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EBI3 regulates the NK cell response to mouse cytomegalovirus infection

Helle Jensen^a, Shih-Yu Chen^b, Lasse Folkersen^c, Garry P. Nolan^b, and Lewis L. Lanier^{a,d,1}

^aDepartment of Microbiology and Immunology, University of California, San Francisco, CA 94143; ^bDepartment of Microbiology and Immunology, Stanford University, Palo Alto, CA 94304; ^cDepartment of Systems Biology, Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby DK-2800, Denmark; and ^dParker Institute for Cancer Immunotherapy, San Francisco, CA 94143

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Natural killer (NK) cells are key mediators in the control of cytomegalovirus infection. Here, we show that Epstein–Barr virus-induced 3 (EBI3) is expressed by human NK cells after NKG2D or IL-12 plus IL-18 stimulation and by mouse NK cells during mouse cytomegalovirus (MCMV) infection. The induction of EBI3 protein expression in mouse NK cells is a late activation event. Thus, early activation events of NK cells, such as IFN γ production and CD69 expression, were not affected in EBI3-deficient (*Ebi3*^{-/-}) C57BL/6 (B6) mice during MCMV infection. Furthermore, comparable levels of early viral replication in spleen and liver were observed in MCMV-infected *Ebi3*^{-/-} and wild-type (WT) B6 mice. Interestingly, the viral load in salivary glands and oral lavage was strongly decreased in the MCMV-infected *Ebi3*^{-/-} B6 mice, suggesting that EBI3 plays a role in the establishment of MCMV latency. We detected a decrease in the sustained IL-10 production by NK cells and lower serum levels of IL-10 in the MCMV-infected *Ebi3*^{-/-} B6 mice. Furthermore, we observed an increase in dendritic cell maturation markers and an increase in activated CD8⁺ T cells. Thus, EBI3 dampens the immune response against MCMV infection, resulting in prolonged viral persistence.

natural killer cell | EBI3 | cytomegalovirus

Natural killer (NK) cells play an essential role in host defense against viral infections, particularly herpesviruses, such as cytomegalovirus (CMV) (1). During infection, NK cell activation is tightly controlled by the integration of signals derived from activating and inhibitory receptors, through the interaction with target or accessory cells, and from cytokine receptors. Several activating NK receptors exist, including the activating killer cell Ig-like receptors (KIRs) in humans, the activating Ly49 receptors in rodents, NKG2D, the natural cytotoxicity receptors (i.e., NKp30, NKp44, and NKp46), and the activating Fc receptor CD16 (2). The activating receptors recognize either stress-induced ligands on viral-infected cells, virus-encoded proteins, or Ig-coated cells. Signals from the activating receptors promote cytoskeletal rearrangements and proliferation, as well as secretion of cytolytic granules and cytokines (2). The inhibitory receptors Ly49 and KIR recognize polymorphic major histocompatibility complex class I ligands that can dampen or prevent the NK cells from attacking self (2).

NK cell-mediated control of viral infections has been studied extensively in mice infected with mouse CMV (MCMV). NK cells contribute directly to the early control of MCMV infection by eliminating the virus-infected cells. In C57BL/6 (B6) mice, Ly49H⁺ NK cells recognize MCMV-infected cells expressing the virus-encoded protein m157. This antigen-specific recognition leads to NK cell activation (3), as well as expansion and differentiation of memory NK cells (4), which is dependent on the DAP12 adapter protein, the costimulatory receptor DNAM-1, and the proinflammatory cytokine IL-12 (4–6). The DAP10 adapter protein and the cytokines IL-33 and IL-18 are required for optimal expansion of Ly49H⁺ NK cells, but not for memory NK cell differentiation (7–9). In addition, optimal activation of both Ly49H⁺ and Ly49H⁻ NK cells and production of IFN γ during MCMV infection is critically dependent on both IL-12 and IL-18 (9, 10). In addition to mediating early control of MCMV infection, NK cells also play

a role in shaping the subsequent adaptive immune responses. Crosstalk between NK cells and dendritic cells (DCs) early during MCMV infection affects the outcome of the T-cell responses. IL-10 secreted by various immune cells, including NK cells, dampens the T-cell response by negatively affecting the maturation of DCs, and in the absence of IL-10 secretion of IFN γ and TNF α by NK cells enhances the maturation of DCs, which boosts the T-cell response (11).

The cytokine Epstein–Barr virus-induced 3 (EBI3) was first identified in B cells infected with Epstein–Barr virus (12), but several other cells from the immune system have also been found to express and secrete EBI3, including activated DCs, regulatory T cells, and regulatory B cells (13–15). EBI3 belongs to the IL-12 family of cytokines that consists of the four heterodimeric cytokines IL-12 (p35/p40), IL-23 (p19/p40), IL-27 (p28/EBI3), and IL-35 (p35/EBI3), which signal through unique pairings of the five receptor chains IL-12R β 1, IL-12R β 2, IL-23R, gp130, and WSX-1 (16). IL-27 and IL-35 lack disulfide linkage and pair poorly and are therefore less stable and secreted in much lower amounts than the disulfide-linked family members IL-12 and IL-23 (16). It has been well-documented that IL-12 and IL-23 function as proinflammatory cytokines. However, studies with IL-27 and IL-35 have been complicated by their instability in solution and the lack of specific reagents. IL-27 has been proposed to possess both proinflammatory and anti-inflammatory properties in that it can promote Th1 polarization (17) but also stimulate the production of IL-10 (18, 19). IL-35 appears to possess anti-inflammatory properties with the predominant mechanism being suppression of T-cell proliferation and conversion of naive T cells into IL-10–producing

Significance

Natural killer (NK) cells play a key role in early viral control of CMV replication and in shaping the adaptive immune response. Despite an early control, CMV persists by exploiting host immune inhibitory pathways. Here, we describe a previously unidentified pathway wherein the cytokine Epstein–Barr virus-induced 3 (EBI3) affects the establishment of mouse cytomegalovirus (MCMV) latency. We also show that both human and mouse NK cells express EBI3 and the EBI3 receptor, gp130, after stimulation. MCMV-infected EBI3-deficient mice showed decreased IL-10 production by NK cells, increased dendritic cell maturation and activation of CD8⁺ T cells, and significantly diminished viral loads in the salivary glands and oral lavage. Together, our results provide insight into how CMV establishes latent infection.

Author contributions: H.J. and L.L.L. designed research; H.J. and S.-Y.C. performed research; S.-Y.C., L.F., and G.P.N. contributed new reagents/analytic tools; H.J., S.-Y.C., L.F., and L.L.L. analyzed data; and H.J. and L.L.L. wrote the paper.

Reviewers: M.A.C., Ohio State University; and D.J.C., Merck Research Laboratory, Palo Alto, CA.

Conflict of interest statement: L.L.L. and the University of California, San Francisco have licensed intellectual property rights regarding NKG2D for commercial applications.

¹To whom correspondence should be addressed. Email: lewis.lanier@ucsf.edu.

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T cells (13). It has been speculated that *EBI3* can be secreted and function as a homodimer; however, this remains to be elucidated (15, 16). Here we identify activated human and mouse NK cells as producers of *EBI3* and show that *EBI3* promotes the persistence of MCMV infection.

Results

Gene Expression Analysis of NKL Cells Stimulated Through Activating Receptors. We investigated the global gene expression profile of human NK cells stimulated through various activating receptors to identify genes involved in the regulation of NK cell functions. We used the human-transformed NK cell line, NKL, stably transduced with either the activating mouse Ly49H receptor or the activating human KIR2DS1 receptor (Fig. S1A). NKL cells, which constitutively express the activating NKG2D receptor (Fig. S1A), were stimulated with saturating amounts of plate-bound isotype-matched control Ig or receptor-specific monoclonal antibodies (mAbs), and the RNA was extracted and used for deep sequencing. Before analysis the quality of the gene expression data was verified using principal component analysis (PCA) and by calculating the Pearson correlation coefficient for the experimental replicates (Fig. S1B and C).

In our gene expression analysis of the activated NKL cells, we scrutinized genes that were up- and down-regulated with at least a 1.5-fold change relative to the control samples. A total of 254, 158, or 596 genes were identified to be up- and down-regulated 4 h after KIR2DS1, NKG2D, or Ly49H stimulation, respectively (Fig. 1A and Dataset S1). We examined the 133 genes that were commonly regulated by all three stimulations (Fig. 1A) by gene set enrichment analysis (GSEA). GSEA is a pathway-based analysis that detects changes in expression of genes in entire pathways or gene sets and is therefore more robust than analyses based upon individual genes. GSEA identified 20 gene sets and pathways that were significantly different relative to the control samples (Fig. 1B). The gene set with the highest enrichment score identified by GSEA was “cytokines,” which included many genes known to be expressed by activated NK cells, i.e., *IFNG*, *FASLG*, *XCL1*, *CCL1*, *CCL3*, *CCL4*, and *CCL5* (2, 20, 21) (Fig. 1C). Interestingly, we detected an up-regulation of *EBI3* in the

activated NKL cells (Fig. 1C). *EBI3* expression has recently been shown to be up-regulated in human NK cells in response to Fc receptor activation, but only in the presence of IL-12 (22), confirming our results that activated NK cells can express *EBI3*; however, to our knowledge no previous studies have examined *EBI3* protein expression and secretion by human and mouse NK cells. Furthermore, the effect of *EBI3* on NK cell function in vivo is unknown.

***EBI3* Protein Expression and Secretion Is Increased in Human NK Cells in Response to Receptor- and Cytokine-Mediated Stimulation.** NKL cells constitutively express *EBI3*, which was significantly increased upon receptor-mediated stimulation (Fig. 2A). *IL12A* (i.e., p35) was also constitutively expressed in NKL cells, but its expression was not increased after receptor-mediated stimulation (Fig. 2A). In agreement with the gene expression data, the majority of or all NKL cells express *EBI3* and p35, respectively (Fig. 2B), and the amount of intracellular *EBI3*, but not p35, proteins was increased following receptor-mediated stimulation for 24 h (Fig. 2C and F, respectively). In addition, the intracellular *EBI3* protein level was increased in response to IL-12 plus IL-18 treatment for 24 h (Fig. 2D). *EBI3* and p35 protein was found in the supernatant of NKL cells after culture, but only *EBI3* secretion was increased after stimulation (Fig. 2E and G), which was consistent with the intracellular protein levels observed. To confirm that *EBI3* is also expressed by primary human NK cells, we examined the intracellular *EBI3* protein level in resting and activated CD56^{dim} (i.e., mature) and CD56^{bright} (i.e., immature) NK cells derived from healthy human blood donors. Resting NK cells did not express *EBI3* (Fig. S2A and B), suggesting that the constitutive expression of *EBI3* observed in NKL cells is due to cell transformation and/or the in vitro culture conditions. Treatment with IL-12 alone for 24 h did not induce *EBI3* protein expression, and only a slight increase was observed after treatment with IL-18 alone (Fig. S2A). However, treatment with IL-12 in combination with IL-18 for 24 h led to a robust induction of *EBI3* protein expression in both CD56^{bright} and CD56^{dim} NK cells (Fig. S2A and B). Priming with IL-2 for 24 h did not induce *EBI3* protein expression; however, a significant increase was observed after stimulation of IL-2-primed NK cells

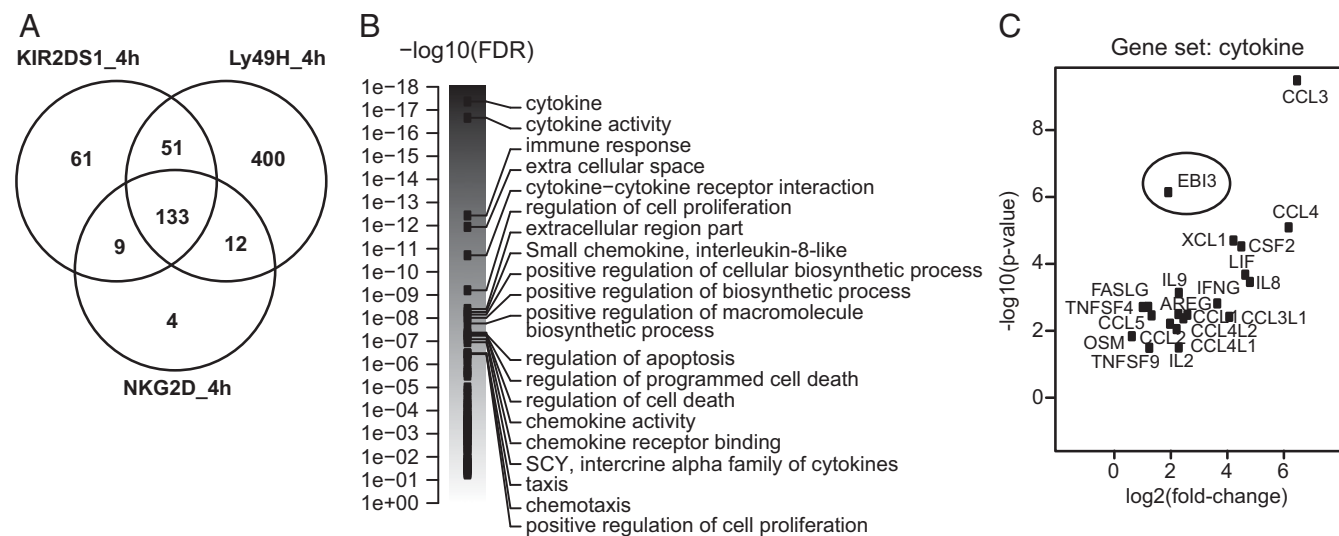


Fig. 1. Gene expression analysis of NKL cells stimulated through activating receptors. (A) RNA was isolated from NKL cells stimulated with the indicated plate-bound Abs for 4 h and then analyzed by deep sequencing. Shown is the number of genes that were up- and down-regulated with at least a 1.5-fold change in the receptor-stimulated NKL cells relative to the control-treated cells ($n = 2-4$ independent experiments). (B and C) GSEA was performed on the deep-sequencing data. (B) Shown are the strongest associated gene sets/pathways when analyzing genes that were significantly differentially expressed between NKG2D-stimulated NKL cells versus control-treated cells at 4 h. The scale is given as DAVID-reported $-\log_{10}$ (false discovery rate) value, i.e., the gene-set score. The score of all gene sets is shown, but only the top 20 are labeled. (C) Shown is a volcano plot of the genes contained in the “cytokine” gene set, which had the strongest enrichment score in B. The x axis indicates a log₂ fold-change, with positive values corresponding to genes for which expression is up-regulated.

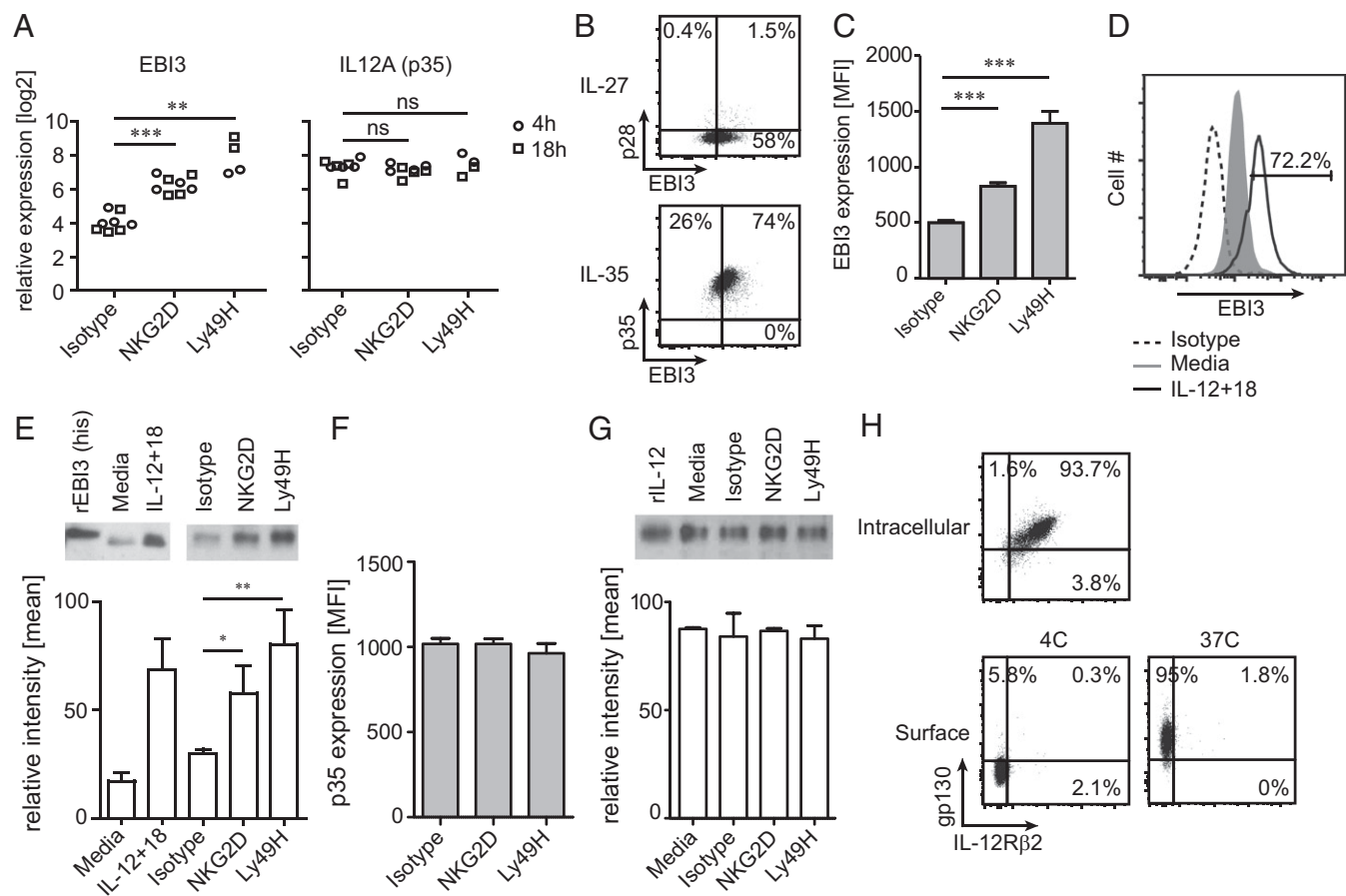


Fig. 2. EB13 protein expression is increased in response to receptor- and cytokine-mediated stimulation. (A) Gene expression data for EB13 and p35 in NKL cells stimulated with the indicated plate-bound Abs for 4 h (circle) or 18 h (square) were obtained from the deep-sequencing analysis ($n = 2-4$ independent experiments; statistical analysis is shown for 4-h samples). (B) Intracellular EB13, p28, and p35 protein expression was examined by flow cytometry in resting NKL cells. Data are representative of three independent experiments. (C and D) Intracellular EB13 protein expression was measured by flow cytometry in NKL cells stimulated with (C) plate-bound Abs or (D) IL-12+IL-18 for 24 h. (C) Data show mean \pm SD from three independent experiments. (D) Data are representative of three independent experiments. (E) NKL cells were stimulated with plate-bound Abs, media, or IL-12+IL-18 for 24 h, and EB13 was detected in the supernatant by Western blot analysis. The graphs show mean \pm SD from two or three independent experiments. (F) Intracellular p35 protein expression was measured by flow cytometry in NKL cells stimulated with plate-bound Abs for 24 h. (G) NKL cells were stimulated with media or plate-bound Abs for 24 h, and p35 was detected in the supernatant by Western blot analysis. The graphs show mean \pm SD from three independent experiments. (H) Surface and intracellular protein expression of gp130 and IL-12R β 2 was examined by flow cytometry in resting NKL cells. Data are representative of three independent experiments. Statistical analysis was performed by two-tailed unpaired Student's t test (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

with plate-bound anti-NKG2D mAb for 24 h (Fig. S2C). In contrast to EB13 expression, the majority of resting NK cells constitutively expressed p35 (Fig. S24). We did not detect any protein expression of p28 (Fig. 2B). NKL cells stained positive for both gp130 and IL-12R β 2 as detected by intracellular protein staining (Fig. 2H, "intracellular"). Furthermore, gp130 was detected at the cell surface of NKL cells, but only after incubating the cells with the anti-gp130 Ab at 37 °C (Fig. 2H, "surface"), suggesting that the gp130 receptor is rapidly recirculated at the cell surface of the NKL cells. Resting primary human NK cells expressed low levels of gp130 at the cell surface, but the expression was increased in response to IL-12 plus IL-18 treatment, with the strongest induction observed in the CD56^{bright} NK cells (Fig. S2D). Furthermore, the majority of CD56^{bright} NK cells and all of the CD56^{dim} NK cells constitutively expressed IL-12R β 2 as detected by intracellular protein staining (Fig. S2E). No cell-surface staining of IL-12R β 2 was detected by flow cytometry (Fig. 2H, "surface").

MCMV Infection Induces EB13 Expression in Mouse NK Cells. Based on our results with human NK cells and the NKL cells transduced to express Ly49H, we examined if MCMV infection could be used

as an in vivo model system to study the functional role of EB13. During MCMV infection we detected an increase in the intracellular EB13 protein level in splenic NK cells (Fig. 3A and B), whereas no difference was detected in T cells, B cells, and DCs from the same samples (Fig. S34). The increase in EB13 protein expression in mouse NK cells was strongest at day 2.5 postinfection (p.i.) (Fig. 3B) with the highest levels observed in MCMV-specific Ly49H⁺ (Fig. 3A and B). The cell-surface expression of gp130 was increased on splenic NK cells in response to MCMV infection (Fig. 3C and D), which indicates a possibility for an autocrine effect. This increase in gp130 surface expression was specific for NK cells, as the surface expression on T cells, B cells, and DCs was either decreased or unchanged (Fig. S3B). p35, but not p28, protein expression was increased in splenic NK cells in response to MCMV infection (Fig. 3E and F), and like EB13, the strongest induction of p35 protein expression was observed at day 2.5 p.i. (Fig. 3E).

EB13-Deficient Mice Show Decreased MCMV Replication in the Salivary Glands and Oral Lavage. To examine the functional role of EB13 in vivo, we infected wild-type (WT) and EB13-deficient (*Ebi3*^{-/-})

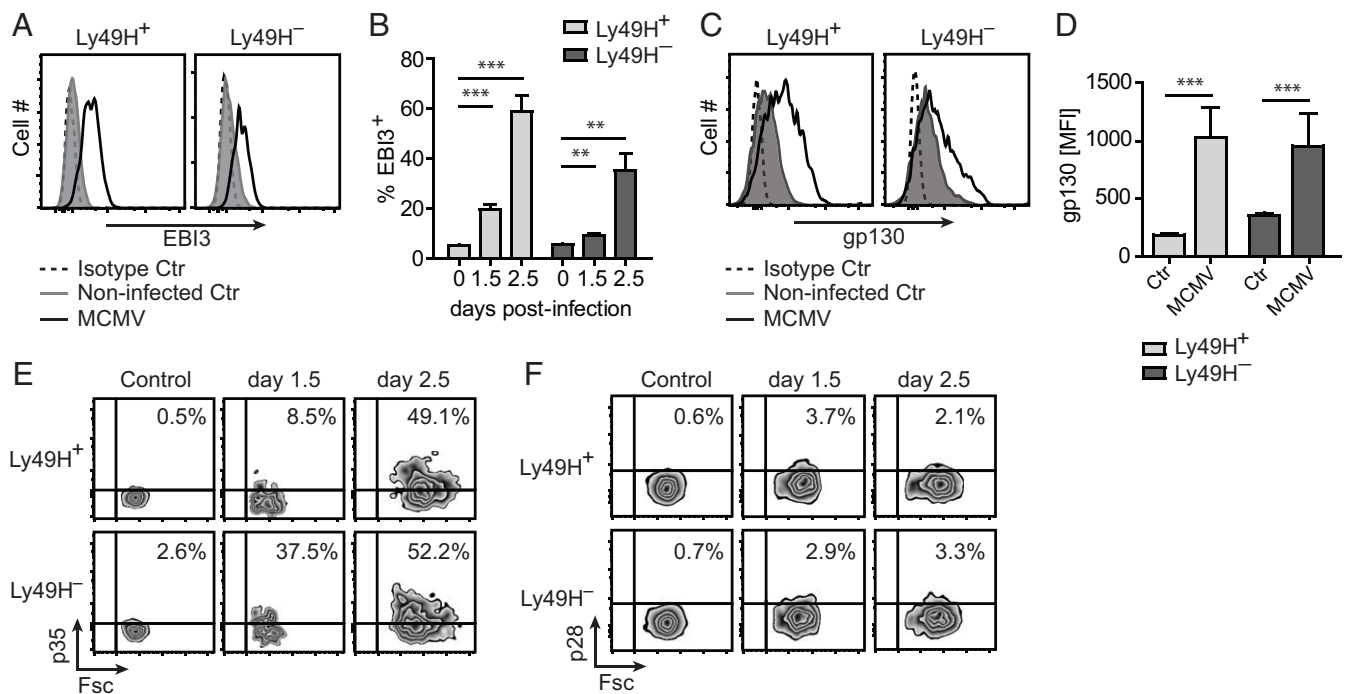


Fig. 3. MCMV infection induces EB13 protein expression in mouse NK cells. Intracellular (A and B) EB13, (E) p35, and (F) p28 protein expression was examined by flow cytometry in splenic Ly49H⁺ and Ly49H⁻ NK cells from noninfected and infected (day 1.5 and day 2.5) WT B6 mice. (A, E, and F) Data are representative of four mice for each time point from two independent experiments. (B) Data show mean \pm SD from four or six mice for each time point from two independent experiments. (C and D) Surface expression of gp130 was examined by flow cytometry in splenic Ly49H⁺ and Ly49H⁻ NK cells from noninfected and MCMV-infected (day 2) WT B6 mice. (C) Data are representative of six mice from two independent experiments. (D) Data show mean \pm SD from six mice from two independent experiments. Statistical analysis was performed by two-tailed unpaired Student's *t* test (***P* < 0.01 and ****P* < 0.001).

C57BL/6 (B6) mice with MCMV. The *Ebi3*^{-/-} B6 mice lack exons 2–5 of the *Ebi3* gene, corresponding to amino acids 24–228 of the EB13 protein (23), which includes the functional fibronectin type 3 domain found at amino acids 128–216 (24). Thus, the truncated version of EB13 likely to be present in the deficient mice would be nonfunctional. No difference was observed between the two mouse strains with regard to the percentages of splenic NK cells and the immature and mature NK cell subsets (Fig. S4 A and B). Furthermore, the splenic NK cells showed similar expression levels of several NK receptors, including KLRG1, Ly49H, Ly49C/I, Ly49D, Ly49A, NKG2A,C,E, NKp46, and NKG2D (Fig. 4A and Fig. S4C). Thus, EB13 deficiency is not associated with any phenotypic or maturational defects in the NK cells. During MCMV infection, the early activation of splenic NK cells, measured by CD69 expression and IFN γ production at day 1.5 p.i., was comparable between the WT and *Ebi3*^{-/-} B6 mice (Fig. 4B and C, respectively). Furthermore, no difference in the viral load in liver and spleen from the mice was observed at day 4 p.i. (Fig. 4D and E). We also observed a similar expansion of peripheral blood KLRG1^{hi} Ly49H⁺ NK cells in the MCMV-infected mice (Fig. 4A). However, the viral load was significantly (*P* < 0.05) decreased in the blood in the MCMV-infected *Ebi3*^{-/-} B6 mice at day 7 and day 14 p.i. (Fig. 4F). Interestingly, we found that MCMV was cleared most efficiently in the salivary glands and oral lavage of the *Ebi3*^{-/-} B6 mice (Fig. 4G and H), which are important sites for virus persistence and dissemination (25).

EB13 Promotes IL-10 Production by NK Cells and Negatively Affects the Maturation of DCs and Activation of CD8⁺ T Cells During MCMV Infection. Several cells in the immune system, including NK cells, produce IL-10 early after MCMV infection. The early production of IL-10 promotes virus replication in the salivary glands by negatively affecting the maturation of DCs, leading to poor priming of

T cells (26, 27). We found that splenic Ly49H⁺ and Ly49H⁻ NK cells from the mice produced similar levels of IL-10 at day 2.5 post-MCMV infection (Fig. 5A). However, the IL-10 production was significantly decreased at day 3.5 p.i. in the *Ebi3*^{-/-} B6 mice (Fig. 5A), suggesting that EB13 plays an essential role in the sustained, but not initial, production of IL-10 by NK cells during MCMV infection. The serum level of IL-10 peaks at day 5 p.i. during MCMV infection (26). We found that the levels of IL-10 in sera were significantly decreased in the MCMV-infected *Ebi3*^{-/-} B6 mice (Fig. 5B), indicating that the overall production of IL-10 was affected in the mice. We further examined the impact of EB13 deficiency on DC maturation and T-cell activation, both of which are affected by IL-10 during MCMV infection (26, 27). We observed higher levels of the maturation markers CD86 and CD40 on splenic DCs derived from the *Ebi3*^{-/-} B6 mice at day 5 p.i. (Fig. 5C). Furthermore, the percentage of activated peripheral CD8⁺ T cells (i.e., NKG2D-positive CD8⁺ T cells) was increased in the MCMV-infected *Ebi3*^{-/-} B6 mice (Fig. 5D). Thus, together these results indicate that EB13 promotes persistent MCMV infection, presumably in part by sustaining IL-10 production, which negatively affects the maturation of DCs and the activation of T cells.

Discussion

In this study, we show that both human and mouse NK cells express EB13 protein after stimulation. Furthermore, we describe a previously unidentified pathway wherein EB13 affects the establishment of MCMV latency. Interestingly, mice deficient in EB13 showed almost no viral replication in salivary glands and oral lavage, which are the main sites for viral persistence and dissemination (25), whereas the early viral replication in the spleen and liver was comparable between the EB13-deficient and WT mice. MCMV-infected mice displayed lower levels of IL-10 in the absence of EB13, an effect that was observed in splenic NK cells at

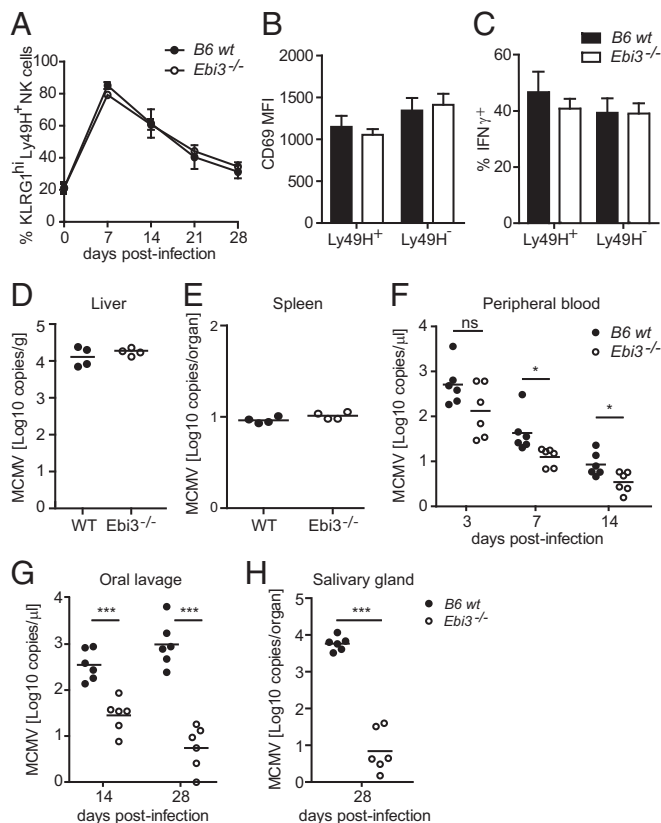


Fig. 4. EB13-deficient mice show decreased MCMV replication in the salivary glands and oral lavage. (A) Expansion of peripheral KLRG1^{hi} Ly49H⁺ NK cells from WT or *Ebi3*^{-/-} B6 mice was measured by flow cytometry at days 0, 7, 14, 21, and 28 p.i. *n* = 6 for each mouse strain and time point from two independent experiments (mean ± SD). (B) CD69 expression and (C) IFN γ production was measured by flow cytometry in splenic Ly49H⁺ and Ly49H⁻ NK cells from WT or *Ebi3*^{-/-} B6 mice at day 1.5 post-MCMV infection. *n* = 4 for each mouse strain from two independent experiments. Data show mean ± SD. MCMV titer in WT or *Ebi3*^{-/-} B6 mice was determined by real-time PCR in (D) liver and (E) spleen at day 4 p.i. in (F) peripheral blood at days 3, 7, and 14 p.i., in (G) saliva (by oral lavage) at day 14 and day 28 p.i., and (H) in salivary glands at day 28 p.i. *n* = 4 or 6 for each mouse strain and time point from two independent experiments. Statistical analysis was performed by two-tailed unpaired Student's *t* test (**P* < 0.05 and ****P* ≤ 0.0003).

day 3.5 p.i. and in the serum at day 5 p.i. As reported previously, production of IL-10 early during MCMV infection is important for limiting DC maturation and T-cell activation to prevent harmful immune-mediated tissue damage in the host (26, 27). During MCMV infection, IL-10 production by NK cells and other immune cells can suppress the maturation DCs, leading to poor priming of CD4⁺ T cells (26). Furthermore, NK cell-mediated IL-10 production during MCMV infection regulates CD8⁺ T-cell activation, where a blockade of IL-10 increases the CD8⁺ T-cell response against MCMV (27). We detected an increase in both the maturation markers of DCs and the percentage of activated CD8⁺ T cells in the MCMV-infected EB13-deficient mice, indicating that the observed decrease in IL-10 production in the EB13-deficient mice was able to enhance the subsequent T-cell response. In this study we found that EB13 affects the production of IL-10 by NK cells during MCMV infection, as measured by direct ex vivo intracellular staining for IL-10 protein. However, it remains to be determined whether the production of IL-10 by other cell subsets, such as myeloid cells and CD4⁺ T cells, is affected by EB13 during MCMV infection. Although beyond the scope of this article, it is also possible that EB13 plays an additional role(s) in MCMV

latency that is independent of IL-10. Production of IL-10 early during MCMV infection depends on the magnitude of viral replication (28). Following a low-dose MCMV infection, viral replication is controlled rapidly within a couple of days and only low levels of IL-10 are produced to limit the immune response. In contrast, during a high-dose MCMV infection, which leads to sustained and elevated levels of viral replication, sustained and higher amounts of IL-10 are needed to limit the immune response and prevent tissue damage (28). Our data suggest that EB13 is essential to sustain IL-10 production during a high-dose MCMV infection.

EB13 can interact with p28 to form IL-27 heterodimers or with p35 to form IL-35 heterodimers (16). We detected p35, but never p28, protein expression in human and mouse NK cells. Both EB13 and p35 were constitutively secreted by the NKL cells. Therefore, in our experimental settings activated NK cells do not produce or secrete IL-27 heterodimers. For several reasons we were not able to distinguish between IL-35 heterodimers and EB13 homodimers and therefore were unable to determine which of the two species are predominantly formed and secreted by the activated NK cells. First, there are no blocking Abs that can distinguish between EB13 and IL-35. Second, IL-35 is very unstable in solution (16), which makes the detection of small amounts by ELISA or immunoprecipitation difficult or impossible. Finally, mice deficient in p35 also lack IL-12, a cytokine essential for NK cell activation and proliferation in response to MCMV infection (6, 10). EB13 protein and the gp130 receptor was up-regulated in splenic NK cells during MCMV infection, whereas no up-regulation was observed in T cells, DCs, or B cells at the time points examined in this study. However, the basal or background level differed between the

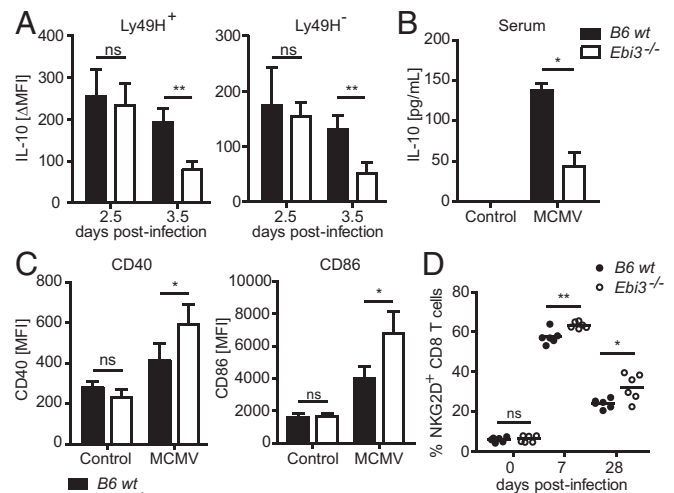


Fig. 5. EB13 promotes IL-10 production by NK cells and negatively affects the maturation of DCs and activation of CD8⁺ T cells during MCMV infection. (A) IL-10 expression was measured by flow cytometry in splenic Ly49H⁺ and Ly49H⁻ NK cells from WT or *Ebi3*^{-/-} B6 mice at day 2.5 and 3.5 p.i. *n* = 4 for each mouse strain from two independent experiments. Shown are the mean ± SD of the induced IL-10 mean fluorescence intensity (MFI) in MCMV-infected compared with noninfected control mice. (B) IL-10 was measured in sera from MCMV-infected (day 5) or noninfected WT or *Ebi3*^{-/-} B6 mice by ELISA. Data are representative of four mice for each time point from two independent experiments. (C) CD40 and CD86 expression was measured by flow cytometry on splenic DCs from MCMV-infected (day 5) or noninfected control WT or *Ebi3*^{-/-} B6 mice. *n* = 4 for each mouse strain from two independent experiments (mean ± SD). (D) NKG2D expression was measured on peripheral CD8⁺ T cells from WT or *Ebi3*^{-/-} B6 mice by flow cytometry at days 0, 7, and 28 p.i. *n* = 6 for each mouse strain and time point from two independent experiments. Statistical analysis was performed by two-tailed unpaired Student's *t* test (**P* < 0.05 and ***P* < 0.01).

various cell types examined. We cannot distinguish between the role of EB13 derived from NK cells versus other cell types during MCMV infection as there presently is no conditional knockout of *Ebi3* to definitively address this issue.

The immune system has established multiple layers of control to ensure effective protection against viral infections, but at the same time to keep the immune system in check to avoid excessive inflammation and autoimmunity. NK cells play a key role in the early control of CMV replication and in modulating the adaptive immune response against the virus (1). Despite the cooperative work between NK cells and T and B cells, CMV can establish persistent infections in mice and humans by exploiting host immune inhibitory pathways to modulate the virus–host balance toward its own benefit (25). Induction of the regulatory cytokine IL-10 during infection represents one such immune inhibitory pathway (29). Our results suggest that the induction of EB13 represents an inhibitory pathway that can be exploited by CMV to establish latent infection. Whether EB13 can affect the persistent infection of other herpesviruses remains to be elucidated. However, it is noteworthy that Epstein–Barr virus, another virus that can establish latency by exploiting the IL-10 inhibitory pathway (25), is also a strong inducer of EB13 expression (12).

Materials and Methods

WT and EB13-deficient ($B6.129 \times 1^{Ebi3^{tm1Rsb/J}}$) mice on a C57BL/6 background were maintained and used in accordance with guidelines of the University of California at San Francisco (UCSF) Institutional Animal Care and Use

Committee. Peripheral blood mononuclear cells (PBMCs) were isolated from blood obtained from the Stanford Blood Center or the Blood Centers of the Pacific under an Institutional Review Board approved protocol (IRB# 10–00265) by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB).

Reagents, vendors, and protocols are included in *SI Materials and Methods*. Details of the mice and cells used, as well as details of the methods used including MCMV infection, in vitro stimulation of NK cells, real-time PCR, flow cytometry, Western blot analysis, IL-10 ELISA, and deep sequencing are presented in *SI Materials and Methods*. Additional questions pertaining to methods, protocols, and reagents are available upon request.

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