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Attenuated immune control of Epstein–Barr virus in humanized mice is associated with the multiple sclerosis risk factor HLA-DR15

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Immune responses to Epstein–Barr virus (EBV) infection synergize with the main genetic risk factor HLA-DRB1*15:01 (HLA-DR15) to increase the likelihood to develop the autoimmune disease multiple sclerosis (MS) at least sevenfold. In order to gain insights into this synergy, we investigated HLA-DR15 positive human immune compartments after reconstitution in immune-compromised mice (humanized mice) with and without EBV infection. We detected elevated activation of both CD4^{+} and CD8^{+} T cells in HLA-DR15 donor-reconstituted humanized mice at steady state, even when compared to immune compartments carrying HLA-DRB1*04:01 (HLA-DR4), which is associated with other autoimmune diseases. Increased CD8^{+} T cell expansion and activation was also observed in HLA-DR15 donor-reconstituted humanized mice after EBV infection. Despite this higher immune activation, EBV viral loads were less well controlled in the context of HLA-DR15. Indeed, HLA-DR15-restricted CD4^{+} T cell clones recognized EBV-transformed B cell lines less efficiently and demonstrated cross-reactivity toward allogeneic target cells and one MS autoantigen. These findings suggest that EBV as one of the main environmental risk factors and HLA-DR15 as the main genetic risk factor for MS synergize by priming hyper-reactive T-cell compartments, which then control the viral infection less efficiently and contain cross-reactive CD4^{+} T cell clones.

☒ See accompanying commentary by Olsson

🖳 Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) that often leads to substantial decline of sensory, motor, autonomic, and neurocognitive functions. While considered to be an autoimmune, CD4$^+$ T cell-mediated disorder, its etiology still remains poorly understood [1].

Genome-wide association studies have identified the human leukocyte antigen (HLA) allele HLA-DRB1*15:01 (HLA-DR15) as the strongest associated genetic risk factor for MS. Other identified loci represent mainly immunologically relevant genes, particularly suggesting a role for helper T-cell differentiation and function in MS pathogenesis [2,3].

As the genetic background confers only partial risk for the disease, the role for environmental risk factors and their synergies with MS genetics have become increasingly apparent, especially in the first two decades of life [4]. Altered immune response to the Epstein–Barr virus (EBV), including immunopathology during the acute primary EBV infection, called infectious mononucleosis (IM), is associated with MS and synergizes with HLA-DR15 resulting in a sevenfold increased risk of the disease [5]. The mechanisms behind this synergy remain unclear.

EBV is a ubiquitous γ-herpesvirus infecting around 95% of the world adult population [6]. Following oral transmission, it starts a latent, growth transforming infection of B cells and persists in their memory compartment [7]. EBV is generally acquired early in childhood and leads to an asymptomatic primary infection at young age. However, if acquired in adolescence or early adulthood, it can manifest as IM [6], an acute illness sparking a vigorous immune response, characterized mainly by immensely expanded EBV-specific CD8$^+$ T cells [8,9]. Immunopathogenic and exaggerated T-cell responses are likely involved in IM being a phenomenon largely of late primary infection [10,11].

Viral and infectious agents have since a long time been thought to play a role in MS [12,13]. Importantly, two studies demonstrated near-complete EBV seropositivity in pediatric MS patients [14,15]. In addition, adult MS patients all became EBV positive prior to disease onset, suggesting that MS risk is extremely low in EBV seronegative individuals, but increases sharply after EBV infection [16] and even further after IM [17,18]. Indeed, higher anti-EBV antibody titers before MS onset [19] are associated with disease risk [20]. The genetic risk for elevated anti-EBV nuclear antigen 1 (EBNA1) IgG titers also correlates with MS development [21] and the risk markedly increases in HLA-DRB1*15:01 positive individuals [22].

However, adequate models are missing to study these interactions. Using a humanized mouse model (huNSG) of EBV infection, we investigated the role of HLA-DR15 in the immune control of EBV. Using data accumulated in our laboratory over several years, we were able to stratify animals reconstituted with HLA-DR15 positive and negative donors. We have demonstrated that T cells from HLA-DR15 positive donors have higher basal activation levels. Despite enhanced T-cell proliferation, EBV viral loads are elevated, which suggests an impaired T-cell-mediated immune control of EBV under the restriction of HLA-DR15. Importantly, we also find evidence for CD4$^+$ T-cell cross-reactivity to the MS autoantigen myelin basic protein (MBP), as well as increased alloreactivity in CD4$^+$ T-cell clones derived from HLA-DR15 individuals. Our data offer insights into the gene–environment interactions that could lead or add to the perturbed T-cell homeostasis in MS.

Results

HLA-DR15$^+$ donors reconstitute hyperactive T cells in huNSG animals

We first wanted to investigate the effect of HLA-DR15 positive donors on T-cell activation at steady state in humanized NOD-scid IL2 receptor γ-chain-deficient (huNSG) mice, as the major histocompatibility complex (MHC) Class II molecule HLA-DR15 carries the strongest genetic risk for MS [2]. To this end, we combined and stratified data from more than 3500 mice spanning 6 years of reconstitution experiments. These data were opportunistically pooled and was obtained by the same flow cytometry panel throughout (Fig. 1A and B, gating strategy is shown in Supporting Information Figs. S1 and S2). Briefly, following intrahepatic injection of human fetal liver-derived human hematopoietic progenitor cells (i.e., donor) shortly after birth, animals were routinely bled after 3 months to check for the reconstitution of human immune system components in peripheral blood at steady state. Animals reconstituted with HLA-DR15 positive donors demonstrated a higher activation of total T cells, CD4$^+$, and CD8$^+$ T cells when compared to animals reconstituted with HLA-DR15 negative donors (Fig. 1A, Supporting Information Fig. S2A and B). This T-cell activation was mainly observed among CD4$^+$ T cells, which constitute around two-thirds of the T cells after reconstitution. Furthermore, a higher activation frequency in the T-cell compartment was also seen upon reconstitution with HLA-DR15 positive donors when compared with donors positive for HLA-DR4 (Fig. 1B), an MHC Class II molecule implicated in other autoimmune diseases, including rheumatoid arthritis and type I diabetes [23,24]. Interestingly, a difference in CD4$^+$ T cell activation was not observed when HLA-DR4 positive versus negative donors (HLA-DR15 exclusive) were compared (Supporting Information Fig. S2C). These findings illustrate that HLA-DR15 hematopoietic progenitor cells reconstitute hyperactive T cells in the huNSG mouse model at steady state.

HLA-DR15 alters CD8$^+$ T-cell frequencies and activation during EBV infection of huNSG mice

One of the hallmarks of IM is the vast expansion of CD8$^+$ T cells, which are key to the immune control of EBV [25]. As the huNSG mouse model has been successfully used to study IM-like EBV infection [26], we examined the expansion of T cells following EBV infection in animals reconstituted with donors positive or negative for HLA-DR15.
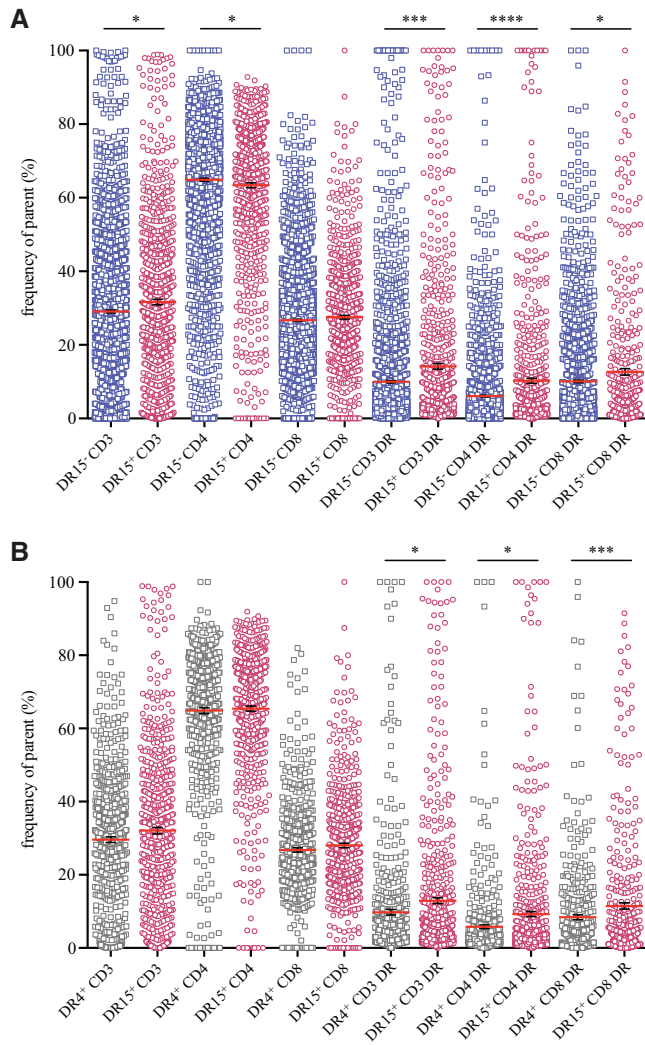
Figure 1. Higher T-cell activation in HLA-DR15$^+$ donor reconstituted huNSG mice. Three months postreconstitution, animals were bled and analyzed for reconstitution of human immune system components in peripheral blood. Data were stratified into animals reconstituted with HLA-DR15 negative (DR15$^-$), HLA-DR15 positive (DR15$^+$) (**A**) and HLA-DR4 positive (DR4$^+$), HLA-DR15 positive (DR15$^+$DR4$^-$) (**B**) donors and collected by flow cytometry. T-cell activation was analyzed by positive staining of HLA-DR (DR). Data shown are combined from >96 donors (*n* of animals = 3579, CD4$^+$ T-cell activation n = 2991) (**A**) and 51 donors (*n* of animals = 1230, CD4$^+$ T-cell activation n = 1135) (**B**). *p < 0.05, ***p < 0.001, ****p < 0.0001; significant comparisons are indicated and significance is based on the Mann–Whitney *U* test. Each symbol indicates one animal (mean ± SEM).

During these experiments, animals were infected with a high dose (10^5 infectious units) of the B95-8 strain of EBV and monitored for 4–6 weeks (Fig. 2A). We first wanted to investigate T-cell expansion following EBV infection at time of sacrifice (Fig. 2B–I). Even though EBV infection induces a large expansion of CD8$^+$ T cells, we observed even higher frequencies (Fig. 2B and C) and total numbers (Fig. 2D and E) in huNSG mice reconstituted with HLA-DR15 positive versus negative donors in both blood and spleen. While we did not observe a difference in the frequencies of CD4$^+$ T cells (Fig. 2F and G) and total numbers in spleen (Fig. 2I),
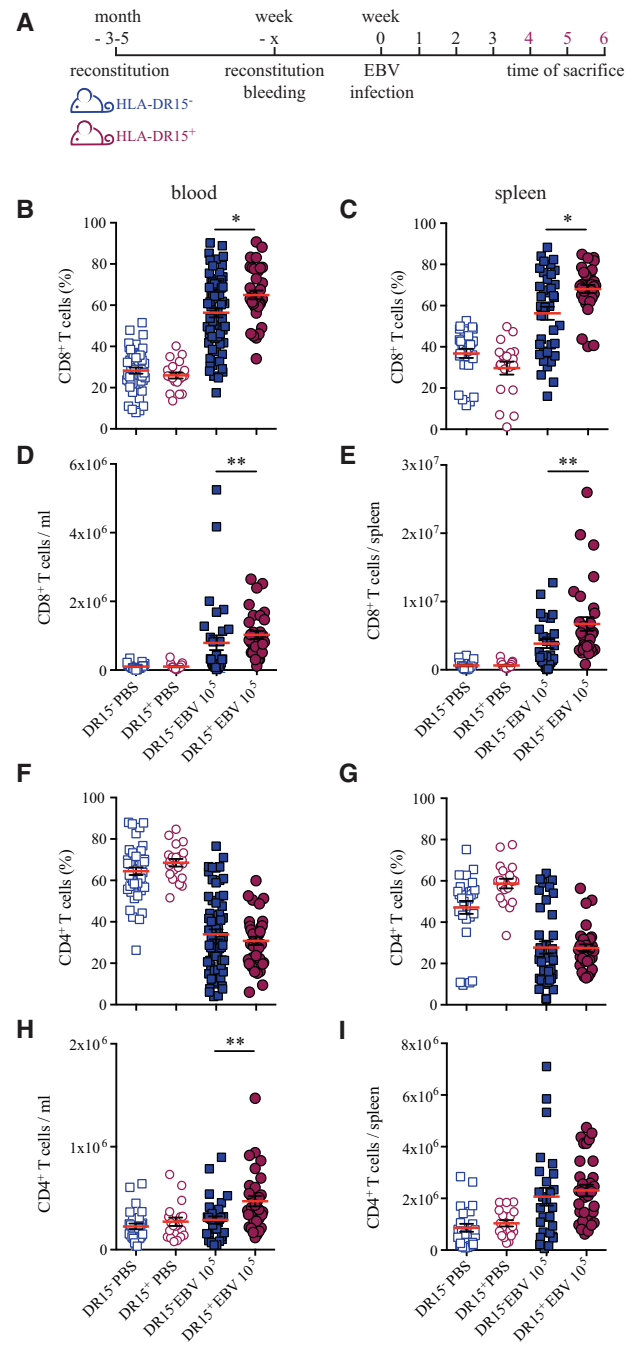
Figure 2. EBV-infected HLA-DR15$^+$ reconstituted mice have higher frequencies of CD8$^+$ T cells. Schematic depiction of analyzed EBV infection experiments. After 3-5 months of human immune compartment reconstitution, animals were injected with 10^5 EBV infectious units or PBS intraperitoneally and monitored for 4–6 weeks by visual examination, weight measurements, and flow cytometry (**A**). At time of sacrifice (4–6 weeks postinfection), frequencies of CD8$^+$ T cells in blood and spleen (**B, C**), total numbers of CD8$^+$ T cells in blood and spleen (**D, E**), frequencies of CD4$^+$ T cells in blood and spleen (**F, G**), and total numbers of CD4$^+$ T cells in blood and spleen (**H, I**), respectively, were analyzed and stratified into animals reconstituted with HLA-DR15 negative (DR15$^-$) and HLA-DR15 positive (DR15$^+$) donors. Data shown are combined from 11 to 24 experiments and contain 19–65 animals per group. *p < 0.05, **p < 0.01; significant comparisons are indicated and significance is based on the Mann–Whitney *U* test. Each symbol indicates one animal (mean ± SEM).
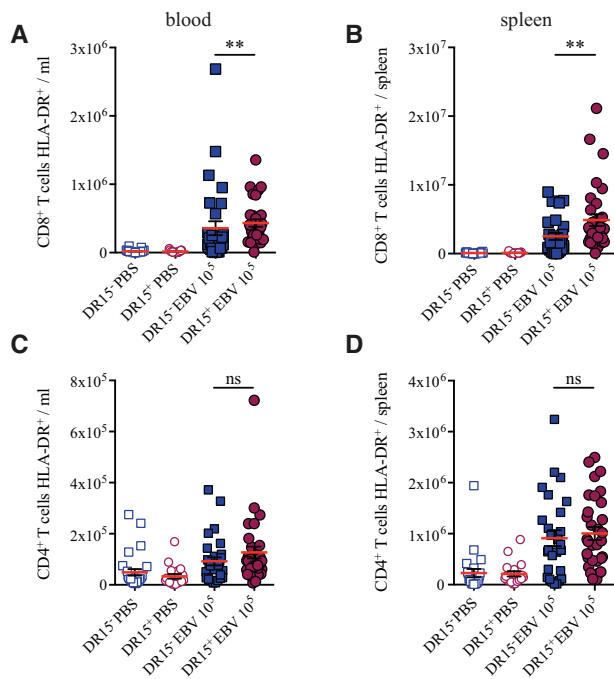
Figure 3. EBV-infected HLA-DR15+ reconstituted mice have higher numbers of activated CD8+ T cells. As indicated, animals were injected with 10^5 EBV infectious units or PBS intraperitoneally. At time of sacrifice (4–6 weeks postinfection), total numbers of activated CD8+ T cells (**A, B**) and activated CD4+ T cells (**C, D**) in blood and spleen, respectively, were analyzed and stratified into animals reconstituted with HLA-DR15 negative (DR15−) and HLA-DR15 positive (DR15+) donors. T-cell activation was analyzed by positive staining of HLA-DR by flow cytometry. Data shown are combined from 11 to 13 experiments and contain 19–32 animals per group. *$p < 0.05$, **$p < 0.01$; significant comparisons are indicated and significance is based on the Mann–Whitney U test. Each symbol indicates one animal (mean ± SEM).

we did see higher total numbers of CD4+ T cells in the blood of HLA-DR15-reconstituted animals (Fig. 2H).

In addition, there were higher numbers of activated CD8+ T cells (Fig. 3A and B) in blood and spleen of HLA-DR15-reconstituted animals, even though no differences were observed in the activation of the CD4+ T-cell compartment (Fig. 3C and D). Similar results were also observed for total T-cell frequencies, numbers, and activation in blood and spleen (Supporting Information Fig. S3). These observations suggest a more robust, HLA-DR15-associated T-cell proliferation and activation following EBV infection.

Reduced EBV-specific immune control in HLA-DR15-reconstituted huNSG

In our huNSG model of EBV infection (10^5 infectious particles), blood viral DNA loads are usually detectable at 3 weeks postinfection [26]. Interestingly, we observed a higher EBV viral load at time of sacrifice in animals reconstituted with HLA-DR15 positive versus HLA-DR15 negative donors (Fig. 4A) or HLA-DR4 positive donors (Fig. 4B), even though their initial reconstitution

was similar (Supporting Information Fig. S4A and B). It has previously been shown that CD8+ T-cell expansion and blood viral load are positively correlated with disease severity in IM patients [8]. Indeed, we observed positive correlations between EBV viral loads in the blood and frequencies of activated CD8+ T cells in the blood (Fig. 4C), as well as EBV viral loads in the spleen and frequencies of activated CD8+ T cells in the spleen (Fig. 4D). Similar correlations of EBV viral loads with total numbers of activated CD8+ T cells were also observed in blood and spleen (Supporting Information Fig. S4C and D). These data, together with increased frequencies (Fig. 2B–E) and activation (Fig. 3A and B) of CD8+ T cells, suggests a reduced EBV-specific immune control despite significant CD8+ T-cell expansion that is associated with HLA-DR15.

The humoral immune response has been challenging to study in the huNSG and other humanized mouse models. These animals lack normal LN development and have poor GC organization, thereby hampering B-cell class switching and affinity maturation [26]. Upon measuring serum anti-EBNA1 immunoglobulin M (IgM), we were, however, able to find a fraction of EBV-infected animals positive for these antibodies (Fig. 4E). The humoral anti-EBNA1 IgM titer from animals with a humoral response above threshold correlated with blood EBV viral loads (Fig. 4F); however, due to the infrequent IgM responses, we were not able to compare between HLA-DR15 positive and negative animals. Thus, EBV is less well immune controlled in the context of HLA-DR15; higher viral loads were observed despite elevated CD8+ T-cell expansion.

Myelin antigen recognition, alloreactivity, and lower specificity by HLA-DR15-restricted T cells

EBNA1-specific CD4+ T cells have been previously shown to cross-react to myelin antigens [27]. We were therefore interested in examining if CD4+ T cells isolated from EBV-infected animals would cross-react to peptide pools spanning MBP and myelin oligodendrocyte glycoprotein (MOG). These two myelin-derived MS autoantigens have been previously studied for their immunodominance in humans and/or encephalitogenic potential in animal models (reviewed in [28,29]) and show comparable mathematically predicted epitope binding affinity to HLA-DR15 and HLA-DR4 molecules [30]. Furthermore, immunodominant high avidity target peptides of MBP are known to be recognized in the context of both HLA-DR15 and HLA-DR4 [30,31]. CD4+ T cells from the splenocytes of EBV-infected animals were expanded before being stimulated with MBP, MOG, or EBV peptide pools. Frequency of IFN-γ-producing cells was then measured in the ELISpot assay. Interestingly, we observed that CD4+ T cells from nearly all HLA-DR15 and from a fraction of HLA-DR4-reconstituted animals produced IFN-γ in response to MBP stimulation (Fig. 5A, Supporting Information Fig. S5A). EBV-derived peptide reactivity was more pronounced in a subset of HLA-DR4-reconstituted animals. These findings suggest MBP recognition by HLA-DR15 positive CD4+ T cells of EBV-infected humanized mice.
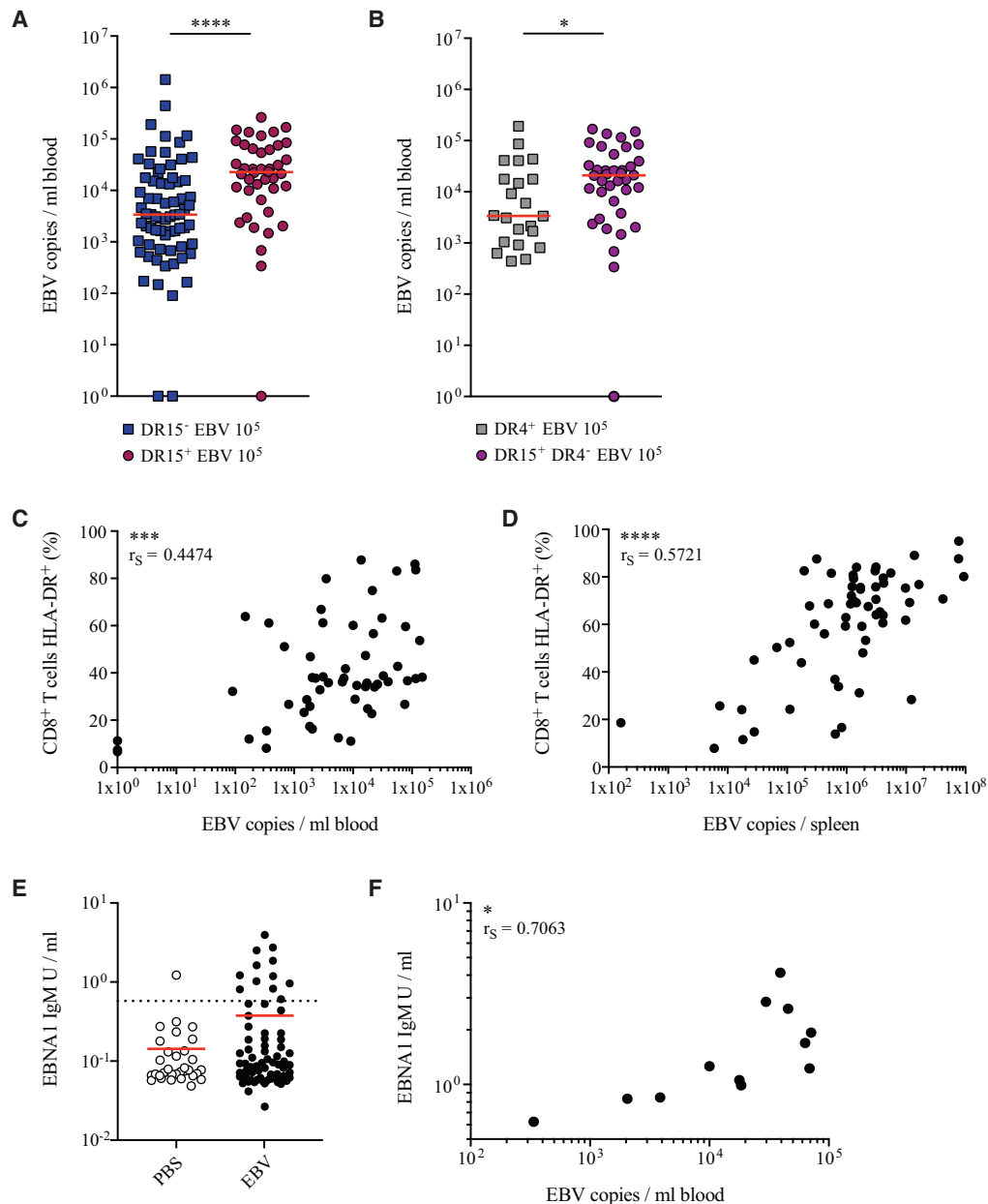
Figure 4. EBV viral loads in huNSG mice correlate with activated CD8$^+$ T cells and humoral IgM responses and are higher in HLA-DR15$^+$ reconstituted animals. Blood EBV viral loads at time of sacrifice (4–6 weeks postinfection with 10^5 EBV infectious units) of animals reconstituted with HLA-DR15 positive (DR15$^+$), HLA-DR15 negative (DR15$^-$) ($n = 42$ and 70, respectively) (A), HLA-DR15 positive DR4 negative (DR15$^+$DR4$^-$) and HLA-DR4 positive (DR4$^+$) ($n = 38$ and 22, respectively) (B) donors, measured by TaqMan real-time PCR. Red lines depict the median and significance is based on the Mann–Whitney U test (A, B). (C) Frequencies of activated CD8$^+$ T cells in blood were plotted relative to blood EBV viral loads ($n = 56$). (D) Frequencies of activated CD8$^+$ T cells in spleen were plotted relative to spleen EBV viral loads ($n = 60$). T-cell activation was analyzed by positive staining of HLA-DR by flow cytometry (C, D). (E) Animals were injected with 10^5 EBV infectious units or PBS intraperitoneally. At time of sacrifice, serum was collected and analyzed for anti-EBNA1 IgM antibodies via ELISA. Red lines depict the mean and dotted line represents threshold set at mean $\pm$ 2$\times$ SD of PBS animals (n PBS $= 33$, EBV $= 71$). (F) Levels of anti-EBNA1 IgM antibodies of animals above threshold were plotted relative to EBV viral loads in blood. Data shown are combined from 11 to 28 experiments. $^*p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$; significant comparisons are indicated. All correlations were analyzed using the nonparametric Spearman correlation, which examines rank correlation. Each symbol indicates one animal.

Considering the essential role of T cells in the immune control of EBV infection [25] and our results suggesting that EBV might be less well controlled under HLA-DR15 restriction, we examined EBV-infected B-cell recognition of individual CD4$^+$ T-

cell clones. For this purpose, CD4$^+$ T-cell clones were isolated from EBV-infected, HLA-DR15- or HLA-DR4-reconstituted animals using bare lymphocyte syndrome lymphoblastoid cell lines (BLCLs; cells isolated from bare lymphocyte syndrome patients
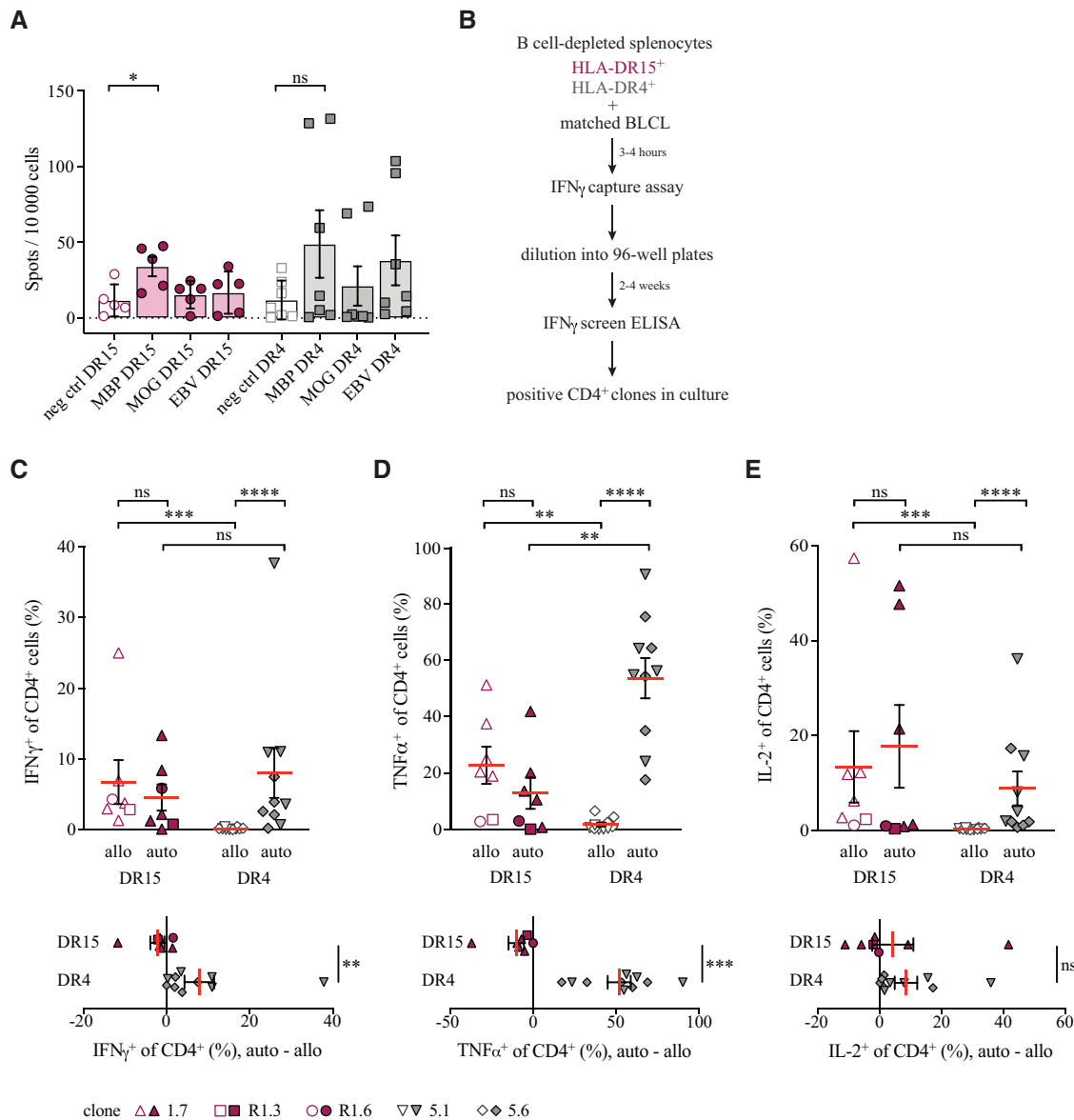
Figure 5. HLA-DR15-restricted EBV-infected B cell-specific CD4+ T-cell clones show cross-reactive cytokine production. Splenocyte-derived and expanded CD4+ T cells from HLA-DR15 (DR15) or HLA-DR4 (DR4) reconstituted animals stimulated by MBP, MOG, or EBV peptides and analyzed for IFN-γ production via ELISpot assay (**A**). No peptide addition served as negative control (neg ctrl) and each point indicates one well. Animals from two experiments were used (eight in total, two per experiment and condition). Schematic depiction of T-cell cloning (**B**). CD4+ T-cell clones were cloned from EBV-infected animals using bare lymphocyte syndrome lymphoblastoid cell lines (BLCLs) transfected with single MHC class II molecules as targets using the IFN-γ capture assay. HLA-DR15 (DR15) or HLA-DR4 (DR4)-restricted CD4+ T-cell clone functionality was examined through the production of the cytokines IFN-γ (**C**), TNF-α (**D**), and IL-2 (**E**) after 8 h of incubation with allogeneic (allo) or autologous (auto) BLCL (HLA-DR15-expressing or HLA-DR4-expressing, depending on the genetic restriction of the T-cell clone). To suggest the specificity of the response, allo values were additionally subtracted from auto values and included in bottom panels (**C–E**). Three HLA-DR15-restricted and two HLA-DR4-restricted CD4+ T-cell clones were used and data from five experiments are included. Each symbol represents one well and individual clones are indicated. Lines indicate mean ± SEM. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; significant comparisons are indicated and significance is based on the Mann–Whitney U test.

lacking MHC Class II expression) transfected with single MHC Class II molecules as targets. CD4+ T cells that produced IFN-γ in response to either HLA-DR15+ or HLA-DR4+ BLCLs were cloned (Fig. 5B). During the initial T-cell clone screening for IFN-γ production via ELISA, we observed BLCL-restricted IFN-γ production in only 27% of HLA-DR15-restricted T-cell clones, versus in

71% of HLA-DR4-restricted T-cell clones tested (data not shown). Three HLA-DR15-restricted and two HLA-DR4-restricted CD4+ T cell clones were used in the assays.

The CD4+ T-cell clones were incubated together with their HLA matched (auto) or mismatched (allo) BLCLs as targets (HLA-DR15 expressing or HLA-DR4 expressing, depending on

the genetic restriction of the T-cell clone). Intracellular cytokine staining for IFN-γ (Fig. 5C), TNF-α (Fig. 5D), and IL-2 (Fig. 5E) revealed stronger alloreactive responses from CD4+ T-cell clones restricted by HLA-DR15. In trying to isolate the specificity of the responses that we observed, the responses toward HLA-mismatched BLCLs were subtracted from the recognition of HLA-matched targets (Fig. 5C–E, lower panels). In contrast, expression of the transcription factors T-bet and EOMES by the investigated T-cell clones was not largely altered by the different stimulations (Supporting Information Fig. S5C and D).

To test their cytotoxic ability, the CD4+ T-cell clones were also incubated together with their HLA-matched or -mismatched BLCLs as targets. While at 6 h of incubation (Fig. 6A), the pattern is less evident; at 18 h of incubation (Fig. 6B), HLA-DR4-restricted CD4+ T-cell clones killed their targets with higher specificity as HLA-DR15-restricted CD4+ T-cell clones showed higher alloreactive responses. In addition, degranulation assessed by CD107a surface expression and the cytotoxic granule marker granzyme B were used to gain insight into the cytolytic potential of the T-cell clones. Interestingly, HLA-DR4-restricted CD4+ T-cell clones degranulated very specifically, while HLA-DR15-restricted CD4+ T-cell clones not only degranulated less, but also demonstrated significant degranulation to the allogeneic target (Fig. 6C). HLA-DR15-restricted clones also displayed high granzyme B expression when incubated with both HLA-matched and -mismatched targets, while HLA-DR4-restricted clones seemed to upregulate granzyme B only upon recognition of the HLA-matched target (Fig. 6D). This is also evident when responses toward HLA-mismatched BLCLs were subtracted from the recognition of HLA-matched targets (Fig. 6A–D, lower panels) and further highlight above-mentioned results.

These data provide evidence for CD4+ T-cell cross-reactivity to myelin antigens. In addition, weaker HLA-DR15-restricted CD4+ T-cell responses against EBV-transformed B cells, but also inflammatory responses that show tendencies toward alloreactivity. Thus, the HLA-DR15 genetic risk factor may stimulate EBV-specific CD4+ T-cell responses that recognize allogeneic target cells as well as myelin antigens, and that provide insufficient help to CD8+ T cells in order to efficiently control EBV infection. This could lead to both IM-like lymphocytosis and promote cross-reactive autoimmune CD4+ T-cell stimulation.

Discussion

Evidence suggests dysregulated adaptive immunity to be at the core of MS [1]. HLA class II molecules, such as HLA-DR15, are expressed on APCs. They present peptides derived from self-proteins or exogenous proteins to CD4+ T cells, which then support and strengthen cellular and humoral immune responses [32]. Indeed, in addition to the importance of CD4+ T cells in MS pathogenesis, evidence from CNS-infiltrating immune cells in patients and B-cell-depleting therapies suggests additional roles for CD8+ T cells and B cells in the disease [33,34], supported by or supporting autoimmune CD4+ T-cell responses.
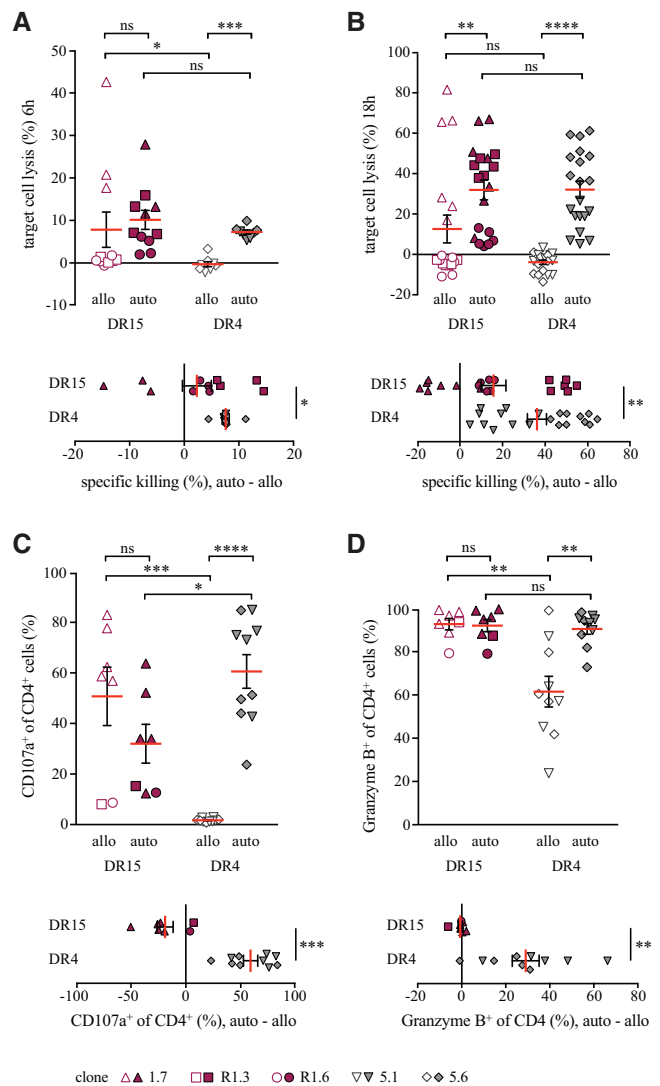
Figure 6. HLA-DR15-restricted EBV-infected B cell-specific CD4+ T-cell clones demonstrate higher alloreactivity in cytotoxicity. CD4+ T-cell clones were cloned from EBV-infected animals using BLCLs transfected with single MHC Class II molecules as targets. HLA-DR15 (DR15) or HLA-DR4 (DR4)-restricted CD4+ T-cell clone responses are measured via target cell killing after 6 (A) and 18 h (B), degranulation by marker CD107a surface upregulation after 8 h (C) and granzyme B expression after 8 h (D) in response to the allogeneic (allo) or autologous (auto) BLCL (HLA-DR15-expressing or HLA-DR4-expressing, depending on the genetic restriction of the T-cell clone). Allo values were additionally subtracted from auto values and included in bottom panels (A–D). Three HLA-DR15-restricted and two HLA-DR4-restricted CD4+ T cell clones were used and data from two to five experiments are included. Each dot represents one well and individual clones are indicated. The mean ± SEM is depicted. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; significant comparisons are indicated and significance is based on the Mann–Whitney U test.

In vivo EBV infection of huNSG mice has allowed us to study the interaction between the main genetic risk factor for MS, HLA-DR15, with the main environmental risk factor, EBV-induced immune responses [4]. Similar to MS patients, we observed elevated EBV-induced T-cell responses in huNSG mice with HLA-DR15 positive immune compartments [27,35]. These seemed to

result from a higher constitutive T-cell activation and increased CD8$^+$ T-cell expansion due to less well-controlled viral loads in connection with this particular MHC class II molecule. Furthermore, we observed CD4$^+$ T-cell cross-reactivity to the MS autoantigen MBP and CD4$^+$ T-cell clones isolated from these EBV-infected huNSG mice that were selected for HLA-DR15-restricted EBV transformed B-cell (LCL) recognition displayed diminished immune recognition of EBV-infected B cells and higher cross-reactivity to HLA-DR-mismatched targets. These findings point to both diminished immune control of EBV and its proinflammatory effects, as well as increased cross-reactivity of the CD4$^+$ T-cell compartment that is more activated by the poorly controlled EBV infection in the context of HLA-DR15. In this way, EBV-induced immune responses could synergize with HLA-DR15 to enhance MS.

Indeed, cross-reactivity with molecular mimicry between EBV and myelin-derived autoantigens has been proposed previously. Primarily, EBNA1 and lytic EBV antigen-specific antibodies, in addition to CD4$^+$ T-cell responses, are elevated in patients with MS and sometimes enriched in the CNS [19,27, 35–39]. Some of these have indeed been suggested to cross-react with CNS autoantigens, like MBP, heterogeneous nuclear ribonucleoprotein L, septin-9, or anoctamin 2 [27,39–42]. This cross-reactivity could amplify T-cell and antibody responses against select EBV gene products, including EBNA1, BFRF3, and BRRF2 [27,39, 41], as well as lead to the local restimulation in the CNS to promote MS-associated lesion formation. Moreover, EBV might induce homing capacities in the respective cross-reactive lymphocyte populations to guide them to the CNS. In line with this, the chemokine CXCL10 was shown to be expressed by EBV-transformed cells, attracting EBV-specific T cells [43] and was also found in the CSF of MS patients [44].

Both B- and T-cell homing of cross-reactive specificities would however be a prerequisite of focusing the resulting autoimmunity on the CNS. Along these lines the α4β1 integrin has been found to be essential for CNS homing of T cells [45]. In addition, the chemokine receptors CXCR3, CCR5, CCR6, and CCR7 have been implicated in brain infiltration of T cells [44,46, 47] and should be investigated for presence of cross-reactive lymphocyte populations. Cross-reactive T and B cells might then restimulate each other in the CNS of patients with MS to promote relapsing-remitting and progressive courses of the disease.

Such T-cell stimulatory capacity of B cells in MS brains could be further augmented by EBV infection that increases co-stimulatory activity for T-cell stimulation [48]. Along these lines, EBV-infected B cells have been detected in the meninges and lesions in the brains of MS patients [49], but these findings are heavily debated [50]. Considering EBV biology and life cycle, B-cell stimulation of T-cell responses in secondary lymphoid organs might be of great importance. As already mentioned, EBV establishes a life-long infection in memory B cells and by doing so modulates their differentiation and function, and promotes their survival [51]. Indeed, memory B cells have recently been shown to mediate the activation of autoreactive CD4$^+$ T cells [52]. It is plausible that EBV infects autoreactive B cells, or B cells that might present antigen to pathogenic, cross-reactive T cells over a long period of time. In addition, in genetically susceptible hosts, acute immune pathologies like IM could serve to decrease the threshold of T-cell activation, facilitating pathogenic B–T cell interactions. The exaggerated EBV-specific T-cell expansion during IM could expand T cells that have been selected in the thymus by self-peptides and will only undergo full activation under acute EBV infection to remain as memory T cells.

Indeed, B-cell depleting therapies, which leave oligoclonal antibody production in the CNS and systemic antibody production by long-lived plasma cells in the BM usually unaffected, have demonstrated beneficial effects in MS patients [34,53]. Furthermore, adoptive transfer of EBV-specific in vitro expanded T-cell lines into patients with MS, even at advanced disease, has demonstrated some encouraging results [54]. These T cells might eliminate an EBV-infected B-cell compartment that stimulates autoreactive T-cell responses, possibly even in the brain. Thus, inefficient immune control of EBV-infected B cells in the context of HLA-DR15 might allow for both priming of cross-reactive T-cell responses and their local restimulation by EBV-infected B cells to propagate autoimmunity in the CNS of patients with MS.

Materials and methods

Animal work

NOD-scid IL2 receptor γ-chain-deficient (NSG) animals with or without an HLA-A2 (NSG-A2) heavy chain transgene (Jackson Laboratory, Bar Harbor, ME, USA) were maintained under specific pathogen-free conditions at the Institute of Experimental Immunology, University of Zürich, Switzerland. Newborn pups (1–5 days old) were sublethally irradiated with 1 Gy and 5–7 h later, intrahepatically injected with around 1.5–3 × 10^5 human fetal liver (Advanced Bioscience Resources) derived and HLA-typed CD34$^+$ hematopoietic progenitor cells. CD34$^+$ cells were isolated by magnetic bead separation based on manufacturer's instructions (Miltenyi Biotech) and frozen in liquid nitrogen. After 12 weeks, peripheral blood was collected from the tail vein and analyzed for human CD45$^+$, CD3$^+$, CD4$^+$, CD8$^+$, and HLA-DR$^+$ cells by flow cytometry. Both female and male animals 3–5 months old and engrafted with human immune cells were intraperitoneally injected with 10^5 EBV Raji infectious units (RIU) or PBS and monitored for up to 6 weeks. Animals used in each experiment were reconstituted from a single donor. NSG-A2 animals were transplanted with HLA-A2 positive CD34$^+$ cells.

Epstein–Barr virus

EBV strain B95-8 was produced in human embryonic kidney HEK293 cells (ATCC) containing a GFP-encoding WT EBV BACmid (p2089; kind gift from H.-J. Delecluse) [55]. Virus concentrates were titrated on Raji cells (ATCC) and GFP-expressing cells were

analyzed 2 days later by flow cytometry (FACSCanto II or LSR Fortessa, BD Biosciences) to determine the EBV RIU.

Enzyme-linked Immunosorbent assay

IFN-γ in T-cell clone supernatants was measured by the human IFN-γ ELISA development kit (Mabtech AB) according to the manufacturer's instructions. EBNA1 IgM in huNSG mouse serum was measured by the EBV EBNA1 IgM ELISA (IBL International) following the manufacturer's instructions. The threshold to determine positive IgM values was set by the mean ± 2× SD of PBS animals.

Enzyme-linked Immunospot assay

CD4+ T cells were isolated from the splenocytes of HLA-DR15 or HLA-DR4 reconstituted, EBV-infected animals (negative magnetic bead separation, Miltenyi Biotec) and expanded by seeding on irradiated allogeneic LCLs as feeder cells (45 Gy) with 1 μg/mL PHA-L (Merck Millipore) and 20 U/mL IL-2 (Peprotech). Fresh IL-2 was added three times per week.

IFN-γ-producing CD4+ T cells were assayed by the human IFN-γ ELISpot BASIC kit (ALP) (Mabtech AB) according to the manufacturer's instructions. On day 15 of expansion, CD4+ T cells were seeded on precoated 96-well plates (MultiScreen Filter Plates, Merck Millipore) at 2.7–5 × 10^4 per well with 2 × 10^4 HLA-matched BLCLs (HLA-DR15- or HLA-DR4-transfected). MBP, MOG, and EBV peptide pools (kind gift from R. Martin, unpubl.) were added at 1 μM concentration for each peptide in the pool (DMSO concentration <0.5%) and incubated with the cells for 28 h at 37°C. CD4+ T cells alone were used as negative control and CD4+ T cells stimulated with PMA/ionomycin as positive control. Wells were counted with the AID ELISpot reader and software (Autoimmun Diagnostika GmbH).

T-cell cloning

CD4+ T cells were isolated from the splenocytes of EBV-infected animals (magnetic bead separation; Miltenyi Biotec) and stimulated with transgenic HLA-matched bare lymphocyte syndrome lymphoblastoid cell lines (BLCLs; HLA-DR15- or HLA-DR4-transfected) for 3–4 h at 37°C. IFN-γ-producing cells were then selected with the IFN-γ Secretion Assay–Detection kit (PE), human (Miltenyi Biotech), and diluted into 96-well plates prefilled with irradiated PBMC feeder cells and HLA-matched BLCLs (20 Gy and 60 Gy, respectively). IL-2 (Peprotech) was added 2 days later and subsequently twice a week at 100–125 U/mL. Two to four weeks later, growing clones were screened with the Human IFN-γ ELISA development kit (Mabtech AB) and IFN-γ-producing clones were placed into culture. CD4+ T-cell clones were fed every 2–3 weeks with irradiated PBMC feeder cells and HLA-matched BLCLs, together with 100–125 U/mL of IL-2.

For T-cell clone killing assays, BLCLs were labeled with PKH26 (Sigma-Aldrich). CD4+ T-cell clones were then incubated alone, or with labeled-matched or -mismatched BLCLs at 5:1 ratio for 6 or 18 h. TO-PRO-3 dye (Life Technologies) was used as a dead cell indicator. For degranulation experiments, CD4+ T cell clones were incubated alone, or with matched or mismatched BLCLs at 1:1 ratio and stained for CD107a (BD Biosciences). Cells were incubated for 2 h before the addition of Brefeldin A (Sigma-Aldrich) and incubated for further 6 h. For transcription factor expression experiments, CD4+ T-cell clones were incubated alone, or with matched or mismatched BLCLs at 1:1 ratio for 8 h.

Flow cytometry

Whole blood was lysed with ACK lysing buffer (Gibco) and washed to obtain PBMCs. Spleens were physically dissociated through a 70 μM strainer and layered on Ficoll-Paque (GE Healthcare) for the separation of mononuclear cells by density gradient centrifugation. Total numbers of leukocytes were calculated from the white blood cell count measured by Beckman Coulter AcT diff Analyzer. PBMCs, splenocytes, or CD4+ T-cell clones were stained with extracellular antibodies for 20 min at 4°C, washed and resuspended either in PBS or fixed in 1% paraformaldehyde. For intracellular staining, extracellular-stained samples were processed with the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) according to manufacturer's instructions. For intranuclear staining, extracellular-stained samples were processed with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, Thermo Fisher Scientific) following the manufacturer's instructions. Live/dead Near IR (Invitrogen) or Zombie NIR (Biolegend) was used for live cell separation.

Antibody clones used in this study were as follows: CD45 (HI30, Biolegend), CD3 (OKT3, UCHT1, Biolegend), CD4 (OKT4, RPA-T4, Biolegend), CD8 (SK1, Biolegend), CD19 (HIB19, Biolegend), HLA-DR (G46-6, BD Biosciences, L243, Biolegend), Eomes (WD1928, eBioscience), T-bet (4B10, eBioscience), IFN-γ (4S.B3, eBioscience), IL-2 (MQ1-17H12, eBioscience), TNF-α (Mab11, BD Biosciences), granzyme B (GB11, BD Biosciences), and CD107a (H4A3, BD Biosciences).

Samples were acquired on the FACSCanto II or LSR Fortessa (BD Biosciences) and analyzed using the Flowjo Software (Becton, Dickinson & Company) following Cossarizza and colleagues [56] whenever possible.

Viral load quantification

Splenic tissue was processed for DNA isolation using the DNeasy Blood and Tissue Kit (QIAGEN) and total DNA from whole blood was extracted using the NucliSENS EasyMAG System (bioMérieux), following the manufacturer's instructions. TaqMan real-time PCR (Applied Biosystems) was used to quantify EBV DNA, with modified primers for the BamH1 W fragment (5′-CTTCTCAGTCCAGCGCGTTT-3′ and

5′-CAGTGGTCCCCCTCCCTAGA-3′) and a fluorogenic probe (5′-FAM CGTAAGCCAGACAGCAGCCAATTGTCAG-TAMRA-3′). All samples were analyzed in duplicates.

HLA typing

DNA was extracted using the QIAGEN GmbH reagent, according to the manufacturer's instructions. Commercial HLA kits (Fujirebio Diagnostics Inc.) were used to identify the HLA alleles using a PCR sequence-specific oligonucleotide reverse assay. Data analysis was performed using the LIPA Interpretation Software (Fujirebio Diagnostics Inc.).

Statistical analysis

Statistical analysis was performed with the GraphPad Prism Software. The Mann–Whitney U test was used to analyze unpaired data with a non-Gaussian distribution. Correlations on non-Gaussian distributed data were analyzed using the Spearman's rank correlation coefficient. The D'Agostino–Pearson omnibus normality test was used to determine normality of data. A p value < 0.05 was considered statistically significant.

Ethics statement

All animal experimentation was approved by the cantonal veterinary office of Zürich, Switzerland (protocols 148/2011, 209/2014, and 159/17) and conducted according to the Swiss Animal Welfare Act, Tierschutzgesetz (TSchG). All human sample studies were approved by the cantonal ethical committee of Zürich, Switzerland, and informed consent of all participating subjects has been obtained (protocols KEK-StV-Nr.19/08 and 2019-00837).

Conflict of interest: The authors declare no commercial or financial conflict of interest.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Abbreviations: **HLA:** human leukocyte antigen · **IM:** infectious mononucleosis · **MBP:** myelin basic protein · **MOG:** myelin oligodendrocyte glycoprotein · **BLCL:** bare lymphocyte syndrome lymphoblastoid cell line · **EBNA1:** EBV nuclear antigen 1

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