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Understanding TLR9 action in Epstein-Barr virus infection

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1. ABSTRACT

The Epstein-Barr virus (EBV) establishes persistent latent infection in peripheral blood memory B cells, may cause infectious mononucleosis, and is associated with cancers including endemic Burkitt's lymphoma (BL). Although latent EBV transforms B cells *in vitro*, additional factors including immunocompromised status or, as in endemic BL, a co-infection with the malaria parasite *Plasmodium falciparum* seem to be required for the development of EBV-associated cancers. Toll-like receptors (TLRs) like TLR9 are capable to recognize EBV and launch innate immune responses, which may limit the spread of the virus and may contribute to control outgrowth of latently EBV-infected B cells. On the other hand, EBV may interfere with the expression and functionality of TLR9, thereby manipulating host immune responses towards favoring long-term survival of the virus. Triggering of TLR9 by bacterial, viral or *P. falciparum* DNA may impact on the proliferation of EBV-infected B cells and on the balance between latent and lytic EBV. Thus, TLR9 signaling in EBV-infected B cells may be beneficial for the host but also for the highly adapted human gammaherpesvirus EBV.

2. INTRODUCTION

The Epstein-Barr virus (EBV) is a human gammaherpesvirus. It infects more than 90% of the world's human population. Despite the host mounting specific humoral and cellular immune responses, EBV succeeds in establishing persistence in peripheral blood memory B cells for a lifetime (1).

EBV was first discovered in endemic Burkitt's lymphoma (BL) around 50 years ago, the most common cancer in children in equatorial Africa (2). Subsequently, EBV was associated with other forms of B cell or epithelial cell malignancies including non-Hodgkin's lymphoma, certain types of Hodgkin's lymphoma, lymphoproliferative disorders in immune suppressed transplant recipients or HIV-infected individuals with immunodeficiency (3), and nasopharyngeal carcinoma (4). More recently, EBV has been also associated with autoimmune diseases including multiple sclerosis (5).

The vast majority of the EBV-infected individuals do not develop EBV-associated malignancies or autoimmunity. This suggests that EBV and the host

Table 1. Patterns of EBV latency gene expression and occurrence

Latency program	EBNA1	EBNA2-6, EBNA-LP	LMP1, LMP2A, LMP2B	EBER1, EBER2	BARTs	Type of tumor
Latency I	+	-	-	+	+	Burkitt's lymphoma
Latency II	+	-	+	+	+	Hodgkin's disease, nasopharyngeal carcinoma
Latency III	+	+	+	+	+	Lymphoblastoid cell line, post-transplant lymphoproliferative disease

EBNA, EBV nuclear antigen; LP, latent protein; LMP, latent membrane protein; EBER, EBV nonpolyadenylated RNA; BART, *Bam* A rightward transcripts

immune system have developed strategies that reduce EBV's potential to become pathogenic, a potential that is mirrored by the unique capacity to transform B cells *in vitro* exhibited by the virus (6).

Most notably, both extremes of immune responses, i.e., deficient immune responses, as in states of immune compromise or immune suppression, and exaggerated immune responses, as in infectious mononucleosis, may contribute to EBV-associated pathology. Immune control of EBV is ascertained by the innate and the adaptive immune systems, with cytotoxic (CD8+) T cells exhibiting a crucial role in adaptive immunity (7). As innate immunity links with adaptive immunity, evading the innate immune system might be particularly important for EBV, given its slow replication cycle and its maintenance of life-long latent infection (8).

Here we review current knowledge on the impact of innate immunity on EBV and vice versa, whereby we focus on B cells that are the main target cell of EBV and the innate immunity element Toll-like receptor 9 (TLR9) that they abundantly express.

3. EPSTEIN-BARR VIRUS (EBV) AND B CELLS

EBV exhibits tropism for epithelial and B cells, the latter being the site for establishing persistent latency (1). How EBV trespasses the nasopharynx epithelial layer to get access to B cells from the nasopharynx-associated lymphoid tissue (NALT) including tonsils is not clear-cut.

The attachment of EBV to B cells is mediated by the direct interaction of EBV glycoprotein gp350/220 with cellular CD21, initiating receptor-mediated endocytosis. After binding to CD21, EBV gp42 can interact with host HLA class II, leading to a conformational change in the viral glycoproteins and triggering fusion with the host cell membrane (9). In polarized oropharyngeal epithelial cells, which lack CD21, interactions between the EBV glycoprotein BMRF-2 via its Arg-Gly-Asp (RGD) motif and the beta1 family of integrins are critical for infection (10).

EBV is mainly present as a latent virus (1). Efficient establishment of latency allows EBV to persist despite host immune responses. Following *de novo* infection of B cells *in vitro*, latency is the default pathway, and primary B cells are often immortalized giving rise to so-called lymphoblastoid cell lines (LCLs) (1).

Depending on EBV gene expression in cell lines and tissues three EBV latency patterns have been described (11). Table 1 summarizes these three latency patterns and their occurrence. The Latency III program is found in LCLs. It involves expression of the full spectrum of latent EBV genes, i.e., EBNA 1-6, latent membrane proteins (LMP)1, -2A, -2B, two small RNAs (EBER1,-2), and Bam A rightward transcripts (BARTs). An EBV gene expression pattern similar to that in LCLs is found in EBV-driven lymphoproliferations of the immune compromised host including post-transplant lymphoproliferative disease of organ transplant recipients with sustained iatrogenic T cell suppression. A distinct EBV expression program that closely matches the Latency III program has been reported in a subset of BL (12). By contrast, in the Latency I and Latency II programs the only EBV nuclear antigen (EBNA) gene expressed is EBNA1 *in vivo*. Both programs express also EBER1,-2 and BARTs. Latency I is characteristic of BL. The latency II program, with the additional expression of LMP1 and LMP2, is seen in Hodgkin's disease, nasopharyngeal carcinoma, and T-cell lymphoma (3).

Studies examining B cells isolated from tonsils found distinct EBV gene expression in the various B cell differentiation stages. These studies led to propose a model in which EBV infects naïve B cells in tonsils (13); upon infection, EBV expresses distinct patterns of its latency genes depending upon the distinct B cell differentiation stages, varying from expression of all 10 known EBV latency genes in naïve B cells to complete absence of EBV gene expression (except non-coding EBER) in resting memory B cells. From this it was inferred that EBV, by virtue of expression of its latency genes, provides cell survival signals in naïve B cells. Data from subsequent studies suggested that EBV might expedite the antigen-driven somatic hyper-mutation and selection process of B cells taking place in germinal centers (GC) (14). Nevertheless, the demonstration in patients with primary EBV infection that EBV avoids GC transit and infects directly memory B cells challenged this model (15). In vitro experiments revealed that EBV is able to infect memory B cells (16, 17) besides the well-accepted susceptibility of naïve and GC B cells to EBV. In addition, we recently demonstrated that tonsillar memory B cells are much more susceptible to EBV infection in vitro than those from the peripheral blood, originating from various lymphoid tissues (17). This suggested that tonsillar memory B cells express properties, which render them more susceptible to EBV infection compared to their counterparts from other lymphatic origin. More recently,

we identified beta1 integrin, which is invariably expressed highest by memory B cells from NALT, and the corresponding signal transduction pathway as mediators of increased susceptibility to EBV infection (18).

Propagation of EBV within the host is linked to proliferation of infected B cells which delivers latent EBV to daughter cells or, more rarely, to switching of EBV to lytic infection (19). Switching to lytic EBV is triggered by the differentiation of infected memory B cells to plasma cells; plasma cells appear to be the main factory of virions, which may subsequently infect new B cells (20). Upon reactivation, the EBV immediate-early genes BZLF1 (encoding the protein Zta) and BRLF1 (encoding the protein Rta) are the very first viral lytic genes expressed (21). The EBV gene expression in the lytic cycle follows a temporal and sequential order. Expression of the immediate-early genes is followed by expression of early and late genes. In EBV virology, these terms are used to describe stages of gene expression in reactivation. Reactivation eventually leads to release of EBV particles and death of the host cell (19).

Transmission of EBV to naïve hosts is thought to occur via droplets loaded with virions (22, 23). Thus, lytic replication of EBV best takes place in nasopharyngeal-associated lymphoid tissue (NALT) that will release EBV into the saliva, generating infectious droplets. Epithelial cells seem to act as amplifier of EBV particles derived from B cells to be shed in saliva (23). The NALT acts, thus, as neuralgic point of EBV transmission, i.e., as portal of entry of EBV as well as portal of exit for further transmission (22, 23).

4. TOLL-LIKE RECEPTORS (TLRs) AND DETECTION OF EBV

4.1. Expression of TLRs in B cells

Pattern recognition receptors including the Toll-like-receptors (TLRs) constitute a major pillar of the first line of defense upon encountering a pathogen. Human B cells express high levels of TLR1, 6, 7, 9, and 10 (24-26). TLR expression patterns of B cells, however, can vary depending on their subset, developmental stage, tissue environment, and malignant transformation (27-29). The specific local environment at human body sites seems to shape the TLR repertoire as TLR9 expression and responsiveness following engagement by the ligand is increased in B cells isolated from tonsils when compared to those isolated from peripheral blood (30). Notably, differences in TLR expression in mouse immune cells compared to human counterparts exist. Human (non-malignant) B cells lack TLR4 in contrast to mouse B cells. Importantly, in humans, only plasmacytoid dendritic cells (pDCs) and B cells respond to TLR9 ligands, whereas in mice, B cells, monocytes, and probably all dendritic cell subsets express TLR9 (31, 32). This differential TLR9 expression pattern in turn affects signaling pathways and results in a unique cytokine and chemokine expression profile. Thus, experimental results from mice cannot be one-to-one extrapolated to humans (33, 34).

4.2. Pattern recognition receptors including TLR9 recognize EBV

Pattern recognition receptors including TLRs have been shown to recognize EBV (for excellent reviews see (8, 38) The EBV structural protein gp350 and the EBV dUTPase are detected by TLR2 in monocytes and macrophages (39, 40). Epstein-Barr virus encoded small RNAs (EBERs) released from EBV-infected cells are sensed by TLR3 which is mainly expressed by dendritic cells (41) In B lymphocyte cell lines, EBERs were shown to be recognized by RIG-I (Retinoic acid-inducible gene-I), a cytosolic protein which does not belong to the TLR family (42, 43). Moreover, EBV is a likely natural ligand for TLR9. The genomic DNA of EBV contains abundantly CpG motifs and is probably the most potent immune-stimulating component of EBV particles (8). TLR9 was reported to contribute to the recognition of EBV in primary monocytes and pDCs (44)(45), but not yet in B cells. Thus, in B cells EBV is proven to being detected only by RIG-I. Notably, the murine gammaherpesvirus 68 (MHV68) is sensed in dendritic cells by TLR9 as well (46). MHV68 has been established as a mouse model for the study of gammaherpesvirus pathogenesis (1), because the restricted host range of EBV has limited *in vivo* pathogenesis studies to clinical investigation of the infection. The nucleotide sequence of MHV68 is similar to that of EBV, and, in particular, MHV68 is very useful to study the role of immunity in gammaherpesvirus infection (1). In addition to engaging bacterial and viral DNA, TLR9 from murine dendritic cells or splenocytes also recognizes the malaria pathogen *Plasmodium falciparum*, but whether the ligand is hemozoin, a metabolic product of Plasmodium, or the DNA of Plasmodium itself is still a matter of debate (47, 48). We have recently shown that hemozoin suppresses induction of lytic EBV in the BL cell line Akata in a dose-dependent manner (49). In view of the epidemiological link between *P. falciparum* malaria and the EBV-associated endemic BL (50) the recognition of both pathogens by TLR9 may be of biological or pathogenic relevance.

TLR9 senses its ligand within endosomes. The ligand consists of a DNA-containing unmethylated cytosine-phosphate-guanine (CpG) motif found mainly in bacterial and viral DNA, but only rarely in mammalian DNA (51-53). Synthetic oligonucleotides (ODNs) containing the CpG motif are used to experimentally stimulate TLR9-expressing cells. B cells can be best stimulated with ODN CpG class B (also known as class K) that have multiple CpG motifs and a phosphorothioate backbone (33). In contrast, dendritic cells are best stimulated with CpG class A (also known as class D) that have mixed phosphodiester-phosphorothiaoate backbones and contain a single hexameric purine-pyrimidine-CG-purine-pyrimidine motif flanked by self-complementary bases (54).

The molecular mechanism of TLR9-mediated sensing of EBV DNA remains to be fully revealed. Reports have shown that ODNs without CpG motifs can be a biologically active ligand for TLR9 as well. Furthermore, the DNA backbone sugar 2' deoxyribose rather than the CpG motif might determine TLR9 activation by synthetic

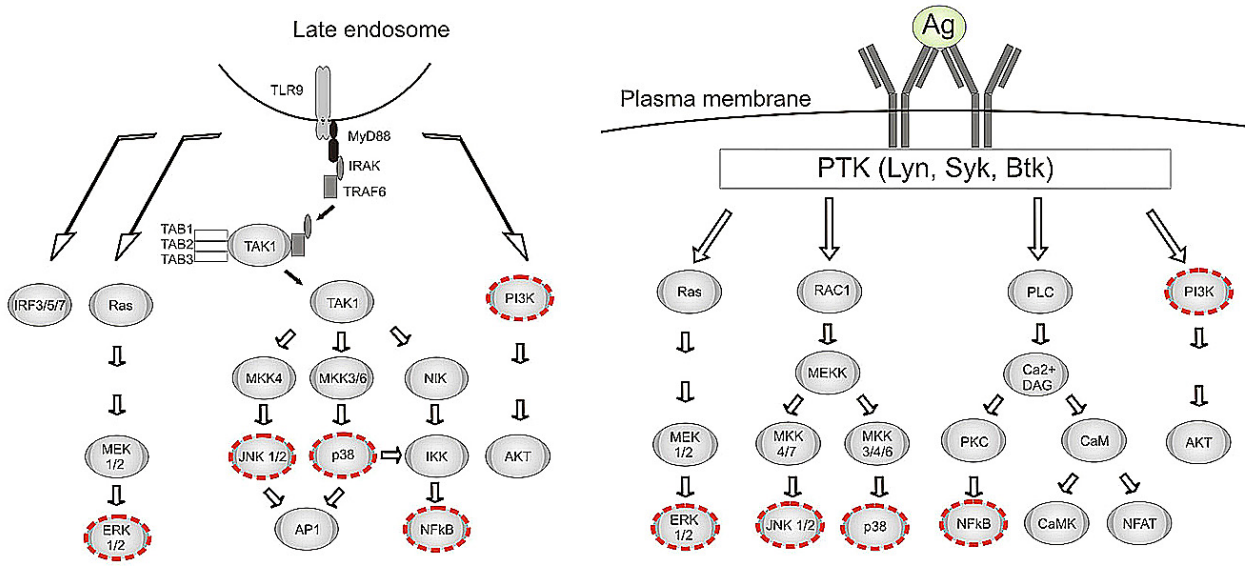

Figure 1. TLR9- and BCR-induced signaling pathways. The main important signaling pathways are shown. Cross-talk between signaling components of the single TLR9 or BCR pathway exists: The PLC-Ca2+/PKC axis can induce MAP Kinases (JNK, p38, ERK); DAG cross-talks to RAS-RAF-ERK; PI3K/AKT cross-talks to IKK-NF-kappaB; the PI3K can induce Ras activation. Possible interactions between TLR9 and BCR signaling might take place at the MAPK and NF-kappaB level (highlighted by red dashed lines), but also downstream of the MAPKs in the nucleus like CREB or ATF-2. Of note, the TLR9-induced signaling pathway to IRFs and Ras-ERK1/2 seems to be less prominent in B-cells, but important in plasmacytoid dendritic cells and mouse macrophages. However, activation of ERK is important in BCR signaling. JNK has a less prominent role in BCR triggering than in TLR9 activation. Abbreviations: Ag, antigen; AP1, activator protein 1; CaMK, calcium-modulin-dependent kinase; DAG, Diacylglycerol; ERK, extracellular signal-regulated kinase; IRF, interferon-regulated factor; IKK, IkappaB kinase; IRAK, IL1R-associated kinase; JNK, c-JUN NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAP kinase; MEKK, MAPK kinase; MyD88, myeloid idfferentiation primary response gene 88; NFAT, nuclear factor of activated T cells; NF-kB, nuclear factor-kappaB; NIK, NF-kappaB inducing kinase; PI3K, Phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PTK, protein tyrosine kinases; TAK1, TGFbeta-activated kinase 1; TLR9, Toll-like receptor 9; TRAF6, TNF receptor-associated factor 6.

DNA (55). Recent studies using natural DNA also suggest that in addition to the CpG content the level of methylation of the motif strongly affects the ability of DNA to trigger TLR9 (56). Thus, TLR9 recognizes not just CpG motifs, but DNA itself with certain structures (37).

The intracellular localization of TLR9 is required for discriminating between self and non-self nucleic acid – the endosomal localization of TLR9 controls its access to different sources of DNA - but not for ligand recognition (57). Moreover, there is a requirement for capsid degradation by lysosomal proteases so that the nucleic acids of viruses become accessible for endosomal TLR9. Purified viral DNA is a poor inducer of TLR9 compared with intact virus (58).

5. TLR9 SIGNALING IN B CELLS

During an infection, bacterial disintegration or cellular invasion via cell-surface receptor-mediated uptake may result in endocytosis of microbial nucleic acids, a process essential for TLR9 engagement (29). Moreover, non-endocytic entry of viruses involving an alternative pathway of virus delivery to the TLR9-containing endosomes can result in TLR9 engagement (8). However, no study has shown this in B cells yet.

Most studies concerning TLR9 signaling have been performed in dendritic cells or macrophages, but not in human B cells. Nevertheless, the known TLR9 signaling components from these studies are considered to be vital also in B cells. After ligand binding within endosomes, TLR9 undergoes conformational changes and recruits MyD88. Signaling of TLR9 in B cells proceeds only through MyD88 (63), which then activates the IL-1R-associated kinase 1 (IRAK1), the TNF-receptor-associated factor 6 (TRAF6), and the TGFbeta-activated kinase 1 (TAK1) pathway (65, 66). TAK1 phosphorylation leads to the activation of NF-kappaB transcription factor (Figure 1). TAK1 also signals through mitogen-activated protein kinases (MAPKs), such as p38 and the c-Jun NH2-terminal kinase (JNK), leading to the activation of activating protein-1 (AP-1) complexes (65). In primary human B cells, these pathways have not been thoroughly investigated. However, upon TLR9 triggering, the activation of the signaling components, e.g. the phosphorylation of p38 and JNK, and activation of NF-kappaB and AP-1 were reported in primary human B cells (67). Thus, NF-kappaB and AP-1 complexes are able to translocate into the cell nucleus where they promote the expression of genes involved in B cell activation, proliferation and production of inflammatory cytokines as a

part of the immune response against pathogens (66). Moreover, activation of CCAAT/enhancer binding protein (C/EBP), cAMP-responsive element-binding protein (CREB) and the Ras - ERK (extracellular signal–regulated kinase) pathway after TLR9 engagement could also be observed (29, 66). Another class of transcription factors, the family of interferon-regulated factors (IRFs, e.g., IRF3, IRF5, IRF7) is activated after TLR engagement, interacts with MyD88 and translocates to the nucleus where they promote the expression of type I interferons (IFNs). Importantly, reports indicate that TLR9-induced ERKs, IRFs and CREBs play less prominent roles in B cells than in macrophages or dentritic cells (own observation, and (67, 68)(66, 69).

Although human B cells are generally considered poor cytokine producers, stimulation with TLR9 ligands results in the secretion of pro-inflammatory cytokines like IL1alpha, IL1beta, IL-6, IL8 and TNFalpha, and to the release of immune regulatory cytokines that might limit the intensity of the inflammatory response, such as IL10 (70-75). Further, endosomal TLR9 stimulation in naïve and transitional B cells induces differentiation in these B cell subsets (76). A proliferative response to TLR9 ligands is predominantly observed in IgM+ memory B cells (27). Moreover, TLR triggering of terminally differentiated plasma cells augments Ig production (28). Thus, the differentiation state of the B cell also defines the possible result of TLR9 activation, most probably through activating distinct signaling pathways and components. Finally, TLR9 plays a role in the induction of T helper 1 (Th1) acquired immune responses (37). .

6. TLR9, EBV AND EPIGENETIC CHANGES

Transcription factors like NF-kappaB, AP1, IRFs and others are crucially important to transduce the signal from the TLR9 to the promoters and thus leading to changes in gene expression. The regulation of activation of NF-kappaB and other transcription factors has been intensively studied and will not be summarized here.

In addition to activation of transcription factors, gene expression can be initiated or modified by the architecture of the chromatin, e.g., DNA and histone modifications. Nuclear DNA is packed into nucleosomes, which consist of a histone octamer core around which DNA (approximately 143bp) is wrapped. The core histones are reversibly modified by acetylation, methylation, ubiquitination, biotinylation and phosphorylation. Modifications occur on the N- and C-terminal tails of the core histones, and more recently analyses of histone modifications by mass spectrometry have revealed several modifications (acetylation and methylation) in the histone fold (77). Among the N-terminal histone tail modifications, acetylation is perhaps the most characterized and has been found associated with actively transcribed regions of chromatin (77).

TLR signaling results in the activation of transcription factors such as AP-1 and NF-kappaB. Histone regulation might be a possibility of how gene expression due to TLR-mediated transcription factor activation can be specifically modified to adapt to the invading organism or to avoid excessive inflammation. First reports described the TLR-induced chromatin remodeling on the IL12p40 promoter in dendritic cells and macrophages indicating that chromatin remodeling is an additional level of TLR signaling specificity (78, 79). Recently, Foster et al. found out that individual host gene promoters can be targeted and modulated in its histone architecture after TLR engagement (80). Repeated triggering of TLRs was shown to repress activation of selected TLR-responsive promoters (TLR tolerance), while other TLR-responsive promoters were not affected (80). Thus, TLRs have multiple ways of inducing or repressing gene expression. However, studies about TLR and histone modifications are very few and were performed using dendritic cells or macrophages, but not B cells.

Importantly, epigenetic changes play a key role in EBV's latent and lytic gene expression as well. Encapsidated EBV contains dublex linear DNA genomes which generally lacks histones. However, upon *de novo* infection of cells, the EBV genome is rapidly chromatized in the nucleus resulting in a closed circular DNA form containing histones (81). Histone modification in the EBV genome is indeed important for regulating EBV gene expression as was shown for the EBV immediate-early gene *BZLF1* (82). Other epigenetic changes in the EBV genome include DNA methylation. Incoming EBV DNA is unmethylated; but as the circularized EBV genome gets more and more chromatized, CpG methylation in the EBV genome proceeds. The core promoter driving *BZLF1* expression is, however, never methylated as it lacks CpG dinucleotides (1). Histone modifications (especially acetylation and phosphorylation) and DNA methylation in the EBV genome play central roles in establishing latency and therefore as well in EBV reactivation (82). We have recently shown that TLR9 affects histone structure on host and viral promoters, e.g. EBV's immediate-early gene *BZLF1* in BL cell lines and thus influences EBV gene expression (49). Thus, various pathogens including EBV or *P. falciparum* might trigger TLR9 leading to pro-inflammatory cytokine production and to suppression of EBV lytic gene expression. TLR9's capability to suppress lytic EBV (and thus hiding in latency) might be beneficial for the virus in a cell which is launching innate immune responses as its avoiding production of viral proteins and virions which might lead to recognition and elimination of EBV. How TLRs modulate chromatin structure of certain promoters and which signaling pathways are required for this modulation needs further investigation. However, Trichostatin A - an Histone-deactylases (HDAC) inhibitor - was shown to reverse the effect of TLR9 engagement on human and viral gene expression (49). Thus, the HDAC inhibitor interfered with the TLR9 action to promote EBV latency, and thus restored EBV lytic gene expression levels. As latency is a requirement for transformation, these findings strengthen the widely accepted fact that HDAC inhibitors have great potential in cancer therapy. The use of

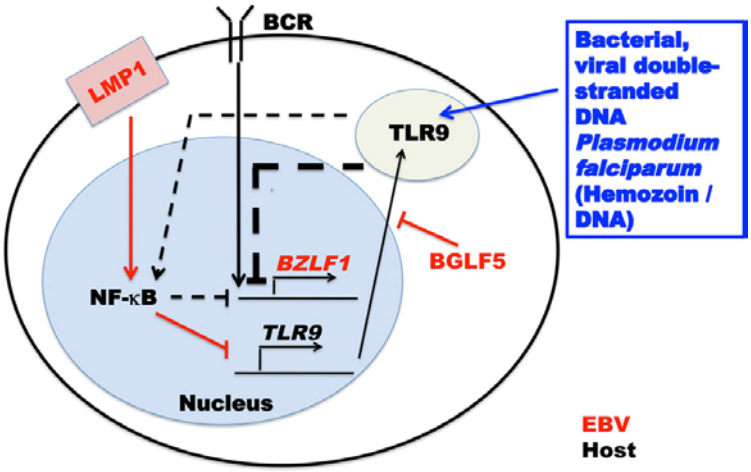

Figure 2. Interactions between EBV and TLR9-mediated signaling. EBV's latent membrane protein 1 (LMP1) inhibits *TLR9* mRNA and protein expression via NF-kappaB activation. The EBV lytic protein BGLF5 degrades TLR9 mRNA and thus TLR9 expression. TLR9 triggering results in NF-kappaB activation that might lead to some inhibition of *BZLF1* expression. Moreover, TLR9 triggering inhibits induction of lytic EBV following BCR cross-linking.

HDAC inhibitors might therefore counteract the detrimental triggering of TLR9 in EBV-associated BL.

7. B CELL RECEPTOR (BCR) AND TLR9 INTERACTION IN THE CONTEXT OF EBV INFECTION

7.1. EBV and BCR-induced signaling pathways

Apart from germline-encoded pattern recognition receptors like TLRs, B cells express clonally rearranged immunoglobulin (Ig) acting as antigen (Ag)-specific receptors on their surface, the so-called B cell receptors (BCRs). They play an important role in the reactivation of EBV (Figure 2). By using cross-linking anti-immunoglobulin G (anti-IgG) antibodies, many of the EBV-positive established BL cell lines react with EBV lytic gene expression and reactivation (19). EBV reactivation in BL cell lines that results from BCR cross-linking with anti-IgG is thought to reflect physiological mechanisms that function when latently infected memory B cells proceed through the germinal center reaction and/or undergo plasma cell differentiation. Thus, the BCR is one of the key elements in analyzing the switch between latent and lytic EBV infection.

When antigen engages the BCR, it will form signaling active microclusters, followed by a conformational change to an 'open form' (83). The first signaling events consist of recruitment of the kinases Lyn and Syk (which phosphorylates the cytoplasmatic signaling domain of the BCR) to the BCR microcluster, and Ca2+ release. The four major signaling pathways activated include phopholipase C (PLC), the Rho family of GTPases like VAV1-3, Ras, and phophatidylinositol-3-kinase (PI3K) (84, 85). They lead to the activation of MAP-kinase signaling cascades and of transcription factors like NF-kappaB, and thus to transcription of a variety of genes depending on the maturation state of the cell (Figure 1). The activated MAP kinases - consisting of extracellular signal-regulated kinase (86), c-Jun NH2-terminus kinase (JNK/SAPK) and p38 MAPK - phosphorylate different sets of transcriptions factors including Elk1 and cMyc by ERK, c-Jun and ATF2 (activating transcription factor 2) by JNK, and ATF2 and MAX by p38 MAPK.

If the B cell is latently infected with EBV, above mentioned activated signaling pathways may eventually lead to the expression of the EBV immediate-early genes BZLF1 and BRLF1, which are transcription factors responsible for the induction of EBV early lytic genes and thus for the initiation of the lytic cascade and ultimately for EBV particle production (19).

7.2. Interaction of BCR and TLR9 affects EBV gene expression

BCR signaling is regulated and fine-tuned by several co-receptors. These include the B cell inhibitory receptor FcγRIIb (recognizes immune complexes) and the stimulatory co-receptor complex CD19/CD21 (recognizes complement coupled antigens). The family of the TLRs seems to be a new player in the field. During an infection, B cells will most probably receive signals from TLRs and BCRs. Investigations how these receptors and their signaling interact are crucial for the understanding of the immune system reacting to a pathogen and, importantly, the development of autoimmune diseases like lupus (90).

Initial reports described the ability of TLR9 signaling to interfere with BCR signaling, leading to synergistic or antagonistic effect depending on the maturation step of the B cell (91). In mature B cells, TLR9 and BCR synergize in B cell proliferation and production of cytokines and Ig production. In immature B cells, TLR9 and BCR synergize with cytokines production (including IL10, IL6, TNFalpha), but TLR9 blocks BCR-mediated growth arrest and apoptosis by interfering with the regulation of c-myc and bcl-xl (68, 92). Moreover, mature memory B cells proliferate and differentiate to Ig-secreting

cells in response to ODN CpG alone, while mature naïve B cells need BCR and TLR9 signaling to achieve the same result. Of note, one has to consider that, the differential expression of TLR9 correlates with its responsiveness to its agonist, ODN CpG (29). Human naïve B cells express most TLRs at low to undetectable levels (28), but BCR triggering rapidly induces the expression of TLR9 (25). Memory B cells express several TLRs at constitutively high levels (28). Thus, TLR9 and BCR can interfere with each other's signaling, but can also interfere with each other's expression.

Importantly, TLR9 was shown to suppress BCR-induced EBV lytic gene expression in BL cell lines. However, the common TLR9 signaling pathways including NF-κB seem not to be solely responsible for the TLR9-induced suppression of the EBV lytic immediate-early gene *BZLF1* (Figure 2). Although TLR9 and MyD88 were responsible for the suppression of lytic EBV, other TLR9 signaling components like p38 or JNK could not held responsible for the suppressive effect on lytic EBV. The BCR pathway, which was activated to induce EBV lytic gene expression in BL cells, also involves common signaling components like p38 and JNK (Figure 1). However, the TLR9 pathway seemed not to interfere or block the BCR pathway. The underlying mechanism seems to be a TLR9-induced modulation of the histone structure of the *BZLF1* promoter (49). Moreover, BCR-induced cytokine expression is synergistically enhanced (hIL10) or suppressed (IL1beta) upon additional TLR9 engagement in these cell lines ((49) and own unpublished observations). These distinct results of BCR and TLR9 engagement in EBV and cytokine expressions show the complexity of the underlying signaling pathways and their interactions.

Little is known on the molecular events integrating TLR signaling into the classical BCR-mediated signaling cascades. Collectively, interactions of signaling pathways of BCR and TLR9 at the level of MAPK and NF-kappaB have been observed (Figure 1). Interestingly, in mice B cells, TLR9 and BCR signaling appear to synergize in autophagosome-like structures where both receptors relocate, and thus hyperactivate signaling through to p38 and JNK (93). Nevertheless, one has to consider that different sets of MAPK and NF-kappaB subunits might be involved. This could explain the diverse outcome depending on the maturation state of the B cell. Activation of ERK is important in BCR signaling but less important in TLR9 signaling in B cells (67); and JNK has a less prominent role in BCR triggering than in TLR9 activation (68). But again, these observations might not hold true in every developmental B cell stage.

8. THE INTERACTION BETWEEN TLR9 AND EBV IMPACTS ON LATENCY AND TRANSFORMATION

8.1. EBV manipulates TLR9 functionality for its survival and latency

TLRs are capable to recognize EBV and initiate an appropriate innate immune response against it. A rapid detection of EBV may be essential to limit the spread of the virus and may contribute to control outgrowth of latently EBV-infected B cells. TLR9-mediated secretion of antiviral cytokines must therefore be regarded of great importance. Nevertheless, EBV exhibits an extremely successfully spreading among man, as it persistently infects more than 95% of the world's population. Thus, EBV has developed strategies to avoid immune detection and manipulate the immune system by altering several cellular functions (94). At least three experimental studies suggest that EBV uses mechanisms to suppress the host innate immune responses (95-97). Two studies link the EBV effect to its latent form of infection. Fathallah et al. (95) disclosed that LMP1 strongly inhibits TLR9 mRNA and protein expression in primary human B cells (Figure 2) and thus inhibits functionality of TLR9 in IL6, TNFalpha and IgG production. Younesi et al. (97) observed that latent EBV infection significantly inhibited TLR9 triggering-induced proliferative human B cell responses. The authors claim that this was not due to down-regulation of TLR9 expression. The mechanism, however, was not elucidated. Remarkably, van Gent et al. (96) found that the lytic phase EBV protein BGLF5 contributes to down-regulation of TLR9 (Figure 2) during the productive phase of infection through RNA degradation in human B cells.

Above quoted studies suggest that EBV proteins impact on TLR9 expression or functionality in certain states of latent and lytic infection, or both. The resulting benefits are reduction of TLR9-induced host immune responses against EBV infection or reactivation and enabling survival of the virus, respectively. Moreover, to make this matter more complex, TLR9 stimulation itself also seems to modulate TLR9 expression. Triggering of TLR9 might lead to an either decreased or increased expression of TLR9 depending on the cell type (monocytes, pDCs, or B cells) and the stimulus (ODN CpG, untreated or UV-inactivated EBV) (26, 45, 98). Further studies are needed to gain more detailed insights on the EBV gene expression impacting on TLR9 expression and signaling.

Importantly, it was also reported for the case of TLR7, that EBV hijacks the TLR7 pathway in order to enhance B cell proliferation, establish latent infection and modify pathways to regulate the activity of antiviral proteins like IRF5 (98). Similar to TLR7, EBV might be able to use TLR9 for its advantage in order to establish latency in the memory B cell pool via promoting latency, driving B cell proliferation, or both. In order for a naïve B cell to get maximally activated and thus transit to a memory B cell, three signals – BCR (mimicked by EBV's LMP2A), CD40 (mimicked by EBV's LMP1) and TLR stimulation (by EBV or other pathogens) are required (99, 100). Thus, EBV can stimulate its target cell to enter the memory B cell pool to establish long-term latency by using TLR9 (or TLR7). On the other hand, both polyclonal TLR and antigen triggering of the antigen-selected and EBV-infected memory B cell may contribute to propagation of latent EBV within the infected host and - by mediating differentiation to

plasma cells - also of lytic EBV that may be transmitted to susceptible hosts

8.2. TLR9 impacts on EBV's gene expression and transformation capability

TLR9 is important to launch innate immune responses against pathogens, for example via expression of pro-inflammatory cytokines. Using the MHV68 model, TLR9 deficiency was found to impair the host response to MHV68 infection, as reduced cytokine expression by dendritic cells and higher MHV68 viral load during both lytic and latent infection was observed in mice lacking TLR9 (46). MHV68 seems to use TLR signaling to condition the target cell for the establishment of viral latency (46). Controversially, ex vivo TLR9 stimulation of latently infected splenocytes using CpG DNA resulted in early B-cell activation, B-cell proliferation and a significant increase in the frequency of latently infected cells reactivating MHV68 (101). With respect to gammaherpesviruses, it has been demonstrated that TLR9 exhibits essential roles during the elimination of acute MHV68 infection and the control of latent viral load following infection through the intraperitoneal route, but not after intranasal infection. Therefore, TLR9 seems to be in particular important for the host immune response to pathogens that reach locations where pDCs are abundantly present (such as the lymphoid organs and blood). Indeed, Fiola et al. (45) recently reported that TLR9 contributes to the recognition of EBV by primary monocytes and pDCs.

Notably, patients with an IL1 receptor-associated kinase 4 (IRAK4) mutation, which abolishes the production of IFNs following stimulation of TLR7, TLR8 and TLR9, do not experience increased susceptibility to herpesviruses infections (102). This suggests that redundancy - at least partly - to TLR9 function may exist.

In the human system, triggering of TLR9 was employed to more efficiently transform B cells following in vitro EBV infection (103). Furthermore, Iskra et al. (100) found that TLR9 triggering resulted in increased EBV-driven B cell proliferation and transformation. Thus, TLR9 triggering may impact on either enhanced expression of EBV latent transforming genes or on the suppression of EBV lytic genes. We demonstrated that immune activation via TLR9 triggering results in suppression of lytic EBV during *de novo in vitro* infection of cord blood B cells (104) and upon BCR-induced reactivation of EBV in chronically infected BL cell lines (49). These findings strongly suggest that TLR9 triggering leads to reinforcement of latent EBV following suppression of lytic EBV and thus may provide an important stimulus towards malignant B cell lymphoproliferation. Importantly, this might explain the development and rapid progression of endemic BL in children co-infected with EBV and the malaria parasite *P. falciparum*. The malaria parasite's hemozoin was shown to trigger TLR9 (47, 48) and suppress EBV lytic gene expression (49), which might lead to B cell transformation due to enforced EBV latent gene expression. Thus, children of the Sub-Sahara African region may develop endemic BL and rapidly progress because of repeated co-infection with *P. falciparum* which triggers innate immune receptors like TLR9, and in turn affect EBV's lytic and latent gene expression promoting B cell transformation. Controversially, other reports show that the *P. falciparum* antigen CDR1alpha is able to induce EBV reactivation from latency in BL cell lines (49), which results in a high viral load and might explain the fact that children in malaria-endemic areas have an elevated EBV load. Moreover, the polyclonal B cell activating capacity of *P. falciparum* might increase proliferation of EBV-positive cells, which may promote the emergence of BL (49). Studies about the interaction of EBV and *P. falciparum* are nevertheless rare, but indispensible for understanding the cause of BL development.

9. CONCLUSIONS AND PERSPECTIVES

EBV infects B cells from the nasopharynx-associated lymphoid tissue (NALT) and subsequently establishes long-term latency in the memory B cell pool. We have elucidated that TLR9 plays a complex role in EBV infection. On the one hand, TLR9 functions to induce pro-inflammatory cytokine expression against pathogens and is therefore be hindered by EBV in order to avoid its own extinction. On the other hand, EBV may use TLR9 to establish EBV latency via gaining access to the memory B cell pool. Although both scenarios may be true, a lot more experiments have to be performed to clarify the role of TLR9 in EBV infection.

EBV is the etiologic agent of infectious mononucleosis and is associated with a variety of cancers including endemic BL. Although EBV exhibits a unique potential to transform B cells in vitro, additional factors seem to be required for the development of EBV-associated malignancies. These factors include a compromised immune system (iatrogenic immune suppression as in organs transplants or immune deficiency as in advanced HIV infection) or as in endemic BL a co-infection with the malaria parasite *P. falciparum*.

The role of TLR9 in tumor development is complex. On the one hand, TLR ligands have a great potential in cancer therapy due to their ability of raising or enhancing immune responses against malignant cells. Among all TLRs, TLR9 ligands are the most intensively studied as they can be rather simply manufactured, administrated through various drug routes, and have very low side effects but exhibit very strong adjuvant effects that polarize helper T cell responses to Th1 (37, 66). In contrast to that, TLR stimulation due to excessive pathogen encounter might lead to high inflammation process over a long period (105). Chronic infection and inflammation are considered two of the most important epigenetic and environmental factors contributing to tumor genesis and tumor progression (106, 107). Chiron and colleagues (29) proposed the model that repeated polyclonal activation of leukemic B cells by microbial molecules during natural infection or inflammation is the initial step in the oncogenic process that lowers the threshold for outgrowth of malignant cell clones.

Evaluating if TLR9 has a positive or negative effect in tumor onset and progression has to be done on the cell type, maturation of the cell, whether an infection is

present, and other factors separately. In the case of EBV-associated malignancies like BL, TLR9 triggering seems to have a detrimental impact in cells as it promotes EBV-induced B cell proliferation and transformation. In endemic BL, this TLR9 trigger may be the repeated infection of children with *P. falciparum*. The interaction of the two pathogens EBV and *P. falciparum* have long been considered as the two main factors causing BL. Recent publications about *P. faciparum's* capability to affect EBV gene expression are the first steps to shed light on this matter. Importantly, HDAC inhibitors were shown to counteract TLR9's impact on EBV gene expression (49), which might yield in new ideas about therapies.

Analyzing the TLR9-activated signaling pathways – sub-pathways, strength and duration of activation – may give important information whether TLRs might have beneficial or detrimental effects on EBV infection and tumor development in each specific case. In this matter TLR triggering gives a 'signaling fingerprint', which has to be carefully examined as it might contain the key for the understanding of the diverse outcome of TLR engagement.

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