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DOI: <https://doi.org/10.1016/j.coviro.2020.07.011>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-198961>

Journal Article

Accepted Version

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Originally published at:

Münz, Christian (2021). Cytotoxicity in Epstein Barr virus specific immune control. *Current Opinion in Virology*, 46:1-8.

DOI: <https://doi.org/10.1016/j.coviro.2020.07.011>

Cytotoxicity in Epstein Barr virus specific immune control

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Highlights

- Persistent oncogenic Epstein Barr virus (EBV) infection is kept in check by cytotoxic lymphocytes.
- Primary immunodeficiencies suggest these to primarily be CD27⁺2B4⁺4-1BB⁺NKG2D⁺CD8⁺ T cells.
- Immune control of EBV in humanized mice is mediated by 2B4⁺CD8⁺ T cells.

Short Title

Cytotoxicity against EBV

Keywords

Natural killer (NK) cells, $\gamma\delta$ T cells, NKT cells, CD8⁺ T cells, humanized mice, primary immunodeficiencies, Hodgkin's lymphoma, Burkitt's lymphoma, KSHV co-infection, HIV co-infection

Abstract

Epstein Barr virus (EBV) is the most common human tumor virus, persistently infecting more than 95% of the human adult population and readily transforming human B cell in culture. Fortunately, only a small minority of EBV carriers develops virus associated malignancies. The majority controls persistent EBV infection with cytotoxic lymphocytes, mainly NK, $\gamma\delta$ and CD8⁺ T cells and the characteristics of the required immune responses get more and more defined by primary immunodeficiencies that affect molecules of these cytotoxic lymphocytes and their investigation in mice with reconstituted human immune system components (humanized mice) that are susceptible to EBV infection and associated lymphomagenesis. The gained information should be able to guide us to develop immunotherapies against EBV and tumors in general.

1. Introduction on EBV and associated malignancies

The human immune system has developed specific capabilities, most likely in response to pathogens that would cause significant morbidity and mortality prior to sexual maturity in the majority of the human population [1,2]. One of these pathogens is probably the common human γ -herpesvirus Epstein Barr virus (EBV) or human herpesvirus 4 [3]. It is arguably the most transforming tumor virus in humans and the only one that can readily immortalize its main host cell, the human B cell, into indefinitely growing lymphoblastoid cell lines (LCLs) in vitro. Furthermore, it is a class I WHO carcinogen and associated with epithelial cell carcinomas and lymphomas [4]. Moreover, it infects and is persistently carried by more than 95% of the human adult human population, reaching this saturation at 2 years of age in developing countries, and establishing seropositivity in 60-70% of the population in the first world at this young age [5]. The remaining 30-40% of the population acquire EBV in Europe and the US then in the second decade of life, often as a symptomatic primary infection called infectious mononucleosis (IM) that is characterized by massive CD8⁺ T cell expansions [6]. Despite this high prevalence of persistent EBV infection only 1-2% of all malignancies in humans are associated with this virus [7]. These include Burkitt's and Hodgkin's lymphoma or nasopharyngeal carcinoma [8]. EBV associated B cell lymphomas are thought to develop from infected B cell differentiation stages with viral gene expression or latency patterns that can also be found in healthy EBV carriers [9]. Latency III of EBV expresses six nuclear antigens (EBNAs), two membrane proteins (LMPs), small non-translated RNAs (EBERs) and micro RNAs (BART and BHRF1 miRNAs), and is found in healthy naïve B cells (Figure 1) as well as lymphomas of severely immune suppressed individuals, like post-transplant lymphoproliferative disease (PTLD) or some diffuse large B cell lymphomas (DLBCLs). Latency II expresses only EBNA1, LMP1 and 2 as viral proteins, and is found in healthy germinal center B cells as well as Hodgkin's lymphoma. These two latency programs are thought to drive differentiation of EBV infected B cells into the memory B cell pool, the site of virus persistence with only non-translated viral RNA (latency 0), or in addition transient EBNA1 expression (latency I) during homeostatic proliferation [10,11]. Latency I is also found in Burkitt's lymphoma, but these tumors are thought to emerge from EBV infected germinal center B cells [12]. Plasma cell differentiation from EBV latency 0 or I expressing B cells leads to lytic replication with infectious virus production [13]. Primary effusion lymphoma (PEL) is the only EBV associated plasmacytoma and contains elevated lytic EBV replication [14••]. This was observed in both a preclinical mouse model of PEL-like lymphoma formation and PEL tissue sections of patients. Infectious EBV particle production is then amplified by lytic replication in mucosal epithelia for efficient shedding into saliva and transmission to new hosts [15]. Thus, all infection patterns of

EBV associated lymphomas are established in persistently EBV infected virus carriers, threatening these with tumor development that fortunately occurs only rarely.

2. Primary immunodeficiencies that predispose for EBV associated pathologies

The rare occurrence of EBV associated malignancies is thought to be due to a nearly perfect immune control of this persistent tumor virus infection, protecting EBV carriers for often more than 70 years [16,17]. This immune control seems to be primarily based on cytotoxic lymphocytes. Four lines of evidence support this notion. Firstly, cytotoxic lymphocytes, primarily natural killer (NK), $\gamma\delta$ and CD8⁺ T cells, expand during IM [5,18•]. Group 1 and 2 individuals have been defined by NK and $\gamma\delta$ T cell or only NK cell expansion during IM. Expanding NK cells consist mainly of immature CD56⁺CD16⁺NKG2A⁺killer immunoglobulin-like receptor (KIR)⁻ NK cells [19,20]. Expanding CD8⁺ T cells with individual specificities reaching 40% of the total repertoire, recognize mainly early lytic EBV antigens [21]. Secondly, some patients with EBV associated malignancies, primarily PTL, Hodgkin's lymphoma and nasopharyngeal carcinoma, benefit from the transfer of EBV specific T cell lines [22]. EBNA1, LMP1 and 2 have been identified as protective EBV antigens in clinical adoptive T cell trials [23,24]. Thirdly, preclinical EBV infection models, mainly mice with reconstituted human immune system components (humanized mice), have identified NK, NKT, $\gamma\delta$ T and conventional $\alpha\beta$ T cells as protective entities against EBV infection and associated lymphomagenesis, and will be discussed in more detail below [25]. Fourthly and possibly most importantly, primary and acquired immunodeficiencies identify T cells and their molecular machineries as crucial for EBV specific immune control [26-31]. Inverse prevalence of EBV associated lymphomas is observed in acquired immunodeficiencies after transplantation and after human immunodeficiency virus (HIV) infection with poorly immunogenic Burkitt's and Hodgkin's lymphoma occurrence early in AIDS and late after transplantation, while highly immunogenic PTL or latency III immunoblastic lymphomas occur late in AIDS and rapidly after transplantation [29,32]. This increased EBV associated lymphomagenesis upon HIV co-infection has recently been modelled in humanized mice [33••]. Co-infection with EBV and HIV led to increased EBV associated lymphoma incidence in humanized mice and antibody depletion of CD8⁺ T cells could no longer increase EBV viral loads in double-infected mice, presumably due to compromised cytotoxic function after loss of T cell help by HIV mediated CD4⁺ T cell destruction. Even more informative are primary immunodeficiencies, in which individual genetic defects predispose for EBV associated pathologies like lymphomas and chronic active EBV infection (CAEBV) that leads due to its continuous myeloid cell stimulation to hemophagocytic lymphohistiocytosis (HLH) [26-28]. The affected molecules (Figure 2) fall primarily into four classes. Firstly, the machinery of cell-mediated cytotoxicity with perforin and Munc13-4 as well as Munc18-2 that are required for cytotoxic granule release [34-36]. Secondly, components of the T cell receptor signaling machinery, including IL2-inducible T-cell kinase (ITK), PI3K 110 δ ,

RasGRP1 and ZAP70 [37-44]. Thirdly, co-stimulatory molecules of cytotoxic lymphocytes, including CD27, CD70, CD137 (4-1BB), SAP as the adaptor of 2B4 and other SLAM receptors, CTLA-4 and the magnesium transporter MAGT1 that affects NKG2D surface expression [45-47, 48•,49•,50•,51•,52-57]. Particularly impressive are the findings that loss-of-function mutations in both CD27 and its ligand CD70 lead to vulnerability against EBV associated pathogenesis in nearly all affected individuals. Altogether, these studies suggest that CD27⁺2B4⁺4-1BB⁺NKG2D⁺CD8⁺ T cells might be particularly important in EBV specific immune control. Fourthly, loss-of-function mutations in molecules that are required for cytotoxic lymphocyte differentiation or expansion also predispose for EBV associated malignancies. These include GATA2, MCM4, CTP1, XIAP and STK4 [53,58-65]. In combination these primary immunodeficiencies point towards NK cells and CD8⁺ T cells as crucial components of EBV specific immune control. Surprisingly, deficiencies in type I and II interferons (IFNs) as well as of antibody responses like in common variable immune deficiency (CVID) do not seem to predispose for EBV associated pathologies [28]. These considerations identify cytotoxic innate and adaptive lymphocytes, primarily NK and CD8⁺ T cells as immune compartments that meet the challenge of B cell transformation by persistent EBV infection and are in most cases victorious.

3. Immune control in humanized mice

In order to interrogate the function of the molecules that are identified by primary immunodeficiencies and which human leucocyte compartments they affect humanized mice are explored [25]. For this purpose human CD34⁺ hematopoietic progenitor cells are neonatally and intrahepatically injected into immune compromised mice that lack innate and adaptive mouse lymphocytes due to RAG, *scid* and γ_c mutations [66-68]. These can then be infected with EBV, develop cytotoxic lymphocyte responses and EBV associated lymphomas in a subset of infected mice [69-73]. Three types of manipulations in this in vivo model of EBV infection have been used to interrogate EBV specific immune control and oncogenesis. These are mutant EBV viruses lacking individual viral gene products or whole programs of the viral life cycle, antibody mediated depletion of lymphocyte subsets or blocking of their surface molecules, and adoptive transfer or stimulation of distinct lymphocyte specificities. Among mutant viruses the most informative were the ones missing the latent EBV antigens EBNA3B and LMP1, the viral miRNAs and the immediate early lytic transactivator BZLF1 [14••,74-76, 77•,78,79]. EBV transformed B cells without EBNA3B do not produce sufficient chemokines, like CXCL9 and CxCL10, to attract T cells into the tumor microenvironment. Therefore, EBV without its tumor suppressor EBNA3B causes increased tumor formation with similarities to DLBCL [74]. In the absence of LMP1, CD4⁺ helper T cells can support rather than restrict EBV associated lymphomagenesis [75], possibly even supporting latency I and II of Burkitt's and Hodgkin's lymphoma [80]. EBV miRNAs suppress MHC class I restricted viral antigen presentation, and in their absence CD8⁺ T cells completely prevent EBV associated lymphoma formation in humanized mice [76]. Finally, BZLF1 deficient EBV revealed that NK cell responses restrict mainly lytic EBV infection [77•] and that early lytic replication is required for efficient lymphoma formation [78,79]. This contribution of lytic EBV infection to virus associated lymphomagenesis becomes also apparent during co-infection with the Kaposi sarcoma associated herpesvirus (KSHV) or human herpesvirus 8 [14••]. Dual infection increases lymphoma formation with similarities to PEL, that carries both EBV and KSHV in 90% of tumors [81]. These PEL-like tumors contain elevated frequencies of cells with lytic EBV infection and their development is compromised upon KSHV co-infection with BZLF1 deficient EBV. Along these lines of viral gene expression variation, different viral isolates have also been used in humanized mice [82-84•], even so most in vivo experiments have so far been performed with B95-8 EBV that was originally isolated from an American IM patient [85,86]. Although some of the tested viral isolates have revealed quite dramatic differences with infection spreading to T cells or significantly elevated lytic replication [83,84•], it has so far been difficult to link these phenotypes to distinct genetic differences in the tested viruses due to the large size of the viral

genome (172kb). In addition to these insights based on EBV genome changes, antibody mediated depletions of T and NK cells revealed that primarily CD8⁺ T and NK cells (Figure 1) restrict EBV infection and associated lymphoma formation in humanized mice [73,76,77•,87]. T cells require 2B4 (Figure 2) and PD-1 for this task, because antibody mediated blocking of both compromises immune control of EBV [88,89]. CD8⁺ T cell depletion did not further increase EBV viral loads and associated lymphomagenesis during antibody blocking of 2B4. Thus, 2B4⁺CD8⁺ T cells seem to control EBV infection in humanized mice. In addition to these loss-of-function experiments by antibody mediated blocking or lymphocyte depletion, adoptive transfer of early lytic EBV antigen specific CD8⁺ T cells or late lytic antigen specific CD4⁺ T cells has been shown to restrict lytic EBV replication, and surprisingly even transformed B cells [79,90]. Furthermore, stimulation or adoptive transfer of V γ 9V δ 2 T cells restricts EBV induced lymphomas in humanized mice [91•,92], preferentially recognizing EBV latency I infected B cells (Figure 1) [18•,93]. Finally, adoptive transfer of NKT cells also restricts EBV associated lymphoma growth [94], preferentially recognizing EBV latency II infected cells (Figure 1) [94,95]. Therefore, humanized mice recapitulate EBV specific immune control by cytotoxic lymphocytes, including NK, NKT, V γ 9V δ 2 T, classical CD8⁺ T and some cytotoxic CD4⁺ T cell populations. Immune modulation of these responses could benefit patients with EBV associated malignancies but should also be watched during immune check-point therapies for effects on EBV specific immune control and possible virus reactivation.

4. Conclusions and outlook on EBV specific vaccination

Primary immunodeficiencies, adoptive T cell therapies and EBV infection of humanized mice characterize cytotoxic lymphocytes, mainly NK and CD8⁺ T cells, as the protective entities that sustain immune control of persistent oncogenic γ -herpesvirus infection. Establishing this immune control therapeutically without individualized adoptive T cell transfer in patients with EBV associated malignancies or prophylactically in seronegative adolescents with a high risk to develop IM would be desirable. Active vaccination against EBV is therefore explored [7,96]. However, most of our vaccines elicit protective antibody responses which are probably of limited value against oncogenic latent and early lytic EBV infection. Therefore, vaccine formulations are explored for the induction of durable cytotoxic T cell responses [97-100], some of them in humanized mice [101-103••]. However, in humanized mice only CD4⁺ T cell responses against EBV have been induced by vaccine candidates so far, even so some of them have protected from EBV infection, such as EBNA1 carrying EBV derived virus-like particles (VLPs) [103••]. Targeting EBV antigens to abundant antigen presenting cell populations in humanized mice for intracellular expression from recombinant viruses might provide a promising avenue to develop CD8⁺ T cell priming vaccination for humans. Such strategies could then be broadened towards other infectious diseases and tumors.

Acknowledgements

Research in my laboratory is supported by Cancer Research Switzerland (KFS-4091-02-2017 and KFS-4962-02-2020), KFSP-Precision^{MS} and HMZ ImmunoTargET of the University of Zurich, the Coronavirusfonds UZH, the Cancer Research Center Zurich, the Vontobel Foundation, the Baugarten Foundation, the Sobek Foundation, the Swiss Vaccine Research Institute, Roche, Novartis and the Swiss National Science Foundation (310030B_182827 and CRSII5_180323).

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This study describes the first successful vaccination in humanized mice that protects from EBV infection.

Figure legends

Figure 1: Innate and adaptive lymphocytes restrict different stages of EBV infection. Innate NKT, $\gamma\delta$ T and NK cells target latency II, latency I and lytically EBV infected B cells, respectively. Latency II is present in germinal center (GC), latency I in homeostatically proliferating memory B cells and lytic EBV replication in plasma cells. Classical $\alpha\beta$ CD8⁺ T cells are able to kill naïve latency III, latency II and lytically EBV replicating B cells. This figure was created in part with modified Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 unported license: <https://smart.servier.com>.

Figure 2: Cytotoxic lymphocyte mediated restriction of EBV transformed B cells. Primary immunodeficiencies identify molecules that are in some cases exclusively essential for EBV specific immune control. These include signaling of the T cell receptor (TCR) like ITK, PI3K, ZAP70 and RasGRP1. Furthermore, mutations in CD27, CD70 and 4-1BB predispose for EBV associated diseases. Loss-of-function mutations in SAP compromise co-stimulation by SLAM protein family receptors, including 2B4 whose blocking by antibodies in humanized mice compromises EBV specific immune control. Mutations in the MAGT1 magnesium transporter decrease NKG2D surface expression and cytotoxic lymphocyte recognition of EBV infected B cells. Mutations in perforin and molecules required for cytotoxic granule exocytosis abolish EBV specific immune control. Mutations in GATA2 and MCM4 inhibit cytotoxic lymphocyte differentiation. Finally, XIAP, CTPS1 and STX4 are necessary for cytotoxic lymphocyte expansion to reach sufficient numbers controlling EBV infection. This figure was created in part with modified Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 unported license: <https://smart.servier.com>.

Figure 1

Figure 2