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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

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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 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 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 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) 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 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-12Rb1, IL-12Rb2, IL-23R, gp130, and WSX1-1 (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 regulatory T cells.


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

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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 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. 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 NK 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 CD56dim (i.e., mature) and CD56bright (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

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 –log10 (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.
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. 2F). 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 recirculated 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 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-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^{−/−})
Fig. 3. MCMV infection induces EBI3 protein expression in mouse NK cells. Intracellular (A and B) EBI3, (F) 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 six or four 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 < 0.001 and **p < 0.01).

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 EBI3 protein (23), which includes the functional fibronectin type 3 domain found at amino acids 128–216 (24). Thus, the truncated version of EBI3 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. 4 A and Fig. S4 C). Thus, EBI3 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 IFNγ production at day 1.5 p.i., was comparable between the WT and Ebi3−/− B6 mice (Fig. 4 B 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. 4 D and E). We also observed a similar expression of peripheral blood KLRG1+ Ly49H+ NK cells in the MCMV-infected mice (Fig. 4 F). 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. 4 F). Interestingly, we found that MCMV was cleared most efficiently in the salivary glands and oral lavage of the Ebi3−/− B6 mice (Fig. 4 G and H), which are important sites for virus persistence and dissemination (25).

EBI3 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. 5 A). However, the IL-10 production was significantly decreased at day 3.5 p.i. in the Ebi3−/− B6 mice (Fig. 5 A), suggesting that EBI3 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. 5 B), indicating that the overall production of IL-10 was affected in the mice. We further examined the impact of EBI3 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. 5 C). 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. 5 D). Thus, together these results indicate that EBI3 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 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 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 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
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.
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 Ebi3<sup>−/−</sup>Flk1+) 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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