Innate immune responses against Epstein Barr virus infection

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ABSTRACT
EBV persists life-long in >95% of the human adult population. Whereas it is perfectly immune-controlled in most infected individuals, a minority develops EBV-associated diseases, primarily malignancies of B cell and epithelial cell origin. In recent years, it has become apparent that the course of primary infection determines part of the risk to develop EBV-associated diseases. Particularly, the primary symptomatic EBV infection or IM, which is caused by exaggerated T cell responses, resulting in EBV-induced lymphocytosis, predisposes for EBV-associated diseases. The role of innate immunity in the development of IM remains unknown. Therefore, it is important to understand how the innate immune response to this virus differs between symptomatic and asymptomatic primary EBV infection. Furthermore, the efficiency of innate immune compartments might determine the outcome of primary infection and could explain why some individuals are susceptible to IM. We will discuss these aspects in this review with a focus on intrinsic immunity in EBV-infected B cells, as well as innate immune responses by DCs and NK cells, which constitute promising immune compartments for the understanding of early immune control against EBV and potential targets for EBV-specific immunotherapies.

EBV INFECTION AND ASSOCIATED DISEASES

EBV belongs to the γ subfamily of herpesviruses and was first identified in cultured lymphoma cells derived from an African BL patient [1]. The global seroprevalence is very high, suggesting that 95% of adults worldwide are infected with EBV [2]. In contrast to diseases that are associated with persistent infection [3], primary infection with EBV usually occurs in childhood and remains asymptomatic during the acute phase, whereas in adolescents and adults, primary infection is symptomatic in >70% of cases [4] and presents as IM. IM is a self-limiting disease with characteristic clinical symptoms of pharyngitis, fever, fatigue, cervical lymphadenopathy, hepatosplenomegaly, and T cell lymphocytosis in the peripheral blood. Although EBV persists in infected individuals, both after asymptomatic primary infection and IM, it is usually immune-controlled but without viral clearance for the rest of the host’s life [5]. However, during the first 5 years after resolving IM, the risk for EBV-positive Hodgkin lymphoma is increased up to five times [6, 7], and a history of IM increases the risk to develop multiple sclerosis by twofold [8, 9]. In rare cases, primary infection is fatal and is the most common cause of infection-associated HLH [10]. Therefore, symptomatic primary EBV infection predisposes for diseases associated with this virus.

EBV is associated with several malignancies of B cell origin, the primary target of EBV. Almost 100% of endemic BL is positive for EBV. Because of the invariant presence of c-myc translocations in BL, however, the exact role of EBV in the transforming process is still not defined clearly [11, 12]. Similarly, classical Hodgkin lymphoma, most frequently of the mixed cellularity subtype, is associated with EBV in 40% of cases. However, as in BL, the culprit for tumorigenesis might not be EBV alone, as this disease is multifactorial [13]. The impact of the host immune response on the control of EBV becomes evident in immunosuppressed patients and patients with primary immunodeficiencies. PTLDs comprise a variety of lymphoid disorders, ranging from polymorphic lymphoid hyperplasia to malignant lymphoma, and are associated mostly with EBV infection [14]. The risk for EBV-associated PTLDs correlates with the degree of immunosuppression, such that the highest incidence is found in solid organ transplant patients with the most intense immunosuppressive regimes. Moreover, virtually all AIDS-related CNS lymphomas and leiomyosarcomas are associated with EBV [15, 16]. Patients suffering from XLP disease are exclusively fragile to primary EBV infection.

Abbreviations: BGLF5=Bam H1 G leftward open reading frame 5, BL=Burkitt’s lymphoma, B2LFL=Bam H1 Z leftward open reading frame 1, cDC=conventional DC, EBER=EBV-encoded small noncoding nuclear RNA, HLH=hemophagocytic lymphohistiocytosis, IM=infectious mononucleosis, KIR=killer cell Ig-like receptor, LMP-1=latent membrane protein 1, pDC=plasmacytoid DC, PTLD=post-transplant lymphoproliferative disorder, XLP=X-linked lymphoproliferative

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but not any other pathogens and develop fulminant and sometimes lethal IM, as well as B cell lymphomas, after severe primary EBV infection. The disease-causing mutations lead to an impairment of T and NK cells to target EBV-infected cells [17–20]. In rare cases, EBV infects T or NK cells, leading to the development of T cell [21] and NK cell lymphomas [22]. EBV is also associated with nonlymphoid malignancies, such as nasopharyngeal carcinoma [23] and EBV-positive gastric carcinoma [24]. In both entities, EBV infection seems to occur before malignant transformation, as EBV is monoclonal in tumor cells [25, 26]. The exact contribution of EBV in this plethora of associated malignancies is still ill-defined, but the presence of EBV gene products capable of inducing cellular transformation on their own, infection of every tumor cell, and epidemiological evidence supports the notion of a pathogenic role for EBV.

**EBV-ASSOCIATED MOLECULAR PATTERNS THAT ELICIT IMMUNE ACTIVATION**

The formidable immune control that keeps EBV in check for >50 years in most infected individuals requires priming of protective T cell responses [5]. For this to occur, EBV-associated molecular patterns need to be recognized by the immune system, probably directly by APCs for optimal T cell priming [27]. APCs carry a number of PRRs, including TLRs, nucleotide-binding oligomerization-like receptors, retinoic acid-inducible gene-like receptors, and C-type lectin-like receptors [28]. Of these, TLR3 and TLR9 have been implicated in sensing of EBV and might complement each other for the recognition of this virus by cDCs and pDCs, respectively (Fig. 1). In addition, monocytes, upon detection of EBV via TLR2, secrete cytokines and chemokines [29–31], whereas TLR7 signaling has been reported to be modulated by EBV [32, 33]. Notably, B cells, the primary target of EBV, also express TLR3 and TLR9 [34]. In contrast to mice, TLR3 and TLR9 are on separate DC subsets in humans. TLR3 is expressed primarily by cDCs, whereas TLR9 is restricted to pDCs [35]. Therefore, both major human DC subsets could detect different cues during EBV infection, whereas the primary target cell of EBV is furnished with both receptors.

TLR3 recognizes dsRNA in endosomal compartments of human cDCs. EBERs have been described to form stem-loop structures, and the resulting RNA was reported to bind to TLR3 [36]. EBERs were found to be released from EBV-infected cells in complex with the La protein, a frequent autoantigen in systemic lupus erythematosus and Sjögren’s syndrome. Sera of patients with IM, chronic active EBV infection, and EBV-associated HLH, diseases whose pathology might be mainly mediated by the release of large amounts of proinflammatory cytokines, were demonstrated to contain high concentrations of EBERs. Indeed, these sera elicited TLR3-dependent cytokine production and DC maturation in vitro. These data suggest that dsRNA structures of EBERs might stimulate cDCs via TLR3 to allow them to prime protective EBV-specific T cell responses.

In contrast, it is much less clear to which extend human pDCs can initiate T cell responses. However, they seem to be able to recognize EBV DNA via the endosomal receptor TLR9 [31, 37], which recognizes unmethylated CpG DNA motifs. Linear dsDNA in EBV particles is unmethylated, but circularizes after cellular infection and then gets hypermethylated successively [38], suggesting that especially noncell-associated virus-encapsulated EBV DNA should be immunostimulatory via TLR9. In addition to pDCs, TLR9 is expressed prominently in human B cells, and its stimulation seems to facilitate B cell transformation by EBV [39]. Once infection, however, has occurred, EBV seems to down-regulate TLR9-mediated signaling [33]. The LMP-1 of EBV down-regulates TLR9 signaling during latent infection [40], whereas the lytic EBV antigen BGLF5 down-regulates TLR9 during lytic replication [41] (Fig. 2). In addition, even UV-inactivated EBV has been reported to down-regulate TLR9 [32]. In this fashion, EBV possibly protects itself from cell death-inducing and lytic reactivation-inhibiting functions of TLR9 signaling [42, 43]. Thus, EBV, possibly primarily the virus-encapsulated DNA, can be sensed by TLR9 in

![Figure 1. Innate immune recognition via identified PRR ligands and innate restriction of EBV. EBV seems to activate pDCs and cDCs with its unmethylated viral DNA and EBER, respectively. These pathogen-associated patterns engage TLR9 on pDCs and TLR3 on cDCs. NK cells, which can be activated by pDCs and cDCs to produce IFN-γ in response mainly to IL-12 and increase cytotoxicity upon encountering IFN-α/β, can restrict EBV infection. NK cells prevent B cell transformation via IFN-γ and kill lytically EBV-replicating cells.](image-url)
SCID mice, this IFN-α sterilizes to down-regulating TLR9 levels by degrading RNA.

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EBV INFECTION

DCs IN INNATE IMMUNE CONTROL OF EBV INFECTION

This TLR-mediated recognition seems to alarm the immune system to EBV infection and matures DCs to form a first barrier against it. Particularly, pDCs have been described as superior sources of the antiviral type I IFN (IFN-α/β) cytokines [44]. In a PBMC transfer model into immunocompromised SCID mice, this IFN-α production by pDCs seemed to be necessary to control EBV infection [37]. PBMCs depleted for pDCs supported disseminated EBV infection, whereas pDC supplementation of PBMCs was able to control infection better after EBV challenge in vivo. The pDC activation by EBV was TLR9-mediated in this system and led to enhanced NK and T cell activation (Fig. 1). As the PBMCs in this model were derived from EBV-seropositive individuals, however, pDCs most likely only supported secondary T cell responses that had been primed in the PBMC donor. It is likely that these secondary responses supported the observed, lasting immune control over EBV infection for 1–2 months, as IFN-α has only been described to block EBV infection of B cells for the first 24 h after infection [45]. The TLR9-mediated detection of EBV by pDCs leading to IFN-α production has been confirmed in independent studies [31, 46]. Thus, pDCs produce the antiviral cytokine IFN-α in response to EBV infection, which restricts the virus initially as well as stimulates other innate immune cells and promotes the development of adaptive immune responses.

These adaptive, particularly T cell-mediated immune responses are, however, most likely not primed by pDCs and also not by EBV-infected B cells [47, 48]. In contrast, inflammatory DCs, modeled in humans by differentiation from monocytes, are able to prime EBV-specific T cell responses, which in turn, can control B cell transformation by EBV in vitro [47]. To fulfill this priming function, monocyte-derived DCs are most likely activated by EBV via TLR2 or TLR3 [31, 56]. However, only TLR3-mediated DC maturation has been reported to lead to the up-regulation of costimulatory molecules and antigen presentation [36], whereas TLR2-mediated monocyte stimulation was shown to lead to the production of MCP1 and the immune-suppressive cytokine IL-10 [31]. In addition, the molecular identity of a TLR2 agonist in EBV is still unknown.

Thus, cDCs or inflammatory DCs are probably involved in the priming of protective T cell responses against EBV.

The major human DC subsets—cDCs, inflammatory, and pDCs—are activated, most likely, by EBV after uptake of viral particles or fragments of EBV-infected B cells. Indeed, human monocyte-derived DCs can cross-present EBV-infected B cell fragments to stimulate CD4+ and CD8+ T cells [49, 50]. Moreover, pDCs get activated by purified viral DNA [31]. However, the possibility still exists that a small subset of inflammatory DCs and pDCs is also infected directly by EBV. Along these lines, entry of the virus and access of the viral DNA to the nucleus have been, at least, demonstrated for monocyte-derived DCs and pDCs [46, 51]. However, viral antigen expression has, so far, not been demonstrated convincingly in DCs. Thus, DCs detect, most likely, virus particles directly or products of EBV-infected cells to mount innate immune responses and initiate adaptive immune control of EBV.

NK CELLS IN EBV RESTRICTION

NK cells are known to be important players involved in immune control of cancer and virus-infected cells [52]. In the case of EBV, observational data in humans support a role for human NK cells during innate immune responses to this virus. During symptomatic, primary EBV infection, IM, numbers, and frequency of NK cells in peripheral blood are increased [4, 53–56]. However, one cohort study reported a direct correlation of NK cell count and blood EBV DNA load [4], whereas another smaller study showed an inverse correlation of NK cell frequency and blood EBV DNA load, as well as an inverse correlation of NK cell count and severity of symptoms [55], suggestive of a direct, beneficial contribution by human NK cells in the latter report. However, during viral infections, distinct, terminally differentiated NKG2C+KIR+ NK cell subsets have been found to be expanded. This was observed during acute

Figure 2. Known interactions between EBV and innate immunity elements in B cells. The published, studied interactions between EBV and innate immunity elements in B cells are virtually limited to TLR9. The unmethylated DNA of EBV triggers TLR9, which suppresses lytic reactivation of EBV by suppressing transcription of the master regulatory lytic gene of EBV, BZLF1, and activates NF-κB, which also suppresses BZLF1 transcription but to a lower extent, whereas triggering the BCR activates BZLF1 and switches infection to the lytic cycle. Conversely, LMP-1 of EBV suppresses transcription of the TLR9 gene via NF-κB activation, and the lytic-phase of EBV protein, BGLF5, contributes to down-regulating TLR9 levels by degrading RNA. pDCs and B cells, but the virus protects itself from innate immune signaling via this receptor by down-regulating it during latent and lytic infection.
and persistent infection with human CMV, hantavirus, or chikungunya virus [57–59]. Therefore, interrogating the bulk NK cell response might not necessarily identify the potentially protective impact of these cells during primary EBV infection.

The antitumoral role of NK cells was highlighted by a recent report of a patient with selective NK cell deficiency, who developed a rare EBV-positive smooth muscle tumor [60]. In another case report, EBV-associated lymphoproliferative disease occurred in a patient with NK cell deficiency [61]. On the molecular level of pathogenesis, mechanistic insight was gained from examining defective immune responses in XLP disease, characterized by extreme susceptibility to EBV [62–64]: blockade of the surface molecules 2B4 and NK-T-B antigen (NTB-A), expressed on normal NK cells and CD8+ T cells, was shown to decrease NK cell cytotoxicity to EBV-positive target cells [17, 18], and both molecules act as inhibitory receptors rather than activating in XLP patients, as a result of mutations in the gene encoding the adaptor protein SLAM-associated protein [65], such that NK and CD8+ T cells are no more able to lyse EBV-infected B cells or respond specifically by cytokine production [17–20]. Furthermore, natural cytotoxicity receptors (i.e., Nkp30, Nkp44, and Nkp46) and NKGD2D, all activating NK cell receptors, have been described to be involved in cytotoxic recognition of EBV-positive cell lines [66]. The activating KIR, KIR2DS1, might also play a part in NK cell recognition of EBV-infected cells, as only EBV, but not other herpes viruses, up-regulate ligands for KIR2DS1 upon infection, and the blocking of this receptor diminishes lysis of EBV-positive target cells to some extent [67]. Interestingly, lytic EBV infection compared with latent infection renders target cells more susceptible to NK cell killing (Fig. 1), probably as a result of down-regulation of MHC-I molecules and increased expression of UL16-binding protein 1 and CD112, ligands for the activating receptors NKGD2D and DNAx accessory molecule-1 (DNAM-1), respectively [68]. Finally, NK cells might function tissue-specifically, as it has been shown that tonsillar NK cells restrict B cell transformation more efficiently than peripheral blood NK cells. This process depends on IFN-γ production by NK cells after their activation by IL-12, produced by DCs [69]. In summary, there is growing evidence that human NK cells are involved in the immune response against EBV in vivo. However, the receptor-ligand interactions that are important for NK cell-mediated immune control of EBV and which infection program is restricted by NK cells in vivo still need to be better defined.

CONCLUSIONS AND OUTLOOK

EBV is one of the examples of a persistent human pathogen that can be controlled by the immune system for extended time periods, suggesting that the respective protective immune responses have been primed to cope optimally with this life-long challenge. Thus, this herpesvirus can teach us how protective immunity has to be shaped and which innate immune compartments must be engaged to achieve this lasting immune control. This knowledge might lead us to the development of novel vaccine adjuvants that stimulate innate immune responses and therefore, promote long-lasting protection against other chronic infectious diseases. Along these lines, we have learned that EBV probably provides different PAMPs that engage pDCs and cDCs for optimal T cell priming. Furthermore, innate lymphocyte compartments, such as NK cells, might have been selected during evolution to restrict the early phases of EBV infection to gain time for the adaptive immune response to build its comprehensive immune control. Finally, EBV has hijacked part of the intrinsic immune recognition mechanisms in B cells to improve infection and regulate reactivation. However, the fact that IM patients already exhibit strong adaptive immune responses when they seek medical care complicates the study of early events during acute EBV infection. Thus, to learn more about EBV-specific innate immunity, experimental systems that allow the dissection of human immune responses in vivo have to be developed further. With the advent of humanized mouse models that support reconstitution of human immune system compartments, such systems become available, which will not only allow dissection of innate immune responses to EBV in more detail but also provide a platform to test vaccine candidates, whose protective efficiency can then be challenged in vivo.

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DISCLOSURES

The authors declare no conflict of interest with the discussed topics.

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