

# Role of the 2B4 Receptor in CD8<sup>+</sup> T-Cell-Dependent Immune Control of Epstein-Barr Virus Infection in Mice With Reconstituted Human Immune System Components

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**Patients with X-linked lymphoproliferative (XLP) disease due to deficiency in the adaptor molecule signaling lymphocytic activation molecule-associated protein (SAP) are highly susceptible to one specific viral pathogen, the Epstein-Barr virus (EBV). This susceptibility might result from impaired CD8<sup>+</sup> T-cell and natural killer cell responses to EBV infection in these patients. We demonstrate that antibody blocking of the SAP-dependent 2B4 receptor is sufficient to induce XLP-like aggravation of EBV disease in mice with reconstituted human immune system components. CD8<sup>+</sup> T cells require 2B4 for EBV-specific immune control, because 2B4 blockade after CD8<sup>+</sup> T-cell depletion did not further aggravate symptoms of EBV infection.**

**Keywords.** XLP disease; EBV; 2B4; CD8<sup>+</sup> T cells; HIS mice.

X-linked lymphoproliferative disease (XLP) type 1 is caused by loss-of-function mutations in the *SH2D1A* gene and results in increased susceptibility to Epstein-Barr virus (EBV)-associated conditions. Affected boys develop fulminant infectious mononucleosis, hemophagocytic lymphohistiocytosis, dysgammaglobulinemia, lymphoproliferative disorders, and malignant lymphoma [1]. The function of the protein encoded by the *SH2D1A* gene, the signaling lymphocytic activation molecule (SLAM)-associated protein (SAP), has been studied to

understand the cause of this disease [2]. The cytoplasmic SAP family of adaptors, including SAP and Ewing's sarcoma activated transcript 2 in humans, interact with SLAM-related receptors (ie, SLAM [CD150], 2B4 [CD244], CD84 [SLAMF5], natural killer, T and B cell antigen [NTB-A; CD352; SLAMF6], Ly9 [CD229] and CD2-like receptor activating cytotoxic cells [CD319; SLAMF7]), which are exclusively expressed on hematopoietic cells. SLAM receptors transmit activating signals on encountering their homotypic self-ligands, with the exception of 2B4, which interacts with its ligand CD48 (SLAMF2), a surface molecule that is also restricted to the hematopoietic lineage. These signals are transmitted via SAP by either coupling SLAM family receptors to SAP-recruited activating signaling cascades or hindering their association with inhibitory effectors [3]. In the context of SAP deficiency and EBV infection, 2 of these receptors, 2B4 and NTB-A, have been demonstrated in vitro to be defective in their signaling function [4–6]. CD8<sup>+</sup> T and natural killer (NK) cells from patients with XLP disease display inhibitory rather than activating signaling after engagement of these receptors [4–6].

In female heterozygote carriers, EBV-specific CD8<sup>+</sup> T cells express only wild-type SAP from 1 X chromosome, whereas CD8<sup>+</sup> T cells specific for other viruses in the very same individuals can also express mutant SAP and seem to have stochastically condensed their SAP-encoding X chromosomes [7]. Therefore, alteration of the signal transduction pathways of SLAM receptors 2B4 and NTB-A in CD8<sup>+</sup> T cells and NK cells is thought to be mainly responsible for the increased susceptibility to EBV infection and its sequelae in patients with XLP disease type 1. Studies investigating the direct contribution of SAP-deficient lymphocyte subsets to EBV control in vivo have not been done. The feasibility of modeling EBV infection in mice with reconstituted human immune system components (HIS mice), mirroring key features of human disease, has been recently described by our laboratory and others, with both CD8<sup>+</sup> T and NK cells being protective [8–10]. Moreover, monoclonal and oligoclonal lymphoproliferative tumors of B-cell origin develop in 15%–25% of EBV-infected HIS mice [9, 10]. This model system allowed us for the first time to specifically address manipulations of SAP-dependent receptor signaling and its consequences for EBV infection in vivo.

## MATERIALS AND METHODS

### Mice and EBV

NOD/LtSz-scid IL2Rγ<sup>null</sup> (NOD-*scid* γ<sub>c</sub><sup>-/-</sup> or NSG) mice were used to generate HIS mice, as described elsewhere [10]. Briefly,

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$1-2 \times 10^5$  CD34<sup>+</sup> cells derived from human fetal livers were injected intrahepatically into irradiated newborn mice and used 3–4 month after reconstitution. Green fluorescent protein transgenic wild-type EBV (B95-8) was titrated on Raji cells to calculate Raji infecting units. Mice were infected intraperitoneally with  $1 \times 10^5$  Raji infecting units and followed up for 5–6 weeks. The EBV load was measured by means of TaqMan (Applied Biosystems) real-time polymerase chain reaction, as described elsewhere [10]. All animal protocols were approved by the cantonal veterinary office of the canton of Zurich, Switzerland (protocol 148/2011). All studies involving human samples were reviewed and approved by the cantonal ethical committee of Zurich, Switzerland (protocol KEK-StV-Nr.19/08).

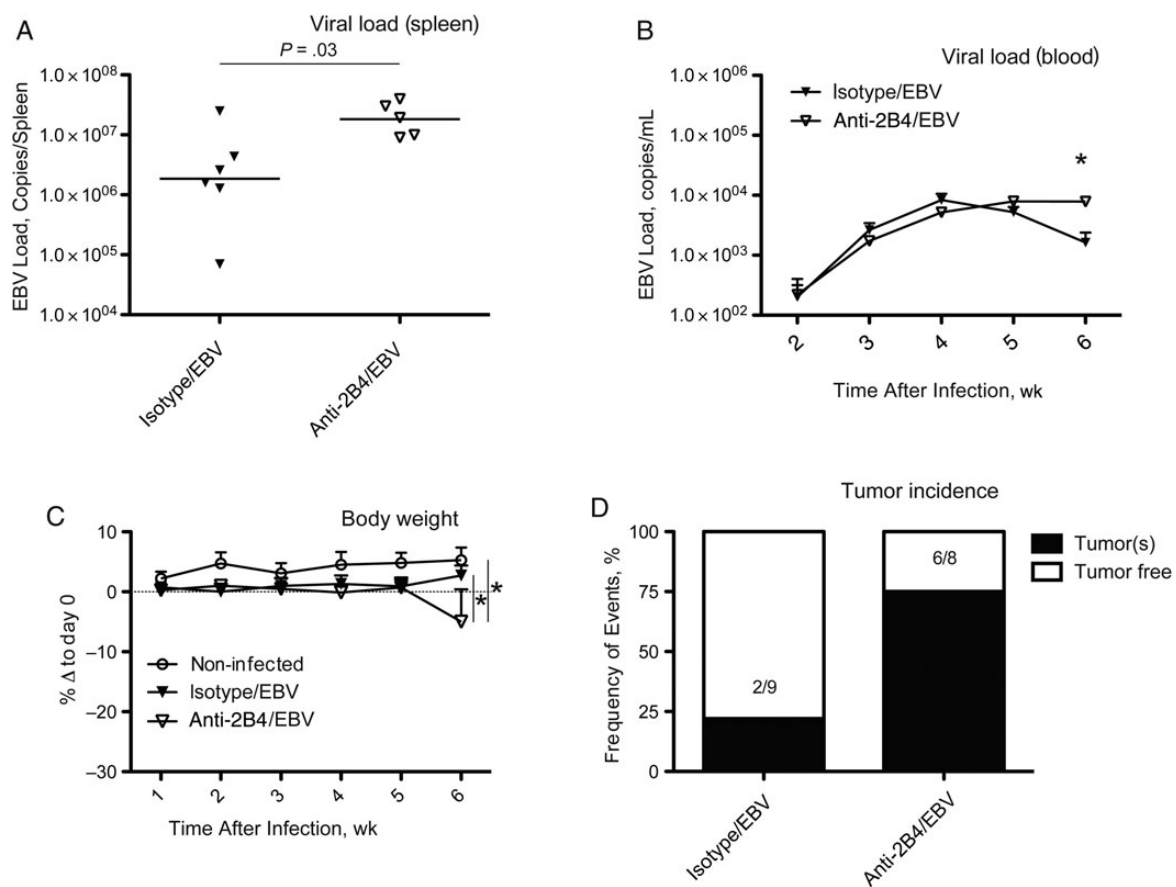
### 2B4 Blockade and CD8<sup>+</sup> T-Cell Depletion

For blocking experiments, mice were treated intraperitoneally with 2  $\mu$ g of anti-2B4 antibody (clone CO54; immunoglobulin

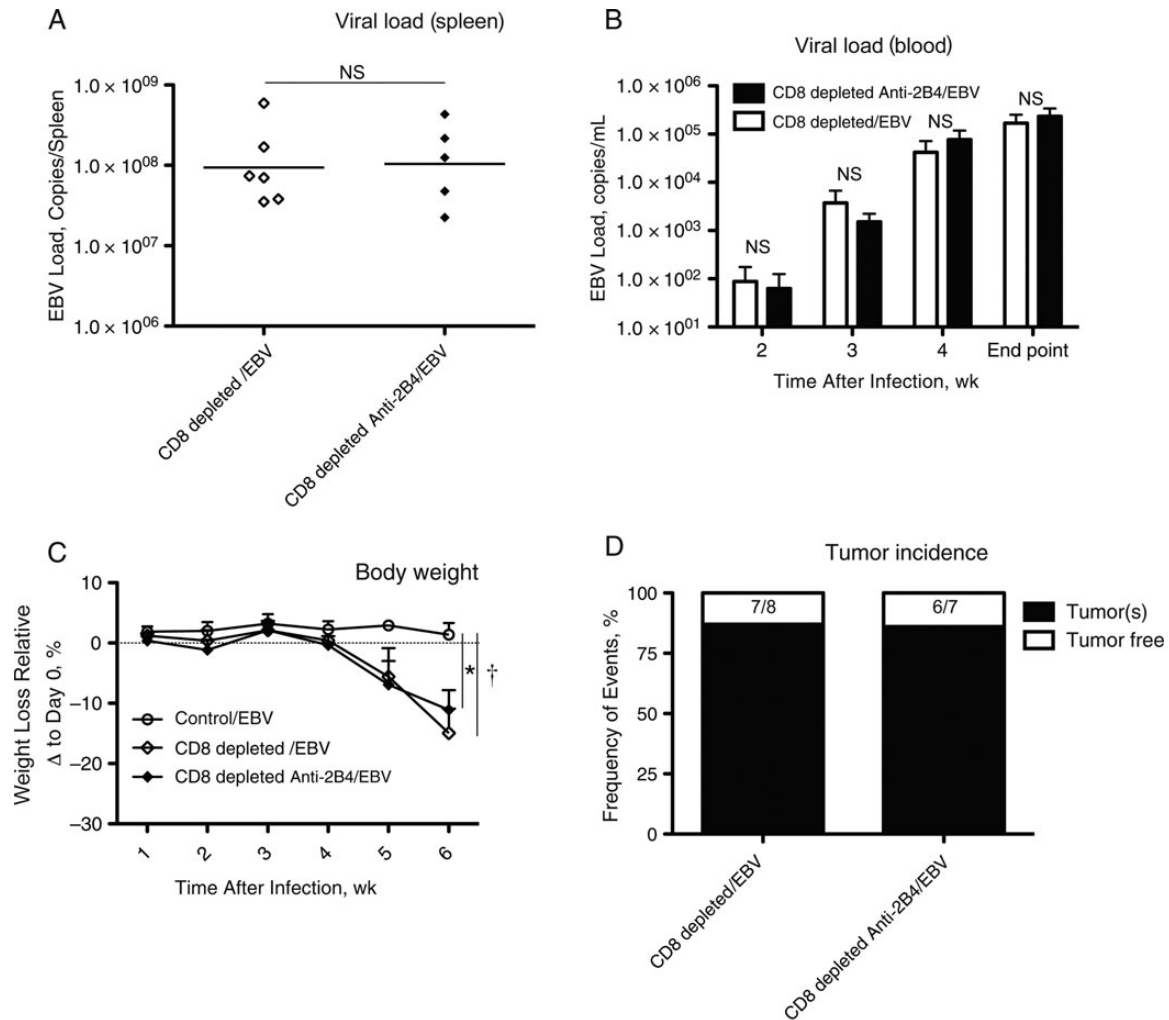
[Ig] M; provided by A. M.), anti-NTB-A antibody (clone ON56; IgG2a; provided by A. M.) or isotype controls in phosphate-buffered saline on the day before infection with EBV and every second day during the course of infection. CD8<sup>+</sup> T cells were depleted with 150  $\mu$ g of monoclonal antibody against human CD8 (clone OKT-8; Bio X Cell) via intraperitoneal inoculation for 3 consecutive days just before EBV infection and every second day (50  $\mu$ g) beginning 2 weeks after infection for the duration of the experiment.

### Flow Cytometry

Peripheral blood mononuclear cells were separated on Ficoll-Paque gradients, preincubated with unlabeled anti-2B4 (clone CO54; provided by A. M.) for 30 minutes on ice, or left untreated and after washing stained with anti-CD56 (clone NCAM16.2; BD Biosciences), anti-CD3 (clone UCHT1; BioLegend), and anti-2B4 (clone C1.7; BioLegend). Analysis was performed with a LSR



**Figure 1.** Human 2B4 signaling is involved in the protective immune response against Epstein-Barr virus (EBV) in mice with reconstituted human immune system components. *A*, EBV load in spleens 6 weeks after EBV infection ( $n = 11$ ). Horizontal bar represents geometric mean; viral loads were compared with 2-tailed Mann–Whitney *U* test. *B*, EBV load over time in peripheral blood in mice treated with blocking antibody to 2B4 or immunoglobulin (Ig) M isotype every other day ( $n = 10-12$  per time point). Results represent mean and standard error of the mean (SEM); \**P* < .01 (2-way analysis of variance [ANOVA] with Bonferroni correction). *C*, Weight loss over time after EBV infection relative to day 0 ( $n = 15-16$ ). Results represent mean and SEM; \**P* < .05 (2-way ANOVA with Bonferroni correction). *D*, Frequency of tumors at death in mice treated with blocking antibody to 2B4 or IgM isotype (numbers in columns indicate mice with tumor/total mice); data are composite data from 2 independent experiments.



**Figure 2.** Blocking 2B4 in the absence of CD8<sup>+</sup> T cells does not alter the course of Epstein-Barr virus (EBV) infection. *A*, EBV load in spleens at the end point (5–6 weeks after infection;  $n = 11$ ). Horizontal bar represents geometric mean; viral loads were compared with 2-tailed Mann–Whitney  $U$  test. *B*, EBV load over time in peripheral blood in mice depleted of CD8<sup>+</sup> T cells and either treated with blocking 2B4 antibody, or not ( $n = 12$ –15 per time point). Results represent mean and standard error of the mean (SEM); NS, not significant (2-way analysis of variance [ANOVA] with Bonferroni correction). *C*, Weight loss over time after EBV infection relative to day 0 ( $n = 13$ –20). Results represent mean and SEM. \* $P < .05$ ; † $P < .001$  (2-way ANOVA with Bonferroni correction). *D*, Frequency of tumors at death in mice depleted of CD8<sup>+</sup> T cells and either treated with blocking 2B4 antibody or not, (numbers in columns indicate of mice with tumor/total mice); data are composite data from 2 independent experiments.

Fortessa cytometer (BD Biosciences), and flow cytometric data was analyzed with FlowJo software v9.8 (Tree Star).

#### Detection of Human Interferon $\gamma$

Human interferon (IFN)  $\gamma$  in serum from HIS mice was analyzed with a human IFN- $\gamma$  enzyme-linked immunosorbent assay kit (Mabtech), in accordance with the manufacturer’s protocols.

#### Statistical Analysis

Mann–Whitney  $U$  test was used to compare viral loads, and 1- or 2-way analysis of variance was used to analyze differences between groups, as indicated in the figure legends. Differences were considered statistically significant at  $P < .05$ .

## RESULTS AND DISCUSSION

To determine the role of 2B4 and NTB-A in EBV infection in HIS mice, we masked these receptors with specific blocking antibodies. Treatment of HIS mice with anti-NTB-A antibody (IgG), however, depleted receptor-carrying leukocytes (reduction by 50% of human CD45<sup>+</sup> leukocytes), whereas anti-2B4 (IgM) treatment did not (data not shown). The antibody used to block 2B4 has been well described for its in vitro blocking activity [11, 12], and it efficiently masked 2B4, thereby showing its specificity (Supplementary Figure 1A and 2B). The expression of the 2B4 receptor is highly up-regulated during EBV infection in HIS mice [10], and blocking 2B4 in EBV-infected HIS

mice led to decreased IFN- $\gamma$  serum levels 4 weeks after infection (Supplementary Figure 1C), consistent with previous in vitro data showing that activation of 2B4 is sufficient to induce IFN- $\gamma$  [4, 5]. This effect was abolished 6 weeks after infection (Supplementary Figure 1D), when anti-2B4-treated mice showed viral loads increased by almost 1 log in both spleen and blood (Figure 1A and 1B).

The increase in IFN- $\gamma$  at this point to levels similar to or even higher than those in isotype-treated mice (Supplementary Figure 1D) might have been provoked by the concurrent higher viral load and subsequently more severe immunopathology. Furthermore, anti-2B4-treated mice showed signs of exacerbated disease, as evidenced by weight loss (Figure 1C), and had more tumors (Figure 1D) than control mice, demonstrating a crucial role for 2B4 signaling in the protective immune response against EBV in HIS mice. Thus, aspects of SAP-deficient XLP disease (ie, impaired viral control and augmented tumorigenesis) can be mimicked in HIS mice by using 2B4 blockade during EBV infection.

NK cells accumulate during acute symptomatic EBV infection in humans [13, 14] and are protective against EBV in HIS mice [10]. Likewise, CD8<sup>+</sup> T cells execute antiviral and antitumor responses in EBV-infected HIS mice [8, 9]. To determine which of these lymphocyte populations requires 2B4 signaling, we depleted CD8<sup>+</sup> T cells during the course of EBV infection with and without additional 2B4 blockade. In the absence of CD8<sup>+</sup> T cells, infected mice demonstrated high viral loads and suffered from severe weight loss and high tumor burden (Figure 2), confirming previous data [8, 9].

Importantly, when we blocked the 2B4 receptor on top of depleting CD8<sup>+</sup> T cells, disease severity was identical to that in CD8<sup>+</sup> T-cell-depleted mice with intact 2B4 signaling capability in terms of viral load, weight loss, and tumor incidence (Figure 2). This suggests that activation of 2B4 signaling on NK cells is not essential during EBV infection. Rather, our results demonstrate that abrogated 2B4 signaling pathways in CD8<sup>+</sup> T cells are responsible for the aggravated symptoms observed in anti-2B4-treated nondepleted mice (Figure 1). Therefore, our studies indicate that CD8<sup>+</sup> T cells are the critical effector cells, being responsible for the impaired immune response to EBV in HIS mice.

This is in good agreement with the finding that somatic reversion of mutated SAP occurs primarily in effector memory CD8<sup>+</sup> T cells in patients with XLP disease [15]. Although 5%–10% of these adaptive lymphocytes revert their mutated SAP gene to become functional, this reversion is very rarely seen in NK cells [15]. These reverted CD8<sup>+</sup> T cells regained normal responses toward EBV-infected cells compared with SAP-deficient cells of the same host, and patients with SAP reversion had a prolonged median survival [15].

These clinical data and our findings in EBV-infected HIS mice imply that compromised CD8<sup>+</sup> T-cell-mediated immune control after SAP mutation is the critical immune deficit of XLP disease. Furthermore, these data suggest that infusion of

autologous CD8<sup>+</sup> T cells with gene therapy-corrected SAP should be explored in patients with XLP disease.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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**Authorship contributions.** O. C. performed the experiments; R. C. determined viral loads; E. M. and A. M. contributed essential reagents; and O. C. and C. M. designed the research and wrote the manuscript.

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**Potential conflicts of interest.** All authors: No potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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