EBI3 regulates the NK cell response to mouse cytomegalovirus infection

Jensen, Helle; Chen, Shih-Yu; Folkersen, Lasse Westergaard; Nolan, Garry P; Lanier, Lewis L

Published in:
Proceedings of the National Academy of Sciences of the United States of America

Link to article, DOI:
10.1073/pnas.1700231114

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
EBI3 regulates the NK cell response to mouse cytomegalovirus infection

Helle Jensen*, Shih-Yu Chen*, Lasse Folkersen†, Garry P. Nolan*, and Lewis L. Lanier*✉,†

*Department of Microbiology and Immunology, University of California, San Francisco, CA 94143; †Department of Microbiology and Immunology, Stanford University, Palo Alto, CA 94304; ‡Department of Systems Biology, Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby DK-2800, Denmark; and §Parker Institute for Cancer Immunotherapy, San Francisco, CA 94143

Contribution by Lewis L. Lanier, January 7, 2017 (sent for review November 1, 2016; reviewed by Michael A. Caligiuri and Daniel J. Cua)

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 IFNγ 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 (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 proteins. Signals from the activating receptors promote cytotoxicity, proliferation, and growth, as well as secretion of cytokines and chemokines (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 IFNγ 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) 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 IFNγ and TNFα 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-12Rβ1, IL-12Rβ2, 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 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.

**Significance**

Natural killer (NK) cells play a key role in early viral control of viral replication in and 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

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1700231114/-/DCSupplemental.
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. S14). NKL cells, which constitutively express the activating NKG2D receptor (Fig. S14), 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 of 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. S1 B 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. NK 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. 2 C 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. 2 E 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 CD56bright (i.e., mature) and CD56dim (i.e., immature) NK cells derived from healthy human blood donors. Resting NK cells did not express EBI3 (Fig. S2 A 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 CD56bright and CD56dim NK cells (Fig. S2 A 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.
with plate-bound anti-NKG2D mAb for 24 h (Fig. S2C). In contrast to EBI3 expression, the majority of resting NK cells constitutively expressed p35 (Fig. S2A). We did not detect any protein expression of p28 (Fig. 2B). NK cells stained positive for both gp130 and IL-12Rβ2 as detected by intracellular protein staining (Fig. 2H, “intracellular”). Furthermore, gp130 was detected at the cell surface of NK 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 recycled at the cell surface of the NK 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 CD56bright NK cells (Fig. S2D). Furthermore, the majority of CD56bright NK cells and all of the CD56dim NK cells constitutively expressed IL-12Rβ2 as detected by intracellular protein staining (Fig. S2E). No cell-surface staining of IL-12Rβ2 was detected by flow cytometry (Fig. 2H, “surface”).

**MCMV Infection Induces EBI3 Expression in Mouse NK Cells.** Based on our results with human NK cells and the NK 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 EBI3. During MCMV infection we detected an increase in the intracellular EBI3 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. S3A). The increase in EBI3 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 EBI3, the strongest induction of p35 protein expression was observed at day 2.5 p.i. (Fig. 3E).

**EBI3-Deficient Mice Show Decreased MCMV Replication in the Salivary Glands and Oral Lavage.** To examine the functional role of EBI3 in vivo, we infected wild-type (WT) and EBI3-deficient (Ebi3<sup>−/−</sup>)
Fig. 3. MCMV infection induces EBI3 protein expression in mouse NK cells. Intracellular (A and B) EBI3, (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 ± 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 ± 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).

Discussion

In this study, we show that both human and mouse NK cells express EBI3 protein after stimulation. Furthermore, we describe a previously unidentified pathway wherein EBI3 affects the establishment of MCMV latency. Interestingly, mice deficient in EBI3 showed almost no viral replication in salivary glands and oral laverage, whereas the early viral replication in the spleen and liver was comparable between the EBI3-deficient and WT mice. MCMV-infected mice displayed lower levels of IL-10 in the absence of EBI3, an effect that was observed in splenic NK cells at
Furthermore, NK cell-mediated IL-10 production by T cells in the MCMV-infected EBI3-deficient mice, indicating that EBI3 is essential to sustain IL-10 production during a high-dose MCMV infection.

EBI3 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 EBI3 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 EBI3 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 EBI3 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). EBI3 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

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 EBI3-deficient mice, indicating that the observed decrease in IL-10 production in the EBI3-deficient mice was able to enhance the subsequent T-cell response. In this study we found that EBI3 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 EBI3 during MCMV infection. Although beyond the scope of this article, it is also possible that EBI3 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 EBI3 is essential to sustain IL-10 production during a high-dose MCMV infection.

Fig. 4. EBI3-deficient mice show decreased MCMV replication in the salivary glands and oral lavage. (A) Expansion of peripheral KLRE1+ 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) IFNγ 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 in (H) 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).

Fig. 5. EBI3 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 EBI3 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 EBI3 represents an inhibitory pathway that can be exploited by CMV to establish latent infection. Whether EBI3 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 EBI3 expression (12).

Materials and Methods

WT and EBI3-deficient (B6.129 x 1Ebi3tm1Flai/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.

ACKNOWLEDGMENTS. We thank the L.L.L. laboratory for comments and discussions; Viola Lam and Tsukasa Nabekura for assistance with MCMV infections; Melissa Loden for generating the Ly49H+ NKl cells; Sandra Laurence Lopez-Verges for generating the KIR2DS1+ NKl cells; Roberto Biasi-soni for providing the cDNA of human KIR2DS1; Hisashi Arase for providing the pMXs retroviral vector; Prometheus Laboratories, Inc., for providing human IL-2; and Novo Nordisk, Inc., for providing the deep-sequencing platform. L.L.L. is an American Cancer Society Professor and funded by National Institutes of Health Grants AI066897 and AI068129 and the Parker Institute for Cancer Immunotherapy. H.J. is supported by a Lundbeck Foundation Postdoctoral Fellowship. In addition, the work was supported by NIH Grants U19AI057229, U19AI100627, R33CA183654, R33CA0183692, R01GM10983601, R01CA184968, R01CA1965701, R21CA183660, R01NS0953301, SU2HAR067676, and R01HL120724; the Northrop-Grumman Corporation; Novartis Grant CMX162AU5067; Pfizer Grant 123214; Juno Therapeutics Grant 122401; Department of Defense Grants OC110674 and W81XWH-14-1-0180; Gates Foundation Grant OPP1113682; and Food and Drug Administration Grant BAA-15-00121.