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Adoptive Transfer of EBV Specific CD8+ T Cell Clones Can Transiently Control EBV Infection in Humanized Mice

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Abstract

Epstein Barr virus (EBV) infection expands CD8+ T cells specific for lytic antigens to high frequencies during symptomatic primary infection, and maintains these at significant numbers during persistence. Despite this, the protective function of these lytic EBV antigen-specific cytotoxic CD8+ T cells remains unclear. Here we demonstrate that lytic EBV replication does not significantly contribute to virus-induced B cell proliferation in vitro and in vivo in a mouse model with reconstituted human immune system components (huNSG mice). However, we report a trend to reduction of EBV-induced lymphoproliferation outside of lymphoid organs upon diminished lytic replication. Moreover, we could demonstrate that CD8+ T cells against the lytic EBV antigen BMLF1 can eliminate lytically replicating EBV-transformed B cells from lymphoblastoid cell lines (LCLs) and in vivo, thereby transiently controlling high viremia after adoptive transfer into EBV infected huNSG mice. These findings suggest a protective function for lytic EBV antigen-specific CD8+ T cells against EBV infection and against virus-associated tumors in extra-lymphoid organs. These specificities should be explored for EBV-specific vaccine development.


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Introduction

Epstein Barr virus (EBV) is a ubiquitous human γ-herpesvirus that establishes persistent infection in more than 90% of the human adult population [1]. Like other herpesviruses, EBV can undergo two infection programs. Latent infection leads to the expression of eight viral proteins and more than forty non-translated RNAs. This program is able to immortalize human B cells and is found in EBV associated tumors [2]. In contrast, lytic EBV infection leads to the expression of more than eighty viral proteins and the production of infectious viral particles [3]. Human T cells recognize both latent and lytic EBV antigens with distinct hierarchies [4]. While the latent nuclear antigen 1 (EBNA1) is a consistently recognized CD8+ T cell antigen [5], latent membrane protein 2 (LMP2) and the EBNA3 proteins are prominent CD8+ T cell antigens [6]. Furthermore, CD4+ T cells often recognize late lytic EBV antigens [7], while CD8+ T cells arise in response to early lytic antigens, including BMLF1 [8]. While EBV transformed B cells (Lymphoblastoid cell lines (LCLs)) can be readily recognized by CD8+ T cells specific for lytic EBV antigens, the protective value of lytic antigen-specific CD8+ T cells has not been readily demonstrated so far. Moreover, the expansion of these lytic EBV antigen-specific CD8+ T cells seems to follow viral load during symptomatic primary infection, called infectious mononucleosis, while latent EBV antigen-specific T cells peak during convalescence consistent with their involvement in viral immune control [4]. Thus, we aimed to evaluate the protective role of lytic EBV antigen specific CD8+ T cells in vitro and in vivo.

Due to its exclusive tropism for human cells, this oncogenic γ-herpesvirus is difficult to study in vivo. However, recent advances have led to the development of two informative in vitro models. One model examines the infection of rhesus macaques with lymphocryptoviruses (LCV), a subgroup of γ-herpesviruses that includes EBV [9], and the other model examines EBV infection in mice with reconstituted human immune system components [10]. In both systems, T cell targeted immunosuppression leads to loss of viral immune control and virus-associated tumor formation [11,12,13]. We have explored EBV infection of non-obese diabetic mice with a severe combined immunodeficiency mutation and with complete loss of the interleukin 2 receptor gamma chain locus...
CD4+ cells were isolated as described previously from human fetal liver tissue [13,18]. The reconstitution of human immune system components in the peripheral blood of humanized NSG-A2tg mice (huNSG-A2tg) was analyzed for each cohort 12 weeks after engraftment and prior to the beginning of experiments.

Ethics statement

All animal protocols were approved by the cantonal veterinary office of Zurich, Switzerland (protocol nos. 116/2008 and 148/2011). All studies involving human samples were reviewed and approved by the ethical committee of Zurich, Switzerland (protocol no KEK-St-Nr 19/08). These protocols follow the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes as well as the Swiss Animal Welfare Act (TSchG; 453) (Amendment of 15 June 2012) and the Swiss Animal Welfare Ordinance (TSchV; 453.1).

Virus infection in vitro

Epstein-Barr virus B95-8 wild-type (WT EBV) and BZLF1 knockout (ZKO EBV) virus were produced in 293 HEK cells (kindly provided by Regina Feederle and Henri Jacques Delecluse, Heidelberg, Germany). Titration of viral concentrates was performed on Raji cells using serial dilution and calculated as Raji infection units (RIU) using flow cytometric analysis of GFP-positive Raji cells 2 days after infection. Bulk peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by centrifugation using Ficoll-Paque, stained with carboxyfluorescein succinimidyl ester (CFSE) and incubated with mock, WT or ZKO EBV at a multiplicity of infection of 0.01 in the presence of cyclosporine A (CsA, 1 μg/ml). Cultures were harvested and counted every 2–3 days. Absolute numbers of cells were counted using trypan blue exclusion and assessed by flow cytometry. In order to maintain cultures in the logarithmic growth phase, they were maintained at a maximal density of 1 × 10^6 cells/ml and supplemented with fresh media every 5 days. Assays were performed on PBMCs from two EBV-seropositive donors in duplicate.

Infection of mice and adoptive transfer experiments

Fourteen to sixteen weeks after engraftment, huNSG-A2tg mice were injected with 10^5 RIU of WT, ZKO EBV or with PBS intraperitoneally, bled weekly starting from week 2 and euthanized 6 weeks after infection. For adoptive transfer studies, mice received 1 × 10^6 of LMP2- or BMLF1-specific CD8+ T cell clones intravenously one day prior to infection, and they were euthanized 4 or 6 weeks post-infection. Clones for adoptive transfer were selected based on their level of IFNγ secretion. All cell transfer experiments were performed with either of two independently obtained LMP2- or BMLF1-specific T cell clones. After termination of the experiments, body and spleen weight were determined. Abdominal organs were analyzed macroscopically for the presence of visible tumors.

Immunohistochemistry

Tissue was fixed using 4% formalin and then paraffin embedded. For immunohistochemistry, anti-CD20 (L26, Cell Marque Lifescreeen), anti-EBNA2 (PE2, Novocastra Laboratories Ltd), anti-NKp46 (BAF 2225, R&D Systems), anti-human CD68 (51H12, Novocastra Laboratories Ltd), anti-neutrophil elastase (NE, ab21595, Abcam), anti-gp350 (OT6, kindly provided by Dr. J.M. Middledorp, VU University Medical Center, The Netherlands) or anti-ZEBRA antibodies (AZ-69, Argene) were used and
4 μm sections were processed using standard procedures on a BOND-MAX automated immunohistochemistry system (Leica Microsystems). Isotype staining served as negative control and EBV-positive specimen of human tonsil or control or M81-infected huNSG-Atg mouse tissue as positive controls. The absolute number of ZEBRA+ (BZLF1+, BZ1+) cells per spleen section was counted in a blinded fashion and normalized to the total area of spleen section. The total area of spleen sections was determined using ImageJ Software. The number of CD20 total area of spleen section. The total area of spleen sections was 10 fields per spleen at ×200 and ×400 magnification, respectively.

**Quantification of EBV load by real-time PCR**

Total DNA from the whole blood was extracted using NucliSens (bioMérieux) according to manufacturer’s instructions. Spleenic tissue was processed using QIAnap tissue Kit (QIAGEN) using the manufacturer’s protocol. Quantitative analysis of EBV DNA was performed by TaqMan (Applied Biosystems) real-time PCR technique as described [20] with modified primers for the BamH1 W fragment (5’-CTTCTTCAGGACCGGTTT-3’ and 5’-CAGTGTCCTCCCCCTGATA-3’) and a fluorogenic probe (5’-FAM-CGTAAGCCAGACAGCCAATTGT-CAG-TAMRA-3’). All PCRs were run on an ABI Prism 7700 Sequence Detector (Applied Biosystems) and samples were analyzed in duplicates. No EBV DNA was detected in the blood of mock-infected animals for the duration of the experiment. Mice were considered uninfected if EBV DNA was not detected in the blood and spleen during the experiment.

**Flow cytometry**

To analyze B cell outgrowth in in vitro assays, cells were labeled using anti-CD19 (HB19, Biolegend), anti-CD3 (UCHT1, Biologend), and anti-CD23 (M-233, BD Biosciences) antibodies. The composition of blood and spleen samples from humanized mice was analyzed using anti-human CD45 (H130, Biolegend), anti-CD3 (MHCD1918, Invitrogen), anti-CD4 (RPA-T4, Biologend), anti-CD8 (SK-L, Biologend), and anti-CD19 (MHCD1917, Invitrogen) antibodies. Spleens were mechanically disrupted and filtered through a 70 μm cell strainer. Erythrocyte lysis in whole blood or in spleen suspensions was done using NHCl. Cell suspensions were stained with antibodies for 20 min at 4°C and washed. To sort EBV-specific CD8+ T cells, PBMCs were isolated using Ficoll-Paque, washed and stained using PE-labeled HLA-A*0201 dextramers complexed with the following peptides: HIV gag87–95 (5’SLYNTVAI5’), LMP226–34 (CLGGGLITMV) and BMLF1259–267 (GLCTLVAML) (Immudex MHC Dextramer). For staining HLA-A*0201 dextramers loaded with the BRLF1109–117, TRAV and TRBV chain repertoire was assessed using a Nucleotide sequencing of PCR products was performed at Eurofins MWG Operon (Balgach, Switzerland) with 30 pmol of reverse C1 primer. TCR gene designations follow the ImMunoGeneTics (IMGT) nomenclature [http://www.IMGt.org].

**Generation of EBV-specific T cell clones**

EBV-specific T cells were sorted from the blood of a healthy HLA-A*0201 positive EBV carrier using LMP2- or BMLF1-specific dextramers. The donor was genotyped as HLA-A*02, A*68, B*44, and B*07. Dextramer positive populations live CD3+CD8+ cells were single-cell sorted and plated onto irradiated PBMCs and LCLs as feeders in the presence of 1 μg/ml phytohemagglutinin L (PHA-L) and 150 U/ml hrIL-2 as previously described [21]. After 2 weeks, T cells were tested in a split well assay for IFNγ secretion upon re-stimulation with 1 μM of relevant peptides. T cells specifically recognizing the tested peptide were re-stimulated with the same protocol. They were tested on day 15 for phenotypic characteristics by flow cytometry and for functional T cell avidity in peptide titration assays.

**Sequence of TRAV and TRBV gene rearrangements**

The TRAV and TRBV chain repertoire was assessed using a primer set as previously described [22]. Primers were obtained from Biomers (Ulmu, Germany). PCR amplification was performed in a 25 μl reaction volume containing Pfu polymerase Buffer, 200 mM deoxynucleotide triphosphate, 0.5 μM C primer, 0.5 U Pfu DNA Polymerase (all reagents were provided by Thermo Fisher Scientific, Reinach, Switzerland), 0.5 μM forward primer, and 100 ng cDNA. The cycling conditions were as follows: initial denaturation for 4 min at 95°C and 35 cycles of 95°C for 30 s, primer annealing at 60°C for 20 s, and primer extension at 72°C for 60 s. The PCR product was validated by electrophoresis on a 2% agarose gel. Nucleotide sequencing of PCR products was performed at Microsynth (Balghach, Switzerland) with 30 pmol of reverse C1 primer. TCR gene designations follow the ImMunoGeneTics (IMGT) nomenclature [http://www.IMGt.org].

**T cell clone epitope avidity, MHC-restriction and degranulation assays**

For peptide titration assays, 1 x 10^6 cells of EBV-specific clones were incubated with synthetic cognate peptides (Shanghai Biochem) for 18 h. LMP2 peptides and BMLF1 peptides at 5 μM concentration served as an irrelevant control for BMLF1- and LMP2-specific CD8+ T cell clones, respectively. For co-culture assays, clones were incubated with HLA-A*02 mismatched or autologous lymphoblastoid cell lines (LCLs) in an effector to target (E:T) ratio of 1.5. LCLs were optionally pulsed with 1 μM cognate peptide for 1 h at 37°C and washed extensively. Supernatants were used for IFNγ ELISA (Mabtech). For degranulation assays, clones were incubated with cognate or control peptide for 0 h and then stained intracellularly with perforin and granzyme B antibodies using the BD Cytofix/Cytoperm Kit.

**In vitro killing assay**

To evaluate the cytotoxic activity of T cell clones against the autologous LCLs, we performed functional in vitro killing assays.
Figure 1. Lytic replication does not significantly contribute to EBV transformation in vitro. Bulk PBMCs were inoculated with WT EBV, ZKO EBV or mock treated in the presence of cyclosporine A. (A) Cells were harvested and absolute cell numbers per well were determined on indicated days. (B) Outgrowth of transformed CD19+ B cells was monitored using flow cytometry. (C) Proliferation of EBV transformed B cells was demonstrated by CFSE dilution and the subsequent increase in percentage of CFSElow B cells. The experiments were performed in duplicate and data is presented as the mean ± SEM. Similar results were obtained in samples from two donors; the average of two experiments is presented (p_mack vs WT/ZKO <0.0001 by two-way ANOVA).

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Autologous LCLs were generated by incubating bulk PBMCs from the T cell clone donor with B95-8 supernatants and used as targets. AKBM cells, genotyped as A*24, A*31; B*35, B*51; C*09, and C*14, were used as a mismatched control target. EBV positive AKBM cells that express GFP upon switching from latent to lytic EBV replication were induced to enter lytic cycle by ligation with F(ab')2 IgG and used directly for co-culture experiments [23]. AKBM or autologous LCLs were incubated with BMLF1- or LMP2-specific CD8+ T cell clones for 18 h at an E:T ratio of 1:1 and stained intracellularly using ZEBRA (BZ.1, Santa Cruz) antibodies. Specific lysis was determined by using the formula: % lysis = 100 x [1 – (experimental ZEBRA- mean isotype)/mean ZEBRA- without effectors – mean isotype)]. Negative value for specific killing indicates an increase in number of ZEBRA+ B cells. Two to four replicates were used for each condition.

HLA-genotyping

Genomic DNA was isolated from cell type of interest using Qiagen DNeasy Kit and genotyped by PCR-SSP method using HLA-A*/-B*/-DRB1* and HLA-DQB1* Kit (Protrans).

Statistical analysis

All data were analyzed with the Mann Whitney test, unless otherwise stated. A p value of <0.05 was considered statistically significant. Statistical analysis and the generation of graphs was performed using Prism software (GraphPad Software).

Accession numbers

The GenBank accession number for the complete B95-8 wild type sequence is AJ507799.2 (Table S3). The UniProtKB/Swiss-Prot identification of BMLF1-protein is Q04360. The UniProtKB/Swiss-Prot identification of LMP2-protein sequence is P13285. The Immune Epitope Database identification numbers of HLA-A*02-restricted BMLF1259–267 and LMP2262–283 epitopes are 20788 and 6568, respectively.

Results

Lytic replication does not significantly contribute to B cell transformation in vitro

To understand the relevance of lytic infection and EBV lytic antigen-specific T cell immune responses in the control of EBV infection and virus-mediated B cell transformation, we used a recombinant EBV virus devoid of the lytic immediate early transactivator BZLF1 that is severely compromised to enter the lytic replication [24]. We first performed in vitro assays to assess if lytic replication itself has an effect on the initial transformation of B cells. Bulk peripheral blood mononuclear cells (PBMCs) from two EBV seropositive donors were infected with either WT or ZKO EBV with equal multiplicities of infection. This was done in the presence of cyclosporine A, an immunosuppressive drug that inhibits T cell responses, so that B cell outgrowth would not be affected by the presence of endogenous EBV-specific memory T cells in the assay. Three weeks post-infection, the absolute number of cells increased in the EBV-containing wells (Figure 1A), as previously reported for another EBV strain [25]. Flow cytometry analysis of the infected cells revealed that elevated cell numbers were due to the outgrowth of EBV transformed B cells (Figure 1B). Transformed B cells up-regulated expression of the cellular activation marker CD23 early after infection as compared to the non-infected control (Figure S1). Both viruses had similar initial transformation kinetics, which was confirmed by comparable CFSE dilution in both WT and ZKO EBV transformed B cells (Figure 1C, Figure S1). Additionally, these transformed cells revealed similar proliferation rates few months after transformation as reported previously (data not shown) [26]. Thus, lytic replication, as demonstrated using ZKO EBV, does not significantly contribute to the transformation of B cells and the subsequent proliferation of EBV-transformed B cell lines, referred to as lymphoblastoid cell lines (LCLs), in vitro.

Effect of lytic replication on infection and extra-lymphoid tumorigenesis in vivo

To address whether lytic EBV replication has an effect on in vivo pathogenesis, we used a mouse model with reconstituted human immune system components (huNSG-A2tg mice). Three independent cohorts of humanized NSG-A2tg mice were infected with WT or ZKO EBV and monitored for 6 weeks after infection (Figure 2A). Whereas infrequent lytically replicating cells, which express BZLF1 protein (ZEBRA, BZ.1), were detected in the spleen sections of the majority of the WT EBV infected animals, no lytically replicating ZEBRA+ cells were detected in the spleen sections from ZKO EBV-infected mice (Figure S2). However, expression of the late lytic EBV antigen gp350 persisted in ZKO EBV-infected animals, which might indicate a low level of weak lytic replication in the absence of BZLF1 (Figure S2). EBV DNA
was detected in the blood starting at week 2. The EBV virus titer rose rapidly until week 4 and moderately from week 4 to week 6 post-infection (Figure 2B). The detection of EBV DNA in whole blood in the first and second week post-infection, however, is limited by method sensitivity (<83 EBV copies/ml). WT and ZKO EBV replicated in the blood of infected animals with similar titers at all the time points, except for week three after infection. Combined data from seven independent cohorts of animals reveals a significantly lower viremia in the ZKO EBV infected animals at that time point (Figure 2B). Notably, while blood viremia in all animals infected with ZKO EBV rarely exceeded 3×10^3 EBV copies/ml in the third week post-infection, half of the WT EBV infected animals reached up to 1×10^4 EBV copies/ml in the blood (Figure 2B). This transient difference was, however, no longer observed at later time points. These findings suggest a low frequency of spontaneous EBV lytic reactivation, which transiently elevated viral loads in half of the WT EBV-infected humanized NSG-A2tg mice.

We observed an expansion of the CD8^+ T cell compartment in the blood of infected animals, which is a hallmark of acute EBV-specific immune responses. Healthy EBV carriers as well as PBS-treated humanized mice maintain CD4^+ cells as a majority of the CD3^+ T cell population, whereas viral infection led to expansion of a subset of cytotoxic CD8^+ T cells and the inversion of the CD8^-CD4^+ ratio, which was more pronounced in WT animals compared to ZKO EBV infected animals (Figure 2C). Expanded T cells may be specific for the antigens expressed during the lytic phase of the EBV life cycle, which is reduced in ZKO EBV infected animals. While EBV-specific T cells could be detected by MHC dextramer staining in the blood of healthy EBV carriers (Figure 3A), the staining intensity for expanded blood and splenic CD8^+ T cell populations in EBV-infected huNSG-A2tg mice was low (Figure S3), as previously reported [13,27]. Interestingly, dextramer staining indicated also lytic EBV antigen specific T cell populations in the spleen of ZKO EBV infected animals, which along with the low level of gp350 staining in splenic sections could indicate a low level of weak lytic EBV replication in the absence of BZLF1. Despite this, the observed CD8^+ T cell expansion suggests that adaptive cell-mediated responses are mounted against EBV antigens, similar to what has been observed in primary EBV infection.

Similar levels of EBV DNA were detected in the spleens of WT and ZKO EBV infected animals after six weeks of infection, while spleens of PBS-treated animals were free of EBV DNA (Figure 2D, data not shown). We also examined mice for the presence of EBV-associated lesions in the abdominal organs, such as spleen, liver and kidneys, 6 weeks after infection. As previously reported, ZKO EBV infection seemed to be less aggressive than WT EBV infection and leads to fewer lymphomas during six weeks post infection [28]. However, the composite data from four and six weeks experiments demonstrated no significant difference in total number of lymphomas between WT and ZKO EBV infected mice: 12 out of 24 WT EBV infected (50%) and 8 out of 21 ZKO EBV infected (38%) animals developed lymphomas in one or more abdominal organs. At least one macroscopically visible B cell tumor was observed in the spleen of 58% of WT EBV infected animals, while ZKO EBV caused splenic tumors in 45% of the infected animals 6 weeks post-infection. Despite this fairly similar frequency in tumorigenesis in the spleen, a detailed analysis of abdominal non-lymphoid organs revealed that ZKO EBV infected animals showed a trend to reduced numbers of lymphomas in the liver (p = 0.08, Figure 2E). A single mouse developed a kidney lymphoma six weeks after WT EBV infection. When combining four and six weeks experiments from 7 independent experimental cohorts with 21–24 animals per group the trend for loss of lymphomagenesis in the liver upon ZKO EBV infection was confirmed (p = 0.06). Histological analysis of the tumors confirmed the B cell origin of the lymphoproliferative lesions. This was concomitant with expression of virus-encoded nuclear antigen EBNA2, which is essential for the establishment of latent EBV infection (Figure 2F, Figure S4). Up to 60% of B cells of WT and ZKO EBV infected animals expressed EBNA2, revealing no difference between those viruses (Figure S4). Furthermore, the number of NK cells, macrophages and neutrophils were comparable in the spleens of WT and ZKO EBV infected animals (Figure S5). Macrophage and less often NK infiltration was a predominant feature of hepatic B cell lesions in WT EBV infected animals.

Most of the infected animals had splenomegaly, and this affected WT EBV infected animals more frequently (Figure 2G). The absolute number of B cells in the spleens remained unchanged upon infection with WT or ZKO EBV as compared to uninfected mice (data not shown). Splenomegaly in WT EBV infected animals resulted from the expansion of CD3^+ T cells, while the expansion of T cells in the spleen was less pronounced in animals infected with ZKO EBV (Figure 2H). Despite moderate T cell expansion in the spleens of ZKO EBV infected animals, the CD8^-CD4^+ ratio was inverted for most of the infected animals independent of lytic replication, indicating the enrichment of cytotoxic CD8^+ T cells irrespective of lytic replication (Figure 2I).

These results indicate that lytic EBV replication affects viremia in the blood only transiently. Despite this, high viral loads lead to the subsequent expansion of CD8^+ T cells. The increased occurrence of tumors in organs other than the spleen indicates that lytic replication might contribute to the establishment of lymphoproliferative disease outside of secondary lymphoid organs in huNSG-A2tg mice.

**Lytic but not latent EBV antigen-specific CD8^+ T cells can eliminate lytically replicating cells**

As we have previously reported, depletion of CD8^+ T cells leads to poorly controlled WT EBV infection in the humanized NSG mouse model [13,14]. In order to assess the role of EBV latent and lytic protein-specific CD8^+ T cells in the control of EBV infection, we established numerous CD8^+ T cell clones specific to representative immunodominant HLA-A*0201 epitopes such as LMP2_263-267 (latent) and BMLF1_259-267 (lytic) from the blood of an HLA-A*0201 positive healthy EBV carrier (Figure 3A). After two rounds of re-expansion using the unspecific T cell mitogen phytohemagglutinin plus interleukin 2 in vitro, the cells were confirmed by dextramer staining to be pure clonal CD8^+ T cell populations. Residual activation-driven CD8 down-regulation was observed for some clones early after re-stimulation (Figure 3B). For further characterization we sequenced T cell receptor variable genes of obtained several clones (Table S1). We observed frequent TRBV20-21 usage by BMLF1-specific CD8^+ T cell. Whereas, each of BMLF1-specific CD8^+ T cell clones had a unique CDR3b and CDR3a sequence, two out of three LMP2-specific clones shared the same CDR3b and CDR3a sequences, indicating their common clonal origin and explaining their similar behavior in vitro. We also observed frequent TRBV3-1 usage by LMP2-specific T cell clones. In peptide titration assays, the functional avidity for LMP2-specific clones was greater than for BMLF1-specific clones (13.8 ± 2.2×10^7 M and 4.4 ± 2.7×10^-6 M, respectively, Figure 3C, Table S2).

To assess if these CD8^+ T clones were able to recognize cognate antigen in the context of MHC class I molecules, we performed co-culture experiments with autologous or allogeneic HLA-A*0201 negative EBV-transformed LCLs. BMLF1-specific clones secreted...
Protection by Lytic EBV Antigen Specific CD8\(^+\) T Cells

A: i.p. 10\(^5\) RIU WT / ZKO / PBS

B: Blood viral load (EBV copies/ml)

C: Blood CD8/CD4 ratio

D: Spleen viral load (EBV copies/mg)

E: Tumor incidence (\%)

F: CD20 and EBNA2

G: Spleen/body weight ratio (mg/k)

H: CD3 T cells (x10/spleen)

I: Spleen CD8/CD4 ratio
limited amounts (less than 260 pg/ml) of IFNγ upon co-culture with autologous WT EBV-transformed LCLs (WT LCLs). In contrast, no IFNγ was detected upon co-culture with control HLA-A*02 negative WT LCLs (nA2 WT LCLs) or LCLs deficient in lytic replication (ZKO LCLs) (Figure 3D, upper panel). LMP2-specific CD8+ T cell clones were capable of recognizing both autologous LCLs (WT LCLs and ZKO LCLs) expressing endogenously processed LMP2 protein, indicating sufficient presentation of LMP2 by cell lines. Interestingly, in two independent experiments the amount of IFNγ secreted by three LMP2-specific T cell clones in response to autologous ZKO LCLs accounted for 49±2% and 20±10% of IFNγ secreted towards WT LCLs (Figure 3D, lower panel). As expected, recognition of autologous WT LCLs by either BMLF1- or LMP2-specific clones was improved dramatically when targets were pre-incubated with cognate but not irrelevant peptide (Figure 3D).

In order to address whether the BMLF1- and LMP2-specific clones were cytotoxic, we first assessed the presence of the pore-forming protein perforin and the cell death inducing serine protease granzyme B by intracellular staining. While incubation with irrelevant peptide resulted in high levels of perforin and granzyme B in these clones, stimulation with relevant peptide decreased the intensity of the staining, indicating the release of cytotoxic granules upon binding of the TCR with the cognate antigen (Figure 3E).

To demonstrate that BMLF1-specific CD8+ T cell clones were able to eliminate autologous lytically EBV replicating LCLs in an MHC class I restricted manner, we co-cultured BMLF1-specific clones with autologous LCLs that spontaneously enter lytic replication (B95-8 EBV strain), or with HLA-mismatched AKBM cells that require the induction of lytic replication (Akata EBV strain) at a 1:1 effector:target ratio. After overnight co-culture, the presence of lytically active LCLs was assessed using intracellular BZLF1 (ZEBRA, Zta, BZ.1) staining. The rate of spontaneous lytic replication in autologous LCLs was very low and reached a maximum of 2.3% of total B cells at high-density growth conditions (Figure 4A), while induced lytically active AKBM cells accounted for 30% of total B cells in culture (data not shown). Several independent BMLF1-specific CD8+ T clones efficiently eliminated ZEBRA+ cells from autologous co-cultures (74–90% of ZEBRA+ LCLs), while specific killing of mismatched AKBM cells did not exceed 24% (Figure 4B). As a control, co-cultures of autologous LCLs with LMP2-specific clones led to increased frequencies of ZEBRA+ cells as compared to control wells without T cells, which is likely due to the killing of latently infected B cells and the subsequent increase in the relative number of ZEBRA+ cells (Figure 4C). Thus, BMLF1-specific CD8+ T cell clones can eliminate LCLs with lytic EBV replication in vitro and might restrict the horizontal transmission of EBV through release of newly synthesized viral infectious particles.

BMLF1-specific clones can transiently control EBV infection in vivo

In order to assess if BMLF1- and LMP2-specific CD8+ T cell clones were able to ameliorate EBV induced pathogenesis in vivo, we performed adoptive transfer experiments in huNSG-A2tg mice. Five independent cohorts of humanized NSG-A2tg mice were infected intravenously with BMLF1- or LMP2-specific CD8+ T cells in order to mimic endogenous circulating effector T cell populations, which could for example be induced by vaccination and indeed the transferred clones carried an effector memory phenotype after expansion (Figure S6). On the next day, mice were infected with either WT or ZKO EBV and the development of lymphoproliferative disease was monitored over 4 or 6 weeks (Figure 5A, Table S2). Adoptive transfer of a BMLF1-specific CD8+ T cell clones tended to reduce blood viremia in WT EBV infected animals 3 weeks post-infection, a time-point when EBV lytic replication results in elevated blood viremia in the blood of WT EBV infected animals (Figure 5B and 5B). Furthermore, ZEBRA+ B cells, containing lytically replicating WT EBV, were diminished in splenic sections of the majority of WT EBV infected animals upon transfer of BMLF1-specific CD8+ T cell clones (Figure 5C). No ZEBRA+ cells were found in splenic sections from ZKO EBV infected animals (Figure S2). However, the amelioration of the EBV viremia in the blood of WT EBV infected animals by adoptive transfer of BMLF1-specific CD8+ T cell clones varied in the experimental cohorts of humanized NSG-A2tg mice (Figure 5D). Taken together, these five adoptive transfer experiments demonstrated that most EBV-infected CD8+ T cells prior to WT EBV infection were less likely to develop high blood viremia (defined as greater than 3×10^7 EBV/ml) at three weeks post WT EBV infection: only 3 animals out of 14 (21%) developed high viremia after BMLF1-specific T cell transfer as compared to 6 out of 14 (43%) after LMP2-specific T cell transfer or 7 out of 16 (44%) animals in the non-treated group (Figure 5E). Adoptive transfer of LMP2-specific T cells did not seem to have an ameliorating effect on EBV infected animals, however ZKO EBV infected animals might have benefitted from the T cell infusion on the third and fourth week after infection, possibly due to elimination of latently infected B cells from blood circulation.

Adoptive transfer of partially mismatched T cells in humans is considered relatively safe [29], despite rare cases of mild graft-versus-host disease [30]. Humanized mice, which received partly matched cytotoxic T cell clones, had no signs of graft-versus-host disease during the experiment. We attempted to identify the
defining characteristics for protective EBV specific T cell clones by examining peptide epitope avidity, functional potency and surface phenotype, but we were unable to identify any in vitro predictors for in vivo protection. Additionally, attempts to find the transferred cells by specific amplification of mismatched MHC class I alleles of the donor using DNA from the blood at weeks 2, 3 and 4 post-infection, as well as in spleens and tumor samples upon termination of the experiments were limited by the sensitivity of our PCR-based method (1% of CD8\(^+\) T cells) and did not detect persistence of the transferred clonal T cell populations (data not shown).

We also assessed the effect of adoptively transferring lytic and latent antigen specific T cell clones on the formation of lymphoproliferative disease induced by WT and ZKO EBV. Consistent with our earlier experiments, EBV infected animals developed splenic EBV-associated B cell lymphomas with...
Protection by Lytic EBV Antigen Specific CD8+ T Cells

A

huNSG-A2tg
n=94, 5 donors

i.v. 10⁶ BMLF1-T / LMP2-T

i.p. 10⁴ RIU WT / ZKO EBV

day (-1) day 0

2 3 4 weeks post-infection

B

2 weeks p.i.

3 weeks p.i.

4 weeks p.i.

Blood viral load (EBV copies/ml)

WT/no T

WT/BMLF1-T

WT/LMP2-T

C

ZEBRA (cells/10⁶ RBCs)

WT/no T

WT/BMLF1-T

WT/LMP2-T

D

2 weeks p.i.

3 weeks p.i.

4 weeks p.i.

Blood viral load (EBV copies/ml)

WT/no T

WT/BMLF1-T

WT/LMP2-T

E

Incidence of high viremia

EBV copies/ml

WT/no T

WT/BMLF1-T

WT/LMP2-T

F

Tumor incidence (%)

spleen

liver

kidney

PBS

WT/no T

WT/BMLF1-T

WT/LMP2-T

ZKO/no T

ZKO/BMLF1-T

ZKO/LMP2-T
Figure 5. Lytic EBV antigen specific CD8+ T cell clones can transiently control EBV infection in humanized mice. (A) Schematic diagram of adoptive transfer experiments. BMLF1- or LMP2-specific CD8+ T cell clones were transferred i.v. into human NSG-A2tg mice. The next day, mice were infected i.p. with WT EBV, ZKO EBV or PBS. Mice were bled weekly starting at week 2 post-infection and were euthanized at week 4 or 6 post-infection (n = 94). (B) Whole blood EBV loads for the second, third and fourth week post-infection of mice, which received BMLF1- or LMP2-specific CD8+ T cell clones prior to WT or ZKO EBV infection, is demonstrated for a representative experiment (cohort 2, n = 3–4 per group, total n = 21). Dotted line represents detection limit. (C) Representative figures and quantification of the ZEBRA+ cells in the spleen sections of WT EBV infected huNSG-A2tg mice, which received no T cells, BMLF1-specific T cell clones and LMP2-specific T cell clones for two initial experiments (n = 6–7 per group, total n = 20). Data represent the mean ± SEM (p E BMLF1-T vs LMP2-T = 0.07). Scale bars, 100 μm. (D) Whole blood EBV loads for the second, third and fourth week post-infection of mice from five independent adoptive transfer experiments (n = 94). Data are represented as the mean ± SEM. (E) Incidence of high blood viremia on the third week post-infection in the WT EBV infected mice, which received no T cells, BMLF1-specific T cell clone and LMP2-specific T cell clone (n.s. by one-way Chi-square). (F) Tumor incidence per organ is presented as the percentage of animals which developed visible tumors in the organs of the abdominal cavity (n = 92).

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comparable frequency irrespective of lytic replication, whereas extra-lymphoid lymphomas were less frequent when EBV lytic replication was impaired (Figure 5F). Lymphomas from both WT and ZKO EBV infected animals contained EBNA2+ B cells, indicating the latency III program of the EBV life cycle. This form of latency is characterized by expression of all latent EBV antigens, namely EBNA proteins 1, 2, 3A, B, C, -LP, and LMP proteins 1 and 2. Latency III is commonly found in the lymphoproliferative lesions of immune-compromised patients.

Our study demonstrates subtle effects of EBV lytic replication on blood viremia and suggests that endogenous EBV lytic antigen-specific T cells might be primed in the huNSG-A2tg mice. Furthermore, we observed that EBV lytic replication may be required for the establishment of extra-lymphoid lymphoproliferative lesions in our mouse model. In healthy EBV carriers, memory CD8+ T cell specific for lytic and latent antigens of EBV are found in significant numbers. In this study, we provide the first evidence that BMLF1-specific CD8+ T cells eliminate EBV-transformed B cells undergoing lytic replication in vitro and in vivo, and are able to transiently control EBV viremia in the blood of infected humanized mice.

Discussion

One form of uncontrolled lytic EBV replication is infectious mononucleosis. This symptomatic primary EBV infection occurs more frequently when primary viral infection is delayed into adolescence. It is characterized by high viral titers, particularly in saliva, and by CD8+ T cell lymphocytosis [31]. Otherwise, pathology by lytic replication of EBV is rare in healthy human carriers and EBV transformed lymphoblastoid cell lines switch only at low frequencies into lytic replication in vitro. Despite these findings, the role of lytic virus replication during EBV infection is only poorly understood. This is in part because the number of experimental systems that are permissive for EBV infection limits studies on EBV biology in vivo. We therefore examined EBV lytic replication in humanized NSG-A2tg mice and the functional role of BMLF1-specific T cells in the control of EBV infection in vivo and in vitro. This in vivo model of EBV infection recapitulates hallmark features of innate and adaptive cell-mediated immune control of EBV, but lacks the development of humoral immune responses and efficient mucosal homing of human immune cells [10].

Our longitudinal in vivo study suggests that EBV lytic replication is limited in our humanized mouse model and plays a transient role in dissemination of the EBV early after infection and prior to innate and adaptive immune control of lytically replicating cells: only mice infected with lytic replication competent EBV, but not lytic replication compromised EBV, developed high viremia in the blood on the third week post-infection.

The massively expanding CD8+ T cells in infectious mononucleosis patients are mostly directed against lytic EBV antigens [32], suggesting that primarily lytic viral replication is ill-controlled during infectious mononucleosis. Particularly, CD8+ T cells that are specific for immediate early and early EBV antigens are observed at high frequencies [33]. These events correlate with more efficient LCL recognition by early lytic EBV antigen specific CD8+ T cells, while late lytic EBV antigen recognition by specific CD8+ T cells is presumably compromised by viral immunoevasins targeting MHC class I antigen presentation [34]. In our humanized mouse model we observed that high blood viral load frequently correlated with the number of EBV-specific T cells found in the spleens of the WT and ZKO EBV infected animals as detected by IFNγ ELISPOT assays (data not shown). Also, we report the inversion of the CD8:CD4 ratio in blood of infected animals, which is less pronounced in animals infected with EBV that is inhibited in its lytic replication. Our lab previously demonstrated that a significant proportion of EBV-specific T cells are primed specifically to lytic antigens rather than latent antigens in humanized NSG-A2tg mice [13]. These data suggest that lytic EBV antigens contribute to CD8+ T cell expansion after infection.

However, in our current study, we observe more EBV-specific T cells among splenocytes from both WT and ZKO EBV infected mice, if we stimulated the splenocytes with LCLs capable of lytic EBV replication (data not shown). Together with the observation in vitro that LMP2-specific CD8+ T cell clones consistently secreted more IFNγ in response to autologous WT LCL than to ZKO LCL stimulation, this might indicate decreased antigen presentation of LCLs deficient in lytic replication. Additionally, detection of EBV-specific T cells using HLA-A*02 dextramers bound to human immunodominant epitopes of EBV lytic and latent proteins was difficult due to low staining intensity (Figure S3). The process of T cell education of the T cells in humanized mice remains unclear. In these mice human T cells are educated on mouse thymic epithelial cells, human bone marrow derived cells or transgenic human MHC class I molecules stabilized by murine β2-microglobulin. We can speculate that this may result in T cells with TCRs, that are suboptimally recognized by human HLA-A*02 dextramers. Alternatively, maintenance of high TCR density on T cells in the mouse host might be compromised by lack of human cytokines and further compromised due to the high antigenic load after EBV infection.

Pudney and colleagues demonstrated direct recognition of lytically infected cells by CD8+ specific cells obtained from infectious mononucleosis patients [34]. However, in these studies, the authors could only demonstrate cytokine production by lytic EBV antigen specific CD8+ T cell clones in response to wild-type, but not BZLF1-deficient LCLs. Extending these findings, we can now document that BMLF1 specific CD8+ T cell clones eliminate ZEBRA expressing EBV infected B cells in LCL cultures. BMLF1-specific clones eliminated EBV lytically replicating autologous LCLs, but not MHC-class I mismatched control LCLs. Thus, lytic EBV antigen specific T cell clones contribute to the immune response.
control of EBV infection by eliminating lytically virus replicating cells.

Another EBV-associated pathological condition in solid-organ and hematopoietic stem cell transplant patients is post-transplant lymphoproliferative disease (PTLD). This disease almost exclusively involves EBV-associated B cell proliferations characterized by expression of all latent proteins of EBV (latency III). In immune-compromised PTLD patients, mostly polyclonal B cell lesions could be found in secondary lymphoid organs and extralymphoid tissues [2]. EBV infection of humanized mice leads to the formation of lymphoproliferative B cell lesions resembling PTLD in histology and expression pattern of viral proteins. EBER+ EBV-transformed B cells were found in similar multifocal sites independently of lytic replication in humanized NSG mice, which were implanted with human fetal thymus and liver fragments in addition to the fetal liver derived CD34+ stem cell transfer of our model [28]. Our study for the first time demonstrates that EBV may require lytic replication to efficiently establish lymphomas in non-lymphoid tissues like kidneys and liver. This could result from decreased expression of growth factors in ZKO-derived LCLs, which may be more critical for the establishment of lymphoproliferative lesions outside of secondary lymphoid organs [26].

One therapy for patients with PTLD is adoptive T cell transfer. Both CD8+ and CD4+ EBV-specific T cells are being explored for adoptive transfer into PTLD patients [35,36,37,38,39]. Here we report, that adoptive transfer of BMLF1-specific CD8+ T cell clones into WT EBV infected humanized NSG mice transiently controlled EBV infection and was able to eliminate BZLF1 expressing cells from huNSG-A2tg spleens. However, given the limitation of detecting lytically replicating cells indirectly by examining blood viremia, this transfer did not result in significant and reproducible long-term control of EBV infection. We addressed whether fluctuations in the control of lytic replication in vivo was due to the characteristics of the transferred clones (epitope avidity, phenotype etc). However, no clear correlations could be found (Table S2). Alternatively, the inefficiency of clonal T cell transfer may result from the overall HLA mismatch – except matched HLA-A2 expression – between reconstituted human immune system components and transferred T cell clones. This might have led to their alloreactive rejection by the host and the limited persistence of the transferred cells in vivo. Clinical studies suggest that a positive outcome of cytotoxic T cell transfer into PTLD patients positively correlates with the matching of donor T cells to the recipient [38]. Despite this, an ameliorating effect of adoptive transfer in PTLD patients could also be observed in partly matched settings [30,39]. Within five cohorts of huNSG-A2tg mice reconstituted with one (Cohorts 2, 3) or more (Cohorts 1, 4, 5) matched HLA alleles, the most pronounced effect of BMLF1-specific clone on viremia in blood was observed irrespective of the number of matched MHC class I alleles (Cohorts 1 and 2 at week 3, cohort 3 at week 4). Likewise, adoptive transfer of partly matched T cell clones in primates revealed no correlation between persistence of the infused cells and genetic backgrounds of donor and recipient [40]. Taken into account that WT EBV infection caused high viremia in half of the infected humanized NSG-A2tg mice, our study indicates that BMLF1-specific CD8+ T cells seem to maintain some immune control and can eliminate B cells undergoing lytic replication. Our data provide a rationale for reconsidering lytic EBV antigens for EBV specific vaccination, particularly during uncontrolled lytic replication and to limit EBV associated tumorigenesis in non-lymphoid tissues, which seems to be supported by lytic replication per se. Accordingly, lytic EBV antigens should be explored as vaccine components to mitigate infectious mononucleosis.

Currently, several EBV specific vaccine candidates are being explored. These include recombinant gp350 protein formulations that aim to elicit neutralizing antibodies against the main attachment protein for complement receptor 1 and 2 (CD35 and CD21) dependent B cell infection by EBV [41,42,43]. Furthermore, recombinant viral vaccines encoding latent EBV antigens, for example modified vaccinia virus Ankar (MVA), encoding a EBNA1-LMP2 fusion protein, are being explored [44,45]. EBV derived virus like particles devoid of viral DNA have been investigated as vaccine candidates [46,47,48,49]. Finally, we have been characterizing EBNA1 targeting to dendritic cells as a vaccine formulation [16,17,50]. However, all of these vaccination schemes include either latent or late lytic EBV antigens. They do not provide any stimulation for the immediate early and early lytic EBV antigen specific CD8+ T cells, which constitute the most frequent EBV specificities in both symptomatic primary infection and healthy EBV carriers. Since these specificities, nevertheless, efficiently recognize and eliminate lytically replicating B cells (34 and our data), they should be included into vaccine formulations against infectious mononucleosis and possibly also against EBV associated malignancies in extra-lymphoid tissues.

Supporting Information

Figure S1 ZKO EBV deficient in lytic replication leads to up-regulation of B cell activation marker CD23 and subsequent proliferation. Bulk PBMCs were inoculated with WT EBV, ZKO EBV or mock treated in the presence of cyclosporine A. Representative FACS plots of mock-treated, WT EBV and ZKO EBV infected PBMCs on the day 3, 5, and 7 after infection and pre-gated on CD191 B cell populations of live CD3− cells. The experiment was done in duplicates. Similar results were obtained for two donors. (TIF)

Figure S2 ZEBRA+ and gp350 cells in the spleen sections derived from WT and ZKO EBV infected animals six weeks post-infection. Spleen sections were screened for presence of ZERBA+ (upper row) or gp350+ (lower row) cells at ×400 magnification and the total spleen area was defined at ×40.
Figure S5 NK and myeloid cell composition in the spleens and livers of WT and ZKO infected mice 6 weeks after infection. (A) Quantification of cells expressing NKP46 (n.s. P WT vs ZKO = 0.44), human CD68 (n.s. P WT vs ZKO = 0.54), and neutrophil elastase (n.s. P WT vs ZKO = 0.72) in the spleen sections from WT and ZKO EBV infected mice. (B) Quantification of cells expressing NKP46 (n.s. P WT vs ZKO = 0.25), human CD68 (P WT control vs tumor = 0.05) and neutrophil elastase (n.s. P WT vs ZKO = 0.11) in the hepatic tumors and control liver tissue in WT and ZKO EBV infected mice. (C) Percentages of CD45+CD19neg lymphocytes (n.s. P = 0.90) and CD45+CD19neg (n.s. P = 0.49) leukocytes, but not lymphocytes within human CD45+ cell population in the spleens of WT and ZKO EBV infected huNSG-A2tg mice. Data represent composite data from three independent experiments as mean ± SEM.

Figure S6 Expression of activation and homing markers by LMP2- and BMLF1-specific CD8+ T cell clones. Flow cytometry plots demonstrate activation and homing markers for an isotype control and two pairs of BMLF1-specific and LMP2-specific CD8+ T cell clones used for adoptive transfer into humanized mice. Pre-gated on the population of live human CD45+CD3neg+ cells in the spleens of WT and ZKO EBV infected mice. Ann Rev Immunol 25: 587-617.

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