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Epstein–Barr Virus-Specific Immune Control by Innate Lymphocytes

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Epstein–Barr virus (EBV) is a potent B cell transforming pathogen in humans. In most persistently EBV-infected individuals, potent cytotoxic lymphocyte responses prevent EBV-associated pathologies. In addition to comprehensive adaptive T cell responses, several innate lymphocyte populations seem to target different stages of EBV infection and are compromised in primary immunodeficiencies that render individuals susceptible to symptomatic EBV infection. In this mini-review, I will highlight the functions of natural killer, γδ T cells, and natural killer T cells during innate immune responses to EBV. These innate lymphocyte populations seem to restrict both lytic replication and transforming latent EBV antigen expression. The mechanisms underlying the recognition of these different EBV infection programs by the respective innate lymphocytes are just starting to become unraveled, but will provide immunotherapeutic strategies to target pathologies that are associated with the different EBV infection programs.

Keywords: natural killer cells, natural killer T cells, Vγ9Vδ2 T cells, lytic replication, infectious mononucleosis, NKG2D, CD27/CD70

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INTRODUCTION ON INNATE LYMPHOCYTES

Epstein–Barr virus (EBV) is a common human γ-herpesvirus that persistently infects more than 90% of the human adult population. At the same time, it was the first human candidate tumor virus that was discovered (1, 2) and remains to date the only human pathogen that can readily transform human B cells into immortal continuously growing lymphoblastoid cell lines (LCLs) (3). Even so EBV contributes with 1–2% to the overall tumor burden in humans (4), the majority of infected individuals carry EBV for life without symptoms. This peaceful coexistence is thought to be maintained by cytotoxic lymphocytes, which massively expand during symptomatic primary EBV infection, called infectious mononucleosis (IM), can be used to treat some EBV-associated malignancies and are affected by primary or secondary immunodeficiencies that predispose for EBV-driven pathologies, such as human immunodeficiency virus-associated lymphomas (5–7).

Among these cytotoxic lymphocytes, adaptive $CD8^+$ T cell responses to EBV have been best characterized and single peptide epitope specificities against early lytic EBV antigens constitute in some individuals up to 40% of the massively expanded $CD8^+$ T cell compartment during IM (8). Much less is known about innate cytotoxic lymphocyte compartments during EBV infection, including natural killer (NK), natural killer T (NKT), and γδ T cells. Nevertheless, they can utilize the same eomesodermin-dependent perforin, granzymes, and death receptor ligands to eliminate EBV-infected cells by cytotoxicity (9). Furthermore, they exist at much higher frequencies than individual $CD8^+$ T cell clones at sites of primary EBV infection, like tonsils (more than 10^{10} more frequent), and therefore can more rapidly respond to pathogen encounter, ensuring the survival of the infected individual until specific T cells have been clonally expanded. However, their target

cell recognition is not directed against EBV protein-derived peptides presented on major histocompatibility complex (MHC) molecules, but instead they recognize infected targets with their germ line encoded receptors or invariant T cell receptors. Activation of innate lymphocytes depend on loss of MHC class I molecules from the surface, stress induced ligand upregulation, glycolipid presentation on non-classical MHC class I molecules, or mevalonate metabolite recognition in the context of butyrophilin (BTN) family members (10–14). As I will discuss below, these different target recognition mechanisms seem to be used to target different stages of EBV infection, thereby achieving a similarly comprehensive immune control over all EBV infection programs as T cells that target antigens of the different EBV life cycles. Thereby, primary immunodeficiencies that affect NK, NKT, or γδ T cells might manifest with different EBV-associated pathologies. A better understanding of which EBV pathology might be targeted by which innate lymphocyte compartment might enable us to utilize these innate cytotoxic lymphocytes in addition to classical T cells for respective immunotherapies.

NK CELLS IN THE PREVENTION OF SYMPTOMATIC PRIMARY EBV INFECTION

Natural killer cells are the preeminent cytotoxic innate lymphocytes, which have been originally described for their spontaneous cytotoxicity against infected and tumor targets (15–17). In particular, deficiencies in NK cells predispose in humans for herpesvirus-driven pathologies (18). It was indeed described early on that NK cells also expand during IM (19–22). IM symptoms are thought to be caused by the associated lymphocytosis of $CD8^+$ T cells, which primarily recognize lytic EBV antigens that are expressed during infectious virus production (23). Indeed, NK cells also preferentially recognize lytically EBV-replicating cells (22, 24, 25). Depletion of NK cells in mice with reconstituted human immune system components (HIS mice) increases viral loads and $CD8^+$ T cell lymphocytosis only for wild-type (wt), but not lytic EBV replication incompetent BZLF1-deficient EBV (25). The respective NK cell-depleted and wt EBV-infected HIS mice also develop more EBV-associated B cell lymphomas and need to be sacrificed due to weight loss 6 weeks after infection (25). HIS mice share an early differentiated NK cell compartment with newborns and young children (26). The respective $NKG2A^+$killer immunoglobulin-like receptor $(KIR)^-$ NK cells preferentially expand during IM and recognize lytically EBV-replicating cells (21, 22). Interestingly, these early differentiated NK cells are continuously lost during the first decade of life and get successively replaced by KIR^+ NK cell accumulation (22, 27). This coincides with an increased risk to develop IM when primary infection is delayed into adolescence (5). Recognition of lytic EBV replication might be mediated by the downregulation of MHC class I molecules and upregulation of NKG2D and DNAM-1 ligands on lytically EBV-replicating B cells (24, 28), tilting the balance of inhibitory, and activating NK cell receptor signaling toward activation. In contrast, EBV transformed B cells with the expression of all latent EBV antigens (LCLs) are only efficiently recognized by NK cells in the allogeneic MHC class I mismatched setting. This allows the recruitment of KIR^+ NK cells to the response and can be harnessed in mixed MHC class I mismatched human immune system reconstitution from two hematopoietic progenitor cell donors in HIS mice (29). Although NK cells in these mixed reconstituted HIS mice have a decreased cytotoxicity against MHC class I negative target cells and are therefore less licensed, they control EBV infection better by NK cells (29). This results presumably from NK cell recognition of the MHC class I mismatched EBV-infected B cells, recruiting KIR^+ NK cells to the innate immune response to EBV. Such allorecognition is currently being harnessed for NK cell-dependent immunotherapies of acute myeloid leukemias (30), but could also be harnessed against persistent infections that reactivate during bone marrow transplantation and home to the hematopoietic lineage. Thus, NK cells preferentially target lytic EBV replication, but might be therapeutically beneficial to target also other stages of EBV infection in the allogeneic setting.

γδ T CELLS AND THEIR RESTRICTION OF EBV LATENCY

Natural killer cells are by far not the only cytotoxic innate lymphocytes that react to EBV infection. In a subset of EBV-positive children (25–50%), Vγ9Vδ2 T cells are also expanded (31). These human T cells with an invariant γδ T cell receptor do not exist in mice and recognize pyrophosphate-containing molecules that are generated in the mevalonate metabolism (32). Interestingly, Vγ9Vδ2 T cell recognition of these phosphoantigens (pAgs) depends on the BTN 3A1 molecule (CD277), but how BTN3A1 supports pAg recognition, remains unclear (32). In addition, γδ T cells can utilize the NK cell receptor NKG2D for target cell recognition (32), which has previously been described to be important in lytic EBV replication recognition by NK cells (24, 28). Interestingly, these Vγ9Vδ2 T cells seem to preferentially recognize EBV-infected B cell lines that express the nuclear antigen 1 of EBV (EBNA1) as the sole viral protein, so-called EBV latency I (31). This latency I is found in Burkitt's lymphoma (BL), the most common childhood tumor in Sub-Saharan Africa and homeostatically proliferating EBV-infected memory B cells (33). Interestingly, such non-transformed EBV-infected memory B cells are thought to be the reservoir of EBV persistence (34), accumulate in the peripheral blood of IM patients (35), and might drive Vγ9Vδ2 T cell expansion in children, which sometimes have viral loads as high as IM patients (36). Indeed, BTN3A1 and NKG2D are required to expand Vγ9Vδ2 T cells with BL cell lines in donors who are susceptible for this expansion (31). Similarly, pAg stimulation of Vγ9Vδ2 T cells in HIS mice was able to prevent outgrowth of adoptively transferred EBV transformed LCLs *in vivo* (37). These activated Vγ9Vδ2 T cells also required their invariant T cell receptor and NKG2D for LCL recognition. In this study, Vγ9Vδ2 T cells seem to eliminate EBV transformed LCLs primarily by FasL- and TRAIL-mediated programmed cell death induction. Moreover, adoptive transfer of Vγ9Vδ2 T cells into HIS mice, in which EBV-associated lymphoma formation was induced by EBV infection, prevented tumorigenesis (38). Even 3 weeks after infection, adoptive transfer of activated Vγ9Vδ2 T cells was still able to reduce tumor burden substantially. These

data suggest that Vγ9Vδ2 T cells preferentially expand to EBV latency I-infected B cells, but, once activated, can also target other EBV latencies, including latency III carrying EBV transformed LCLs. However, it remains unclear why this Vγ9Vδ2 T cell expansion can only be achieved in some donors and how pAg presentation or mevalonate metabolism is regulated during the different EBV latency programs. Nevertheless, Vγ9Vδ2 T cells seem to complement NK cells by recognizing latent EBV infection, while the latter innate lymphocyte subset preferentially controls lytic EBV replication. A combination of both cytotoxic innate lymphocyte subsets could be beneficial to target EBV infection.

NKT CELL-MEDIATED IMMUNE CONTROL OF EBV-DRIVEN B CELL TRANSFORMATION

Similar to our lack of understanding of how EBV regulates the mevalonate metabolism for Vγ9Vδ2 T cell recognition, also NKT cell recognition of EBV-infected B and epithelial cells is poorly understood, even so cytotoxicity of $CD8^+$ NKT cells against EBV latency II Hodgkin lymphoma (HL) and nasopharyngeal carcinoma (NPC) cells was previously reported (39). NKT cells carry the invariant Vα24-Jα18/Vβ11 T cell receptor and recognize glycolipids that are presented on the non-classical MHC class I molecule CD1d (11). CD1d has been reported to be downregulated on fully EBV transformed LCLs (40). Nevertheless, EBV infection of primary human B cells and LCL outgrowth can be restricted by NKT cells, and restoring CD1d expression on LCLs allows NKT cells to recognize EBV latency III (40). These data suggest that during B cell infection and transformation CD1d ligands are produced and presented on CD1d that allow for NKT cell recognition. Therefore, NKT cells can also restrict EBV-induced tumorigenesis *in vivo* (39). In particular, $CD8^+$ NKT cells can directly lyse EBV positive HL and NPC cells and produce IFN-γ, which augments protective Th1 responses against EBV infection (39). $CD4^+$ NKT cells, which mainly produce IL-4 and bias immune responses toward Th2 polarization, do not seem to be able to control EBV on their own, but synergize with $CD8^+$ NKT cells for improved immune control (39). While NKT cells are reduced in the peripheral blood of HL patients (39), they seem to be enriched in the tumor tissue (41). The HL and NPC associated EBV latency II with expression of three EBV latent antigens, namely EBNA1 and the two latent membrane proteins 1 and 2 (LMP1 and 2), can also be found in germinal center (GC) B cells of healthy EBV carriers (42). Therefore, NKT cells might play a role in restricting EBV latency II in GC B cells and epithelial cells. The latter might, however, only occur during NPC tumorigenesis, because EBV seems to mainly induce lytic replication in epithelial cells of healthy EBV carriers (43).

PRIMARY IMMUNODEFICIENCIES THAT COMPROMISE EBV-SPECIFIC IMMUNE CONTROL

The above discussed studies seem to indicate that several human innate lymphocyte subsets target different stages of EBV infection with NK cells recognizing lytic replication, Vγ9Vδ2 T cells reacting to EBV latency I and maybe III, and NKT cells providing restriction of EBV latency II. Can further evidence for this differential targeting of EBV by innate lymphocytes be gleaned from primary immunodeficiencies that predispose for EBV-associated pathologies (7, 44) and compromise these innate lymphocyte compartments?

The selective loss of NK, NKT, or γδ T cells is rare in primary immunodeficiencies. Usually, the respective mutations affect multiple immune compartments like the GATA2 mutation that was later characterized in the original patient with susceptibility to herpesvirus infections and decreased NK cell activity (18, 45). This mutation results in low numbers of B, $CD4^+$ T, NK, dendritic, and monocytic cells. The associated uncontrolled EBV infection manifests in fulminant IM, hemophagocytic lymphohistiocytosis (HLH), and chronic active EBV (CAEBV). Similarly, mutations in the cytotoxic machinery (perforin, Munc13-4, and Munc18-2) that predispose for HLH and CAEBV affect all cytotoxic lymphocytes (46–48). Furthermore, the mutations in SLAM-associated protein (SAP) and X-linked inhibitor of apoptosis that result in X-linked lymphoproliferative diseases (XLP) 1 and 2 affect many lymphocytes and also result in fulminant IM and HLH (49–53), even so also NKT cell development is compromised in XLP1 patients (54, 55). Therefore, overall loss of cytotoxic lymphocyte control of EBV infection seems to result in uncontrolled IM, CAEBV, and HLH. However, other primary immunodeficiencies seem to be more selective, both with respect to clinical manifestation and loss of cytotoxic lymphocytes. In this regard, patients with mutations in IL-2 inducible T cell kinase (ITK) lack all NKT cells and present sometimes with HL (56–63). Similarly, CD70 deficiency predisposes for HL (64, 65), but so far only the deficiency of $CD8^+$ T cells in recognizing EBV transformed B cells has been characterized. While in four of the five patients with CD70 deficiency no information about NKT cell numbers were given (64), in one patient NKT cell numbers were at least fivefold decreased. Thus, it is tempting to speculate that primary immunodeficiences, resulting from ITK and CD70 mutations, more prominently predispose for loss of NKT cell-mediated innate immune control and thereby favor uncontrolled EBV latency II, as in HL.

Even so CD70 is so far the only known ligand of CD27, CD27 mutations predispose for a much larger spectrum of EBV-associated pathologies, including HLH and different EBV-associated lymphomas, and also increase the mortality of affected individuals (66–68). It has been speculated that this results from ligand-independent signaling events of CD27 that are compromised in addition to T and NK cell recognition of LCLs (44). In addition to CD27, mutations in the magnesium transporter MagT1 and the transcription factor NFκB1 compromise NK cell recognition and predispose for EBV-induced lymphoproliferations and lymphomas (28, 69–74). These have been suggested to compromise NKG2D, TNF receptor (e.g., CD27), and SLAM receptor family (SAP dependent) signaling (28, 73). These receptors are crucial costimulatory molecules and activating receptors on $CD8^+$ T and NK cells, respectively. More selective NK cell deficiencies have been reported for mutations in the minichromosome maintenance complex component

4 (MCM4) and the Fcγ receptor 3A (CD16) (75–78). Both types of mutations diminish or functionally impair the $CD56^{dim}CD16^{+}$ NK cell compartment, which contributes to the early differentiated $NKG2A^{+}KIR^{-}$ NK cells that were found to restrict lytic EBV replication (22, 25). In addition, they could mediate further restriction of lytic EBV replication by CD16-mediated antibody-dependent cellular cytotoxicity against late lytic viral glycoproteins. Patients with MCM4 and CD16 mutations present with EBV-induced lymphoproliferative diseases, including EBV-positive Castleman's disease in the case of CD16 mutations. These selective NK cell deficiencies could point toward ill controlled lytic EBV infection that stimulates these lymphoproliferations.

In contrast to NKT and NK cells, Vγ9Vδ2 T cells have not received much attention in the characterization of primary immunodeficiencies that predispose for EBV pathologies. However, from the above described pathways that are affected by these, several are predicted to affect also Vγ9Vδ2 T cell function. Downstream of the TCR signaling, which in the case of Vγ9Vδ2 T cells engages pAgs in the context of BTN3A1, ITK phosphorylates PLCγ1, which elicits Ca^{2+} flux and phosphatidylinositol-4,5-bisphosphate cleavage to release diacylglycerol that in turn activates the guanine nucleotide exchange factor RasGRP1, whose mutations also predispose for EBV-associated B cell lymphomas (79). PLCγ1 activation is also Mg^{2+} dependent and thereby influenced by MagT1 function. Thus, mutations in ITK, MagT1, and RasGRP1 affect T cell receptor signaling and predispose for EBV-associated pathologies. Furthermore, NKG2D is a prominent coreceptor on Vγ9Vδ2 T cells and elicits NFκB1-dependent gene transcription (31). NKG2D and NFκB1 are affected by primary immunodeficiencies with EBV pathologies that result from mutations in the magnesium transporter MagT1 and the transcription factor NFκB1, respectively (28, 73). Finally, cytotoxicity of Vγ9Vδ2 T cells is also affected by the perforin, Munc13-4, and Munc18-2 mutations. These considerations suggest that T cell receptor signaling, costimulation, and effector functions of Vγ9Vδ2 T cells are compromised in some primary immunodeficiencies that predispose for EBV pathologies.

Apart from these immunodeficiencies whose genes can be related to innate lymphocyte function, other more general

TABLE 1 | Primary immunodeficiencies that are associated with loss of immune control by innate lymphocytes and EBV-associated pathologies.

Affected protein	EBV-associated pathology	Affected innate lymphocytes	Reference
Cytotoxic machinery			
Perforin	CAEBV, HLH	NK, NKT, γδT	(46)
Munc13-4	CAEBV, HLH	NK, NKT, γδT	(47)
Munc18-2	CAEBV, HLH	NK, NKT, γδT	(48)
DNA-binding proteins			
GATA2	CAEBV, HLH	NK	(18, 45)
MCM4	EBV lymphoma	NK	(75, 76)
NF-κB1	EBV lymphoma	NK	(73, 74)
Costimulatory receptors and their ligands			
CD27	EBV lymphoma	NKT	(66–68)
CD70	EBV-positive Hodgkin's lymphoma	NKT	(64, 65)
CD16	EBV-positive Castleman's disease	NK	(77, 78)
NKG2D and TCR (because of MagT1 deficiency)	EBV lymphoma	NK, γδT	(69–72)
Signaling molecules			
SAP	EBV lymphoma, IM, HLH	NKT	(49–51, 54, 55)
ITK	EBV lymphoma	NKT	(56–63)
RasGRP1	EBV lymphoma	NKT	(79)
PI3K 110δ	EBV viremia	NK	(82)
Others			
XIAP	IM, HLH	NKT	(52, 53)
Coronin 1A	EBV lymphoma	NKT	(80)
CTP synthase 1	IM, EBV lymphoma	NKT	(81)

CAEBV, chronic active EBV; HLH, hemophagocytic lymphohistiocytosis; IM, infectious mononucleosis; NK, natural killer; EBV, Epstein–Barr virus; NKT, natural killer T; SAP, SLAM-associated protein; XIAP, X-linked inhibitor of apoptosis; ITK, inducible T cell kinase; MCM4, minichromosome maintenance complex component 4; PI3K, phosphatidylinositol-3-kinase.

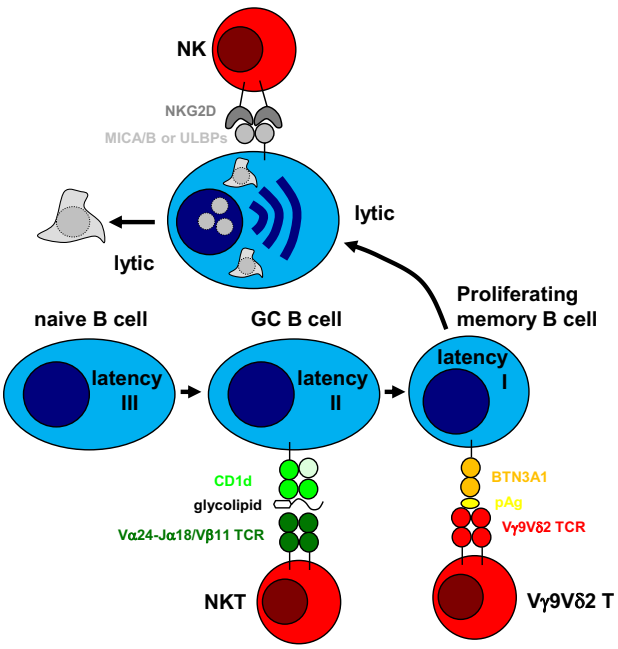

FIGURE 1 | Innate lymphocytes target different stages of Epstein–Barr virus (EBV) infection. EBV was suggested to drive B cell differentiation by expressing all eight latent EBV proteins (latency III) in tonsillar naïve B cells and rescuing germinal center (GC) B cells with the expression of three latent EBV proteins (latency II) toward memory B cells. In homeostatically proliferating memory B cells, only one latent EBV protein is expressed for viral genome maintenance (latency I). From this infected memory B cell pool, EBV can reactivate into virus producing lytic replication, most likely after B cell receptor engagement. Natural killer (NK) cells have been shown to preferentially recognize lytic EBV replication and NKG2D has been suggested as an activating receptor involved in this recognition after upregulation of its MICA/B and ULBP ligands. In a subgroup of infected individuals, Vγ9Vδ2 T cells can be stimulated by EBV latency I Burkitt's lymphoma cell lines and recognize these by mevalonate metabolite recognition in a butyrophilin (BTN) 3A1-dependent fashion. Finally, natural killer T (NKT) cells have been suggested to recognize EBV latency II in Hodgkin's lymphoma cell lines, presumably by recognizing glycolipid presentation on CD1d. Thus, cytotoxic innate lymphocytes can target different stages of EBV infection.

deficiencies like the mutations in the actin-binding protein coronin 1A and CTP synthase 1 are associated with NKT cell loss and EBV-associated lymphoproliferative diseases (80, 81). Furthermore, loss-of-function mutations in phosphatidylinositol-3-kinase subunit 110δ diminish NK cell killing and results in EBV viremia (82). Thus, primary immunodeficiences in the perforin machinery of cytotoxic lymphocytes, their costimulatory molecules, DNA-binding proteins that are required for their differentiation, and some less well-mechanistically understood gene products diminish innate lymphocyte activity and predispose for EBV-associated pathologies. These are summarized in **Table 1**.

CONCLUSION AND OUTLOOK

The above outlined arguments suggest a division of labor among innate lymphocytes in targeting different programs of EBV infection. While NK cells might preferentially eliminate lytically EBV replicating cells, and immunodeficiencies that affect them could primary result in lymphoproliferations, NKT cells might be superior in restricting Hodgkin's lymphoma and especially affected by ITK and CD70 deficiencies. Finally, Vγ9Vδ2 T cells might be able to target BL cells and LCLs. In combination, NK, NKT, and Vγ9Vδ2 T cells could therefore restrict EBV latencies I–III and lytic replication (**Figure 1**). This comprehensive immune control by innate lymphocytes might be especially important during early primary infection before protective $CD8^+$ T cell responses have been primed. A better understanding of how these innate lymphocyte subsets collaborate during primary EBV infection could provide insights why IM preferentially develops in adolescence and which subgroup of these are especially at risk. Furthermore, characterizing how NK, NKT, and Vγ9Vδ2 T cells recognize EBV-infected cells and which infection programs in virus-associated malignancies are especially susceptible to this recognition could suggest immunotherapeutic approaches against the respective tumors, harnessing these innate lymphocytes.

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The author confirms being the sole contributor of this work and approved it for publication.

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