulate NFκB activity, because LMP1 constitutively activates NFκB. Besides a role in inhibition of IKKε function, miR-155 contributes to stable genome maintenance as inhibition of this miRNA reduces EBNA1 expression, which supports a role for miR-155 in maintaining EBV persistence. In addition to cellular miR-155, LMP1 induces expression of another cellular miRNA, miR-146a, presumably via NFκB activation[78]. The action of cellular miR-146a in EBV infection is unclear, but Cameron and co-workers pose a role for this miRNA in acting as a negative feedback mechanism of LMP1 to modulate NFκB-induced effects, including type I IFN response.

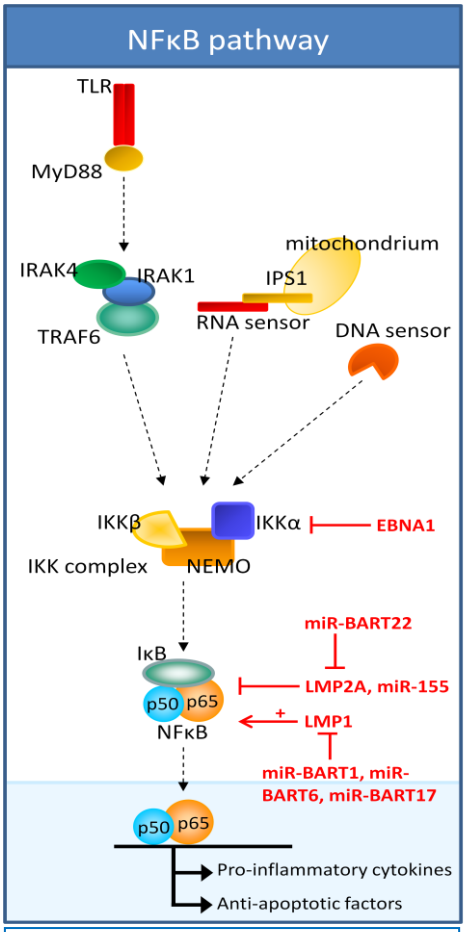

Figure 5: Overview of EBV latency proteins modulating NFκB signaling.

miR-BART1/miR-BART6/miR-BART17/miR-BART22. Latency protein LMP1 is targeted by multiple EBV-encoded miRNAs (Figure 5)[79, 80]. In EBV-infected epithelial cell lines, LMP1 protein levels are dramatically reduced by miR-BART1, miR-BART6, and miR-BART17, which results in decreased NFκB signaling[80]. Recently discovered miR-BART22 is present in EBV-associated malignancy nasopharyngeal carcinomas (NPC) and causes reduced levels of latency protein LMP2A. Given that LMP2A is an inhibitor of NFκB activation, inducing degradation of LMP2A by EBV miR-BART22 promotes NFκB activation[74, 80, 81]. The decrease in LMP2A levels is accomplished presumably via direct interaction of miR-BART22 with LMP2A, as only LMP2A protein levels and not mRNA levels were decreased in various cell lines[80]. Thus, it is clear that EBV produces various miRNAs

that modulate EBV latency proteins, presumably to regulate NFκB activation. When and to what extent EBV-encoded miRNAs are exactly expressed during EBV infection is unknown.

To speculate about the dual role of latency gene products in modeling NFκB signaling, we have to go back to the start of EBV infection. Upon infection of B cells, EBV induces naïve B-cell activation, proliferation and differentiation into memory B cells[3, 6, 10]. For this process signaling via NFκB is required, for which LMP1 is essential as it mimics a constitutive active TNF receptor[75, 76]. Besides the production of pro-inflammatory cytokines, NFκB is involved in induction of anti-apoptotic factors, and induction of B-cell proliferation and differentiation[19]. These findings imply an essential role for LMP1 in establishing EBV latency in B cells by preventing B-cell apoptosis and by inducing B-cell proliferation and differentiation. This is consistent with the finding that LMP2A, which is simultaneously expressed with LMP1 and inhibits NFκB signaling, is suppressed by EBV miR-22[74, 80]. Once latency is established, it has to be maintained to persist in humans for life. It may therefore be that EBV primarily focuses on inhibiting innate immune signaling to prevent antiviral responses and thus its elimination. This is consistent with the fact that the majority of EBV latency proteins inhibit NFκB signaling, and that LMP1 activity is suppressed by multiple miRNAs and extracellular secretion of LMP1 into exosomes[73, 74, 77, 79]. Thus, EBV may modulate NFκB signaling by enhancing NFκB activity to establish latency and inhibit NFκB activity to maintain latency by preventing antiviral responses. This hypothesis may also be a plausible explanation for the contrasting findings observed for EBV at the level of PRRs.

EBERs induce resistance against type I IFN via interaction with cellular proteins

The exact role of EBERs in EBV infection is unclear. However, more and more evidence points towards a role for EBERs in evading innate immunity. In EBV positive tumor cells, EBERs are responsible for protection against type I IFN-induced apoptosis[71, 72, 82]. EBERs interact with cellular RNA-activated protein kinase (PKR), one of the ISGs induced upon type I IFN signaling. PKR is a protease which cleaves eIF2α, resulting in a translational block, but also IκB, the inhibitor of NFκB[71]. Evidence suggests that interaction of EBERs with PKR inhibits its protease activity and that this inhibition is critical for protection against IFN-induced apoptosis[71, 72]. However, others prove that PKR inhibition by EBERs does not occur and thus may not be responsible for resistance against type I IFN[82]. Overall, it seems evident that EBERs confer resistance against type I IFN-induced apoptosis of EBV-infected cells, but whether inhibition of PKR by EBERs is required for this resistance remains unclear.

During latency many EBV latency proteins as well as EBV-encoded and cellular miRNAs modulate innate immune signaling pathways through IRF3/IRF7 and NFκB, and IFN-induced signaling. However, why EBV modulates innate immune signaling differentially is unknown. A plausible hypothesis is that PRR-induced signaling is required for EBV to establish latency and that EBV may suppress these signaling pathways to prevent antiviral responses and to persist in humans for life. To confirm this hypothesis, knowledge of the time and extent of expression and activation of the various EBV and cellular gene products in the infected host cell is necessary.

4. Modulation of innate immunity during EBV lytic phase

Lytic infection is characterized by expression of the entire EBV genome, which is tightly regulated by the sequential expression of immediate-early, early and late lytic genes essential for *de novo* production of viral particles[3, 10]. In total more than 80 lytic genes are expressed coding for structural and non-structural factors, from which some may be recognized by PRRs resulting in the initiation of an antiviral response. Recognition of lytic gene products by the adaptive immune system has already been demonstrated. EBV lytic proteins are immunodominant antigens for $CD4^+$ and $CD8^+$ T cells derived from patients with acute IM[83, 84]. In addition, clonal analysis of $CD8^+$ cytotoxic T cells (CTLs) revealed that the T cell receptors of these CTLs recognize epitopes of immediate-early proteins BZLF1 and BRLF1 and early lytic proteins BMLF1, BMRF1, and BALF2[84]. Modulation of innate immune signaling by EBV may be essential to complete *de novo* production of EBV virions. Some EBV lytic proteins have been discovered to modulate innate immune signaling during EBV replication, which seem to primarily focus on targeting PRR expression and PRR-induced signaling via IRF3/IRF7 and via type I IFN.

EBV lytic proteins target Toll-like receptors

Currently, two EBV lytic proteins, dUTPase and BGLF5, are known to interfere with Toll-like receptors at the level of expression and signaling (Table 4). These proteins may modulate innate immune signaling to allow EBV to persist without being cleared[85, 86].

TLR2. There are implications for EBV non-structural protein dUTPase, present in the cytoplasm and required for EBV genome replication, in modulating NFκB activity[85]. As mentioned earlier, extracellularly added dUTPase is able to specifically trigger TLR2 on the surface of monocytes resulting in activation of NFκB signaling. Ariza and coworkers suggest that EBV-encoded dUTPase can trigger TLR2, as this protein might be secreted from infected cells[85]. In addition, they suggest that secreted dUTPase, via TLR2 activation, induces production of anti-inflammatory cytokines, like IL-10, thereby dampening the immune system, which may result in an environment favorable for production of EBV virions without being noticed by the immune system[85]. Again it should be kept in mind that dUTPase may not be secreted and that it may not interact with TLR2 during EBV infection. Considering this protein is present in the extracellular environment, it may be that this is a consequence of dying EBV-infected cells and that activation of TLR2 by dUTPase may be an undesired side-effect, as upon TLR2 triggering, activation of NFκB and IRF3/IRF7 contribute to the initiation of antiviral responses. Prevention of TLR2-induced signaling may therefore be more useful during EBV infection. This idea is supported by the observation that α-herpesvrius HSV-1 reduces TLR2

expression during lytic infection[87]. In HEKF293 cells infected with HSV-2, lytic protein viron host shut off protein (vhs) decreases expression of TLR2, but also of TLR3 and cytoplasmic RNA sensors RIG-I and MDA-5. Likewise, EBV may lower expression of TLR2, TLR3, RIG-1 and MDA-5 during infection, which may be mediated by lytic protein BGLF5, the host shut off protein encoded by EBV[26].

TLR9. TLR9 senses EBV dsDNA and upon recognition induces a robust antiviral response in certain cell types, including pDCs and monocytes[29, 37]. TLR9 triggering may be detrimental during lytic infection and EBV has developed ways to counteract TLR9-induced signaling. In EBV-infected B cells, TLR9 expression was markedly decreased at both mRNA and protein level that was specifically caused by EBV lytic protein BGLF5[86]. EBV infection also reduces expression of other Toll-like receptors, including TLR1, TLR6, TLR7, and TLR10 although to a lesser extent[86]. As EBV lytic protein BGLF5 induces host shut off, meaning that in the host cell all protein synthesis is inhibited, it may be that the decreased expression of other TLRs is caused by BGLF5 as well. Another possibility is that reduction of TLR expression is an effect of other lytic proteins, which are not revealed yet.

Pattern Recognition Receptor	PRR signaling enhanced + or inhibited -	Role of EBV in modulating PRR signaling
Toll-like receptor 2	+	TLR2 triggering by dUTPase secreted by EBV-infected cells
Toll-like receptor 9	-	TLR9 expression decreased by BGLF5

Table 4: Overview of EBV modulation with PRR signaling during lytic phase. Dual roles of EBV on TLR expression and signaling.

EBV lytic proteins hamper type I IFN response by targeting IRF3/IRF7 signaling

Most of the lytic proteins currently known to modulate innate immunity interfere with IRF3/IRF7 signaling (Figure 6). Both immediate-early proteins BZLF1 and BRFL1 are involved in dampening type I IFN production[88, 89]. BZLF1 interacts with IRF7, a transcription factor essential in type I IFN expression[88]. Interaction of BZLF1 with IRF7 does not affect IRF7 translocation to the nucleus, but does suppress IRF7 activity and thereby production of type I IFN. The exact mechanism underlying this observation is unclear. However, it is observed for the γ-herpesvirus KSHV that immediate-early lytic protein RTA promotes proteosomal degradation of IRF7 via ubiquitination mediated by an intrinsic ubiquitinase E3 ligase of RTA[90]. This suggests that EBV BZLF1 may use this mechanism of ubiquitination to decrease IRF7 expression during EBV infection. Immediate-early protein BRLF1 inhibits type I IFN production by decreasing expression of IRF7, but also of transcription factor IRF3[89]. Downregulation of IRF3 and IRF7 by BRLF1 does not depend on localization of these transcription factors, as in both the cytoplasm and nucleus, mRNA and protein levels of IRF3 and IRF7 are reduced.

However, the exact mechanism underlying diminished expression of IRF3 and IRF7 caused by BRFL1 is unknown.

Two other EBV lytic proteins, BGLF4 and LF2, interfere with type I IFN production by targeting IRF3 and IRF7 respectively[91, 92]. EBV lytic protein BGLF4, a virion-associated kinase conserved among herpesviruses, affects IRF3 activity, but not by interfering with IRF3 nuclear transition. BGLF4 reduces the amount of IRF3 recruited to its promoter, but how exactly is not known[91]. A plausible mechanism is that BGLF4 may interact with the activated form of IRF3 in the nucleus and thereby prevent IRF3-induced gene expression, a strategy which has been discovered for murine herpesvirus-68 ORF36, a conserved herpesviral kinase[93]. On the other hand, EBV lytic protein LF2, a structural protein found in the tegument of EBV virions, affects IRF7 activity[92]. LF2 specifically interacts with the transactivation domain of IRF7, preventing dimerization and thus production of type I IFN.

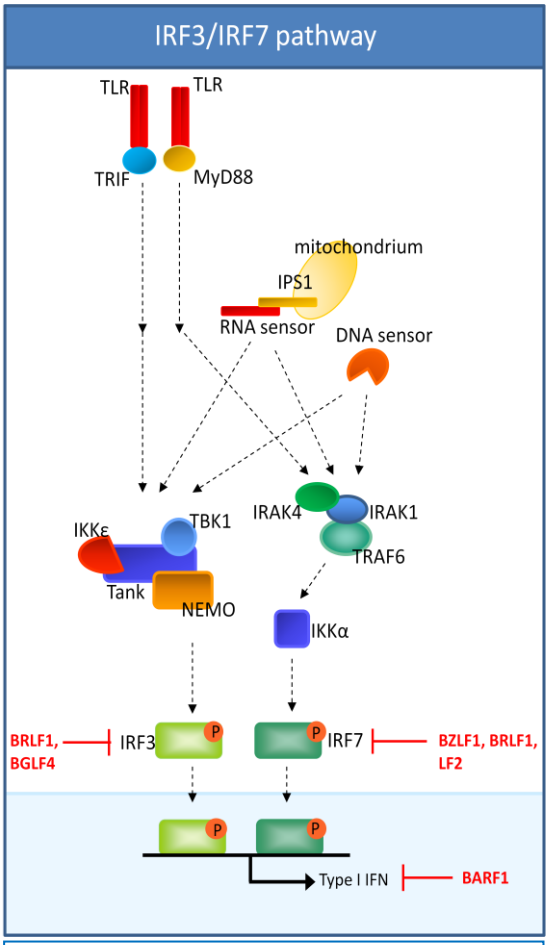

Figure 6: Overview of EBV lytic proteins involved in modulating IRF3/IRF7 signaling.

EBV lytic gene BARF1, which encodes a soluble colony-stimulating factor-1 receptor (CSF-1R), decreases expression of type I IFN in monocytes upon stimulation with colony-stimulating factor-1 (CSF-1) in monocytes[94]. Although the mechanism of inhibition by BARF1 is not yet identified, a plausible strategy is that EBV secretes BARF1 in the extracellular compartment where it sequesters CSF-1 and thus results in suppression of CSF-1-induced type I IFN production.

EBV inhibits type I IFN-induced signaling during lytic infection

Upon infection of monocytes, EBV increases expression of cellular protein Suppressor of Cytokine Signaling 3 (SOCS3), an inhibitor of type I IFN-induced signaling[95]. Increased expression of SOCS3 is accomplished by EBV transcription factor BZLF1, which regulates expression of early genes during lytic infection. In EBV-infected monocytes, host protein SOCS targets the JAK/STAT pathway and prevents activation of STAT proteins by IFNAR-associated kinases, Jak1 and Tyk2 (Figure 7). STAT activity is induced upon type I IFN binding, produced upon pathogen recognition, to its receptor

IFNAR on host cells resulting in the production of ISGs to create an antiviral state. This process is hampered by EBV as it induces expression of cellular protein SOCS, which inhibits STAT activity[95]. Exploitation of this host regulatory mechanism is a way for EBV to suppress host innate immune responses during lytic infection.

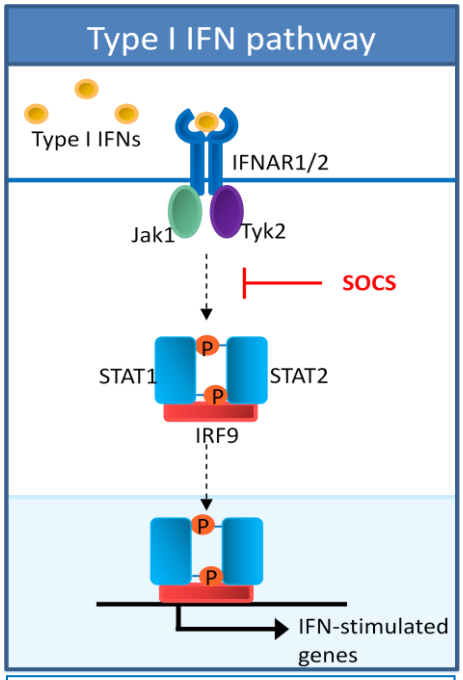

Figure 7: EBV inhibits expression of ISGs by inducing expression of SOCS.

Remarkably, EBV lytic genes are not solely expressed during lytic phase, but appear to be expressed also during initial EBV infection[10]. Expression of lytic genes is presumably induced as in newly infected host cells EBV DNA is exposed to the cellular transcription machinery. Examples of lytic genes expressed during initial infection are viral homologues of cellular BCL-2, proteins involved in preventing apoptosis, BCRF1, and immediate-early gene BZLF1, all proteins involved in modulating immune signaling[10]. Given this, it may be that the lytic proteins discussed above exert their modulatory function on innate immunity during initial infection as well and may contribute to establishment of EBV latency.

Taken together, EBV has various strategies to modulate innate immunity during lytic infection, which primarily interfere with PRR expression and PRR-induced signaling via IRF3/IRF7. Given that the majority of these lytic proteins suppress PRR-induced signaling, the prime focus of EBV during lytic infection may be to evade innate immunity. Furthermore, EBV is recognized by various other PRRs than only TLR2 and TLR9, which suggests that the virus also interferes with the expression of these PRRs to hamper innate immune responses. There are no publications concerning EBV lytic proteins interfering with the NFκB pathway and it could be that NFκB modulation by EBV is not important during lytic infection. However, in order to complete viral replication, death of the infected host cell and antiviral immune responses has to be suppressed long enough. Given that activation of NFκB is involved in preventing both processes, suggests that NFκB plays an important role during lytic infection as well.

5. Discussion

Almost the entire human population is infected with EBV, mostly carriers are asymptomatic for life[3]. To establish and maintain latency, and to allow enough time to complete virion synthesis in order to infect other host cells and new hosts before clearance of the infected cell by the immune system, EBV has to prevent antiviral responses during both latent and lytic phase. Multiple proteins have been discovered for EBV in evading adaptive immunity[11, 24, 26]. Nowadays, the role of EBV in evading innate immunity, which is key in regulating antiviral responses, receives more attention[1]. The induction of antiviral responses starts with recognition of viruses by pattern recognition receptors, which upon triggering induce signaling routes via IRF3/IRF7 and NFκB to drive both innate and adaptive immune responses[1, 2]. Upon B-cell infection, EBV virion components, but also EBV gene products expressed during both latent and lytic phase, may be recognized by PRRs that initiate antiviral responses. In recent years, various strategies of EBV to modulate innate immune signaling induced upon PRR triggering during both latent and lytic infection have been discovered. PPR-induced signaling is targeted by multiple EBV induced gene products interfering with PRR expression and with the signaling pathways through activation of IRF3/IRF7 and NFκB, but also more downstream with type I IFN-induced signaling.

How to interpret innate immune modulation by EBV during latent phase

During latency various EBV gene products interfere with both IRFR3/IRF7 and NFκB signaling cascades, although with different outcomes. Modulation of innate immunity does not per se imply that EBV evades innate immunity to prevent initiation of antiviral responses. It could be that EBV modulates IRF3/IRF7 and NFκB signaling pathways to serve another purpose in EBV infection.

Aim of EBV modulating IRF3/IRF7s and type I IFN-induced signaling. EBV proteins EBNA2, LMP1 and LMP2A/B, inhibit type I IFN-induced signaling pathway and suppress expression of ISGs to evade innate immune responses. Remarkably, EBNA2 and LMP1 also stimulate signaling through IRF3/IRF7. As activation of IRF7 is required for expression of EBV latency protein LMP1, it seems that EBV does not so much modulate IRF7 signaling to evade innate immunity, but rather exploits this signaling route in order to establish latent infection. Apparently, IRF7 activation is necessary for EBV infection and thus the production of type I IFN is an undesired side-effect and impossible to prevent. It seems therefore logical that EBV proteins EBNA2, LMP1, and LMP2A/B act more downstream of type I IFN by counteracting type I IFN-induced signaling and thus to evade establishment of an antiviral state.

Aim of EBV modulating NFκB signaling. Also multiple EBV gene products are involved in modulating NFκB signaling with differential effects. EBNA1, LMP2A, cellular miR-155 and BARTs miR-1, miR-6 and miR-17 inhibit signaling through NFκB, while LMP1 and miR-BART22 stimulate this signaling route. However, the effects of NFκB signaling are not that straightforward as for IRF3/IRF7 signaling. The effects of NFκB activation are quite complex as this transcription factor induces expression of a wide variety of genes involved in many different cellular processes, including production of pro-inflammatory cytokines, and B-cell survival and differentiation[19]. It is therefore difficult to dedicate a role for EBV in evading innate immunity by modulating NFκB. EBV presumably promotes NFκB activation to induce B-cell survival and differentiation required for establishment of EBV latency immediately after entering a new host cell. Inhibition of NFκB signaling may be a mechanism for EBV to regulate constitutive activation of NFκB by LMP1 and thus to suppress B-cell activation and differentiation, to allow EBV persistence in resting memory B cells. Inhibition of NFκB may also be a mechanism for EBV to evade the immune system by preventing production of pro-inflammatory cytokines. Another possibility is that EBV activates NFκB to prevent initiation of lytic infection, as was shown for γ-herpesvirus KSHV[96].

Role of miRNAs. Viral miRNAs are abundantly expressed by all herpesviruses and it seems clear that they have multiple functions in EBV infection[14, 79, 80]. During latency, innate immune signaling is regulated by various viral and cellular miRNAs with dual roles. EBV promotes expression of cellular and EBV-encoded miRNAs, which modulate both cellular and EBV latent protein activity, thereby playing a role in the stimulation and inhibition of innate immune signaling. It has been noted for EBV that miRNAs are secreted and can be exported to neighboring cells where they can specifically target cellular proteins[97]. The secretion of miRNAs by EBV may also be a mechanism to make neighboring uninfected cells more susceptible to EBV infection.

Hypothetical model of innate immune modulation. Overall, modulation of PPR-induced innate immune signaling during latency seems to serve two purposes for EBV. The first goal of EBV, upon entering a host cell, may be to enhance innate immune signaling to induce B-cell proliferation, differentiation, and anti-apoptotic effects to establish latency. At the same time, this signaling may be suppressed at various levels by EBV to maintain latency and to avoid antiviral effects and its elimination. This hypothetical model, including EBV induced gene products modulating innate immune signaling during latency discovered so far, is depicted in figure 8.

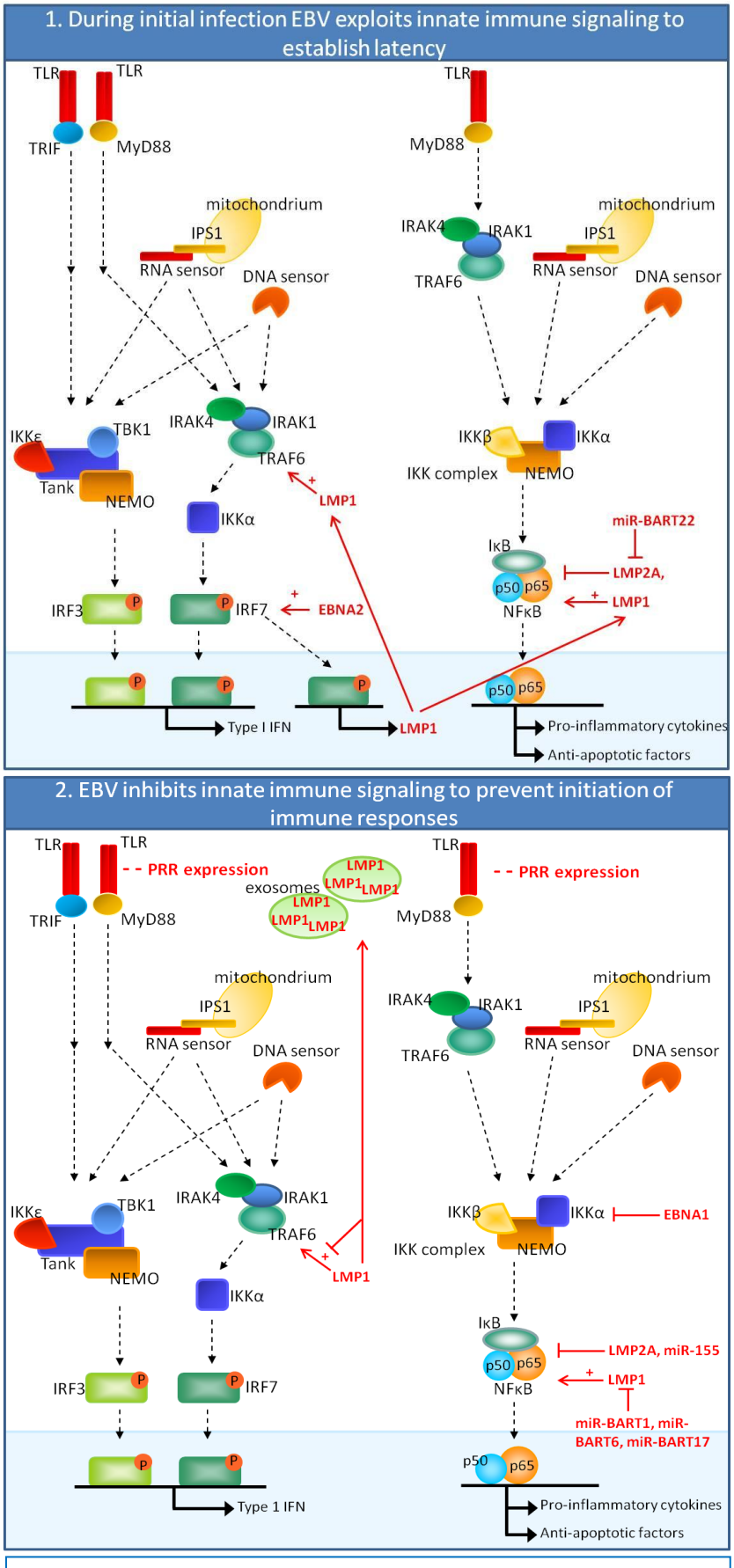

Figure 8: Hypothetical model. 1. EBV enhances PRR-induced signaling, 2. EBV suppresses these signaling routes to prevent clearance.

Innate immune modulation during lytic phase: Prime focus on inhibiting type I IFN production

It seems obvious that all EBV lytic proteins discovered so far contribute to inhibition of PPR-induced innate immune signaling. These proteins target IRF3/IRF7 and type I IFN-induced signaling, implying that the main purpose is to evade the immune system during lytic phase. There is no evidence for a role of EBV in modulating the NFκB pathway. NFκB singaling is quite complex as it induces expression of genes involved in a wide variety of processes, and thus has a lot more implications than only stimulating immune responses. Most EBV-infected host cells have to survive for 5 days after reactivation [98]. NFκB activation may be of crucial importance by stimulating host cell survival and thus to provide the time window needed to complete the production of new virus particles. In addition, as more than 80 lytic genes are expressed and the function of many lytic proteins is not known, it may be that more EBV proteins are involved in modulating innate immunity than discovered so far[3]. The lytic EBV proteins known to be involved in inhibiting PRR-induced innate immune signaling are only immediate-early and early proteins, suggesting that only initially during lytic infection evasion of innate immunity may be required. One could wonder whether EBV late lytic proteins are involved in modulating innate immunity, as they may only contribute to production of EBV viral particles because they are structural components of the virion[3]. Taken into account what has been discovered so far, it is clear that during the lytic phase EBV aims to prevent type I IFN production and thus to evade innate immunity.

Lessons from innate immune modulation by other human herpesviruses

Other members of the herpesvirus family have also developed tricks to modulate innate immunity. Some strategies similar to those employed by EBV, but some strategies have not been seen for EBV yet. Human herpesviruses share quite some homology, such as genome similarities and similar host cell tropism, and have all co-evolved with their host[3]. They may have developed mechanisms to counteract innate immune signaling by interfering with similar targets. For example, similar to EBV, ICP0 of α-herpesvirus HSV-1 targets IRF3 to suppress type I IFN production[2, 99]. In addition, both α-herpesvirus VZV and β-herpesvirus HCMV encode for proteins that inhibit cytoplasmic DNA sensor-induced signaling[2]. This implies that EBV may also interfere with DNA sensor-induced signaling upon triggering to modulate innate immune signaling.

KSHV. KSHV belongs, similar to EBV, to the γ-herpesviridae and has a similar infection strategy as EBV[3]. KSHV and EBV infect both B cells and are associated with development of various tumors. Furthermore, both viruses encode host shut off proteins which reduce MHC I expression in order to evade $CD8^+$ T cell responses and thus adaptive immunity[26, 100]. These are only few of multiple features KSHV and EBV share. It seems therefore likely that KSHV and EBV have evolved similar ways

to modulate innate immune signaling (reviewed by Sathish *et al.*). Indeed, just like EBV, KSHV proteins target the IKK complex, which activates NFκB by phosphorylating IκB for degradation[101]. Other KSHV proteins target transcription factor IRF7, inhibit IFNAR1/2 associated kinase Tyk2, and TLR-induced signaling, all mechanisms to evade innate immune signaling similar to EBV. KSHV protein ORF50 targets IRF7 and induces its degradation, presumably by an intrinsic ubiquitinase activity. As multiple EBV proteins also reduce IRF7 protein levels, but not at the mRNA level, this mechanism of ubiquitination found for KSHV may also be involved in IRF7 degradation during EBV infection. Although there is no evidence that EBV proteins bear an intrinsic ubiquitinase activity, it has been shown that EBV lytic protein BZLF1 acts an adaptor for ECS (Elongin B/C-Cul2/5-SOCS-box protein) ubiquitin ligase complex that targets p53 for degradation[102]. This similar strategy of BZLF1 recruiting ubiquitinase activity may be involved in downregulating IRF7 levels during lytic infection. K8 and kinase ORF36 interfere with IRF3 binding to its promoter region to inhibit type I IFN production. In addition, KSHV expresses viral homologs of IRF which can bind to or degrade cellular IRFs and suppress their activity[101]. As KSHV and EBV are quite alike, it seems plausible that EBV also codes for proteins that interfere with IRF3 promoter binding and for IRF homologues to prevent type I IFN expression, which remain to be discovered.

Thus, it seems that all human herpesviruses have developed various tricks to counteract innate immune signaling during infection. Some herpesviral strategies are also found for EBV, others are not. As herpesviruses share many features and in particular KSHV and EBV share quite some homology, it seems likely that EBV has evolved more strategies to modulate innate immune signaling then currently discovered, which are similar to other herpesviruses.

In conclusion, EBV evolved multiple ways to manipulate innate immunity by both enhancing and inhibiting innate immune signaling, which probably contribute to establish and maintain persistence in humans for life. Knowledge of the multiple strategies EBV has evolved to modulate innate immunity will broaden our view on EBV infection and provides more insight into complex EBV-host interactions. In addition, this understanding will reveal potential therapeutic candidates to interfere with in order to abrogate EBV infection. For instance targeting several gene products that evade innate immunity by interfering with innate signaling pathways, or proteins critical for establishment of latency, like LMP1. Subsequently, this may allow for sufficient antiviral responses and may reduce resistance of EBV-infected (tumor) cells to type I IFN, thereby making these (tumor) cells more susceptible to elimination by the immune system. Thus, understanding of the complex interplay between EBV and host will help us a step further in the development of therapeutic interventions for EBV-associated diseases, such as PTLD, IM, and several malignancies.

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