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EBI3 deficiency leads to diminished T helper type 1 and increased T helper type 2 mediated airway inflammation

Elif Dokmeci,[1] Lan Xu,[2] Eve Robinson,[2] Kseniya Golubets,[2] Kim Bottomly[3] and Christina A. Herrick[2]

[1]*Department of Pediatrics, Section of Pediatric Pulmonology, Yale University School of Medicine, New Haven, CT,* [2]*Department of Dermatology, Yale University School of Medicine, New Haven, CT, and* [3]*President's Office, Wellesley College, Wellesley, MA, USA*

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Correspondence: Dr E. Dokmeci, Department of Pediatrics, Section of Pediatric Pulmonology, Louisiana State University Health Science Center, 1501 Kings Highway Shreveport, LA 71130, USA.
Email: edokme@lsuhsc.edu
Senior author: Christina Herrick, email: christina.herrick@yale.edu

Summary

Despite extensive investigation of the signals required for development of T helper type 1 (Th1) and type 2 (Th2) immune responses, the mechanisms involved are still not well-defined. A critical role for Epstein–Barr virus-induced gene 3 (*EBI3*) in these responses has been proposed. *EBI3*, initially discovered as a transcriptionally activated gene in Epstein–Barr virus-infected B lymphocytes, codes for a subunit of the cytokine interleukin-27 (IL-27). While initial studies suggested that it had an important role in promoting Th1 responses, subsequent studies have revealed that EBI3 receptor signalling influences a variety of immune cell types and can inhibit both Th1 and Th2 responses. In the present study, we evaluated EBI3$^{-/-}$ mice for their ability to mount both Th1-mediated and Th2-mediated airway inflammatory responses. The EBI3$^{-/-}$ mice sensitized by exposure to inhaled ovalbumin plus a high dose of lipopolysaccharide, which normally results in Th1 responses in wild-type (WT) mice, instead developed Th2 type airway inflammation, with increased numbers of eosinophils. The EBI3$^{-/-}$ mice that were exposed to inhaled ovalbumin with a low dose of lipopolysaccharide, which induces Th2 responses in WT mice, showed a marked enhancement of these responses, with increased airway eosinophils, increased serum IgE levels and increased levels of Th2 cytokines (IL-4, IL-5 and IL-13) in culture supernatants of mediastinal lymph node cells. Increased production of Th2 cytokines was also seen when naive CD4$^+$ T cells from EBI3$^{-/-}$ mice were stimulated *in vitro* compared with cells from WT mice. These results provide the first evidence that EBI3 may play an inhibitory role in allergic asthma development.

Keywords: allergic asthma; CD4/helper T cells; cytokines; EBI3; mice

Introduction

Epstein–Barr virus-induced gene 3 (*EBI3*) was initially discovered as a transcriptionally activated gene in Epstein–Barr virus-infected human B lymphocytes.[1] It encodes a 34 000 molecular weight haematopoietin receptor that lacks a membrane-anchoring motif and has a similar structure to the p40 subunit of interleukin-12 (IL-12).[2,3] The EBI3 protein forms heterodimers with either IL-12p35 or p28, a novel IL-12p35-related polypeptide.[4] The heterodimeric cytokine composed of EBI3 and p28 subunits is called IL-27, which is a member of the IL-12 family,[5] and is known to be an early product of activated antigen-presenting cells (APCs) that is produced upon Toll-like receptor ligation. It drives a rapid clonal expansion of naive, but not memory CD4$^+$, T cells, and synergizes with IL-12 to trigger interferon-γ (IFN-γ) production via T-bet from naive CD4$^+$ T cells.[1,4,6–10] Interleukin-27 promotes T helper type 1 (Th1) differentiation through the WSX-1/TCCR receptor which is associated with gp130.[11] Studies of WSX-1/TCCR-deficient mice found impaired IFN-γ production and Th1 differentiation and increased susceptibility to infections with intracellular pathogens.[12,13] It has also been reported,

Abbreviations: APC, antigen-presenting cell; BAL, bronchoalveolar lavage; EBI3, Ebstein–Barr-induced gene 3; IFN, interferon; IL-12, interleukin-12; LN, lymph node; LPS, lipopolysaccharide; OVA, ovalbumin; Th, T helper; WT, wild-type.

however, that production of EBI3 can be associated with a predominant Th2 response, such as in the placenta or in Hodgkin and Reed–Sternberg cells of Hodgkin lymphoma.[2,7,14] In addition, production of EBI3 can be higher than that of p28 in some instances, suggesting that EBI3 may have functions other than the Th1-promoting role described for IL-27, acting either as a homodimer or in association with other heterodimeric binding partners.[1,8] For example, it has been reported that levels of *EBI3* expression were higher in mucosal samples of ulcerative colitis compared with Crohn's disease.[14,15] Consistent with these data, EBI3-deficient ($EBI3^{-/-}$) mice displayed an impaired ability to mount Th2 immune responses and were protected against oxazolone-induced colitis.[16] Hence, EBI3 could have distinct functions and play independent roles from those described for the EBI3/p28 heterodimer, IL-27.

Allergic asthma is a chronic airway disease characterized by airway hyper-responsiveness, eosinophilic infiltration, mucus hypersecretion, and elevated serum IgE.[17] Both augmented Th2 responses and impaired Th1 responses have been described in this condition,[18,19] with Th2-type cytokines playing a critical role in the inflammation seen in asthma.[20] Interleukin-4 participates in Th2 cell differentiation,[21,22] IgE isotype class switching,[23] and airway remodelling containing enhanced collagen synthesis by fibroblasts.[24] Interleukin-5 promotes differentiation and migration of eosinophils[25–27] and IL-13 plays a major role in mediating both airway hyper-responsiveness and mucus production.[28] Inappropriate development of Th2 responses therefore appears to play a central role in the development of allergic asthma. In contrast, Th1 cells have been shown to prevent allergic disease by inhibiting the activity of Th2 cells.[29–31]

The gene *EBI3* is expressed by tissue macrophages and dendritic cells, two cell types that play important functional roles in asthma.[5,32] Lipopolysaccharide (LPS) -stimulated dendritic cells showed expression of both p28 and EBI3 before expression of IL-12 p35/p40 subunits, suggesting that IL-27 may act very early in the generation of Th1 immunity. However, recent studies demonstrated that the biological function of IL-27/WSX-1 signalling is more complex, because it is also critically involved in the negative control of both Th1 and Th2 inflammatory responses.[33,34] As a result, to gain a better understanding of the role of EBI3 in asthma, we evaluated the ability of $EBI3^{-/-}$ mice to generate both Th1-mediated and Th2-mediated airway inflammation. Our previous work has shown that either Th1 or Th2 responses can be induced in response to inhaled ovalbumin (OVA), depending on the dose of inhaled LPS used during the initial exposure.[35] Exposure to inhaled OVA along with a low dose of LPS results in Th2-mediated allergic airway inflammation upon subsequent inhaled OVA challenge, whereas exposure to a higher dose of LPS during inhalational sensitization to OVA results in Th1-mediated airway inflammation upon challenge. By using these two established lung inflammation models we studied the role of EBI3 in the generation of Th1 or Th2 responses in the airway. The data presented here show that in the absence of EBI3, exposure to inhaled OVA plus a high dose of LPS (OVA + LPS^{high}) fails to generate the Th1 response normally seen in wild-type (WT) mice, but instead results in Th2 type airway inflammation. Further, exposure to inhaled OVA plus a low dose of LPS (OVA + LPS^{low}) results in Th2 responses that are markedly greater than those seen in WT mice. It was not clear, however, whether the enhanced Th2 response seen in $EBI3^{-/-}$ mice indicated an inhibitory role for EBI3 in Th2 generation normally, or whether it was simply a reflection of the release from Th1 inhibition. To address this question, we evaluated the effect of EBI3 deficiency in another model of Th2 generation involving initial epicutaneous exposure to OVA.[36] In this model, highly skewed Th2 responses are seen, with no evidence of a Th1 component. We show here that $EBI3^{-/-}$ mice sensitized by epicutaneous exposure to OVA mounted Th2 responses, as measured by Th2 cytokine production in skin-draining lymph nodes, that were equivalent to those seen in WT mice, with no enhancement of the response as was seen in the mice sensitized by exposure to OVA inhalation. The data suggest that the robust Th2 responses seen in the airways of $EBI3^{-/-}$ mice were probably the result of the loss of the normal Th1 response seen in these mice, and not a direct result of the Th2 inhibitory effect of EBI3 on Th2 development.

Materials and methods

Mice

BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). A breeding pair of EBI3 knockout mice (on a BALB/c background) was kindly provided by Dr R. Blumberg (Boston, MA) and was bred in our facility.[16] Mice were housed in the pathogen-free animal facility at Yale University Animal Care Facility. Six- to 8-week-old female mice were used in all experiments. All studies were approved by the Yale University School of Medicine Institutional Animal Care and Use Committee (IACUC). (IACUC #: 2004-10393, approved 16 February 2006).

Inhalational and sensitization challenge protocols

The WT and $EBI3^{-/-}$ mice were anaesthetized with Isoflurane and then exposed intranasally to 100 μg OVA (Grade V; Sigma-Aldrich, St Louis, MO) in 50 μl PBS on days 0, 1 and 2. To induce a Th2 response, mice received 100 μg intranasal OVA with a low dose (0·05 μg) of

Escherichia coli LPS O55:B5 (Sigma-Aldrich); for Th1 generation mice received 100 μg intranasal OVA with a high dose of LPS (20 μg). All mice were challenged on days 14, 15, 18 and 19 intranasally with 25 μg OVA in 50 μl PBS.

Epicutaneous sensitization

BALB/c and EBI3$^{-/-}$ mice were sensitized by epicutaneous exposure to OVA, as previously described.[36] Briefly, mice had their backs shaved with electric clippers, and then 1–2 days later (day 0), 50 μl of either OVA (Grade V; Sigma Chemical Co., St Louis, MO) (2·0 mg/ml) in PBS or PBS alone was applied to gauze in the centre of an occlusive patch (BAND-AID® Clear Spots; Montreal, QC, Canada), which was then affixed to their backs. Patches were left intact for 4 days.

Analysis of bronchoalveolar lavage fluid

Mice were killed on day 21 by exposure to CO_2 and a cannula was inserted into the trachea. Cells from the airways were collected by infusing three 1-ml washes of cold PBS. Cytospin preparations of the bronchoalveolar lavage (BAL) fluid cells (up to 1×10^5 per slide) were generated for individual mice, stained with Diff-Quik (Baxter Healthcare, Miami, FL), and differentials were performed on 200 cells based on morphology and staining characteristics.

Lymph node cytokine production

Mediastinal lymph nodes (LN) were harvested from mice that had been previously sensitized and challenged intranasally as described above, at the time of death on day 21. Cells from individual mice were isolated and stimulated *in vitro* by culture with OVA and APCs (syngeneic T-cell-depleted splenocytes). A total of $2{\cdot}5 \times 10^5$ CD4$^+$ T cells/ml, $2{\cdot}5 \times 10^5$/ml freshly isolated APCs and pOVA (5 μg/ml) in Click's/5% fetal calf serum were cultured at 37° for 48 hr. Levels of IFN-γ, IL-4, IL-5 and IL-13 from cell supernatants were determined by Luminex (Beadlyte Mouse 21-Plex cytokine detection system, Upstate Cell Signaling Solutions, Lake Placid, NY), a bead-based multiplex immunoassay. Bio-Plex Manager program was used as the data analysis software. In some experiments, skin-draining (axillary) LNs were harvested on day 4 after epicutaneous exposure to OVA, as described above, and restimulated *in vitro* as above for mediastinal LN cells.

OVA-specific antibody measurement by ELISA

Serum was collected at the time of death on day 21. The OVA-specific antibodies (IgG1, IgG2a, IgE) were measured by ELISA as previously described,[26] using biotin-labelled rat anti-mouse antibodiebs [anti-IgG1 (Biosource International, Camarillo, CA), anti-IgG2a (R19-15; BD PharMingen, San Diego, CA) and anti-IgE (Biosource International)]. The OVA-specific antibody concentrations were calculated by comparison with the following standards: monoclonal anti-OVA mouse IgG1 (Sigma); monoclonal anti-OVA mouse IgE (kindly provided by Dr E. Gelfand, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO); and IgG2a, pooled hyperimmune serum generated by repeated intraperitoneal injection of BALB/c IL-4$^{-/-}$ mice with OVA in alum (concentration arbitrarily set at 200 000 U/ml).

In vitro *stimulation of T cells*

CD4$^+$ T cells were isolated from BALB/c and EBI3$^{-/-}$ mice by negative selection as previously described[27] using monoclonal antibodies to CD8 (clone 53-6.72, clone 2.43),[28] class II MHC I-Ad (212.A1),[29] and anti-immunoglobulin-coated magnetic beads (Collaborative Research, Bedford, MA). Naive CD4$^+$ CD44^{low} CD62L^{high} T cells were purified under sterile conditions from spleens of WT and EBI3$^{-/-}$ mice by FACS sorting. All staining procedures were performed on ice. Cells were incubated with anti-FcR (24G2) antibody in combination with mouse immunoglobulin for 20 min on ice then stained with antibodies against CD44, CD62 and appropriate isotype controls (BD Bioscience, Bedford, MA) for 30 min on ice. Cells were purified by FACS sorting using FACSCalibur. Syngeneic T-cell-depleted splenocytes were used as APCs and prepared by negative selection using antibodies to CD4 (GK1.5),[30] anti-CD8, anti-Thy1,[31] and treatment with rabbit complement. The APCs were mitomycin-C-treated. All cultures were set up in flasks containing a 1 : 2 ratio of CD4$^+$ T cells and APCs at a concentration of 1×10^6 CD4$^+$ T cells/ml. Cells were stimulated by anti-CD3 and recombinent IL-2 (25 U/ml) for 4 days. Production of Th1 and Th2 cytokines was determined by Luminex from culture supernatants. In some experiments, IL-12 (5 ng/ml) was added to the naive T-cell cultures.

Effector T cells

Cultured T cells were harvested after 4 days, washed with PBS and restimulated with platebound anti-CD3 for 48 hr. Production of Th1 and Th2 cytokines was determined by Luminex from culture supernatants.

Statistical analysis

Differences were evaluated for significance ($P < 0{\cdot}05$) by Student's two-tailed *t*-test for independent events (Excel). Data are given as mean values ± SEM. Unless indicated otherwise, five mice were used for each condition studied in an individual experiment. Each treatment condition was repeated at least three times.

Results

Increased airway inflammation with elevated eosinophils and reduced neutrophils in BAL fluid of EBI3$^{-/-}$ mice

We used our previously established models of Th1-mediated and Th2-mediated airway inflammation to evaluate the effect of EBI3 deficiency on airway responses. Our previous studies have shown that WT mice exposed to OVA with a low dose of LPS initially, will generate Th2 responses, with characteristic Th2-mediated allergic airway inflammation, including increased eosinophil numbers in the BAL fluid, seen upon subsequent inhaled antigen challenge. On the other hand, mice exposed to OVA plus a high dose of LPS initially, generate Th1 responses, including elevated BAL neutrophil numbers following inhaled antigen challenge. The WT or EBI3$^{-/-}$ mice were exposed to OVA intranasally with either a low or high dose of LPS, or to PBS alone, on days 0, 1 and 2. Mice were then challenged on days 14, 15, 18 and 19 with intranasal OVA and killed on day 21. As shown in Fig. 1, total BAL cell counts in EBI3$^{-/-}$ mice were higher in both the OVA + LPS^{low} (Th2) and OVA + LPS^{high} (Th1) groups compared with WT mice, indicating a greater inflammatory response in the absence of EBI3. More specifically, EBI3$^{-/-}$ mice showed significantly higher numbers of eosinophils in BAL fluid, the hallmark of Th2-mediated airway inflammation, in both OVA + LPS^{low} and OVA + LPS^{high} sensitized groups (Fig. 1b). In addition, we observed that neutrophil infiltration of BAL fluid was significantly lower in EBI3$^{-/-}$ mice that had been exposed to OVA + LPS^{high}, indicating a decreased response. Taken together, the data indicate that EBI3 deficiency caused an overall increase in allergic airway inflammation, with both a decrease in Th1 generation and an increase in Th2 responses.

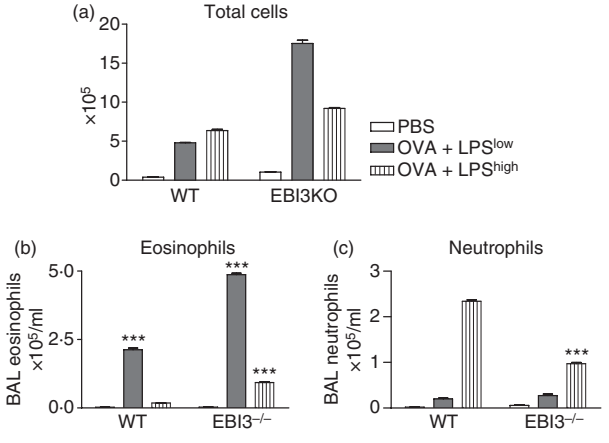

Figure 1. Comparison of airway inflammation by analysing bronchoalveolar lavage (BAL) cell counts and differential analysis in BALB/c wild-type (WT) mice and BALB/c EBI3-deficient (EBI3$^{-/-}$) mice. WT mice and EBI3$^{-/-}$ mice were exposed intranasally to PBS alone, or ovalbumin (OVA) with low-dose lipopolysaccharide (LPS; 0·05 μg) in PBS, or OVA with high-dose LPS (20 μg) in PBS during priming. They were challenged 2 weeks later with intranasal OVA. After killing, BAL was performed and inflammatory cells recovered from individual mice were counted. All data are reported as mean ± SEM from individual mice. Data are pooled from three different experiments (WT, $n = 15$ and EBI3$^{-/-}$, $n = 15$). Statistical significance was determined by unpaired Student's t-test; $^{***}P < 0{\cdot}001$. (a) Total number of BAL cells in WT and EBI3$^{-/-}$ mice in three different groups; (b) absolute number of eosinophil; (c) neutrophil counts by BAL cell differential analysis.

Augmented production of Th2 and reduced production of Th1 cytokines by mediastinal LN cells from EBI$^{-/-}$ mice

Next we investigated cytokine production by mediastinal LN cells isolated from OVA + LPS^{low} and OVA + LPS^{high} sensitized mice. As shown in Fig. 2, the levels of IL-4, IL-5 and IL-13 were all greatly enhanced in cultures of LN cells from both WT and EBI3$^{-/-}$ mice that had been exposed to OVA + LPS^{low} compared with PBS-exposed controls. However, levels of these cytokines were significantly higher in EBI3$^{-/-}$ mice compared with those in WT mice. Moreover, IFN-γ levels, which indicate Th1-mediated inflammation, were significantly lower in cultures of LN cells from EBI3$^{-/-}$ mice compared with those from WT mice that had been exposed to OVA + LPS^{high}. These data clearly demonstrated augmented Th2, and decreased Th1, cytokine production in the LN of EBI3$^{-/-}$ mice, compared

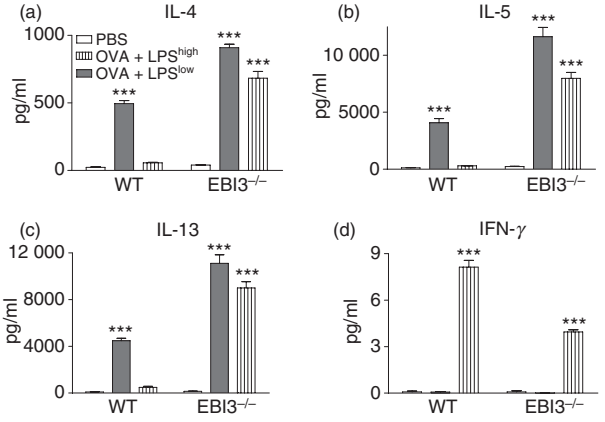

Figure 2. Cytokine production in mediastinal lymph nodes. Wild-type (WT) BALB/c and EBI3-deficient (EBI3$^{-/-}$) mice were sensitized and challenged with intranasal ovalbumin (OVA) and on day 21 mediastinal lymph node cells were isolated and stimulated *in vitro* with OVA and antigen-presenting cells (APC) from naive BALB/c mouse spleens. Cytokines in culture supernatants were measured at 48 hr (a–d). Data are reported as mean ± SEM cytokine levels from three separate experiments. Statistical significance was determined by unpaired Student's t-test; $^{***}P < 0{\cdot}001$.

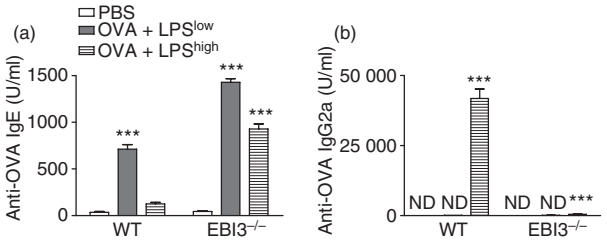

Figure 3. (a) Augmented antigen-specific IgE production in EBI3-deficient (EBI3$^{-/-}$) mice. (b) Undetectable levels of anti-ovalbumin (OVA) IgG2a production in EBI3$^{-/-}$ mice. Anti-OVA IgE and anti-OVA IgG2a levels were measured in sera from wild-type (WT) and EBI3$^{-/-}$ mice. Sera were collected from OVA + LPS^{low}, OVA + LPS^{high} and PBS-sensitized WT and EBI3$^{-/-}$ mice during harvest and antibody levels were measured by ELISA as described in the Materials and methods. Data are the mean ± SEM (n = 5 mice/group). Statistical significance was determined by unpaired Student's t-test; ***$P < 0{\cdot}001$. Experiments were repeated three times with similar results. N.D., none detectable.

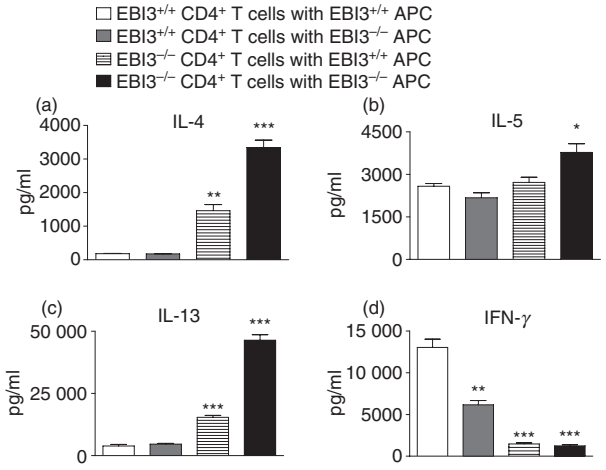

Figure 4. *In vitro* cytokine responses: CD4$^+$ CD44^{low} CD66L^{high} T cells were purified from spleens of BALB/c and EBI3-deficient (EBI3$^{-/-}$) mice through FACS sorting and cultured for 4 days with soluble anti-CD3 antibody and antigen-presenting cells (APC) in the presence of interleukin-2 (IL-2). Expanded CD4$^+$ T cells from EBI3$^{-/-}$ and BALB/c mice were washed after 96 hr and cultured with plate-bound anti-CD3 antibody for 48 hr. Concentrations of IL-4, IL-5, IL-13 and interferon-γ (IFN-γ) in the culture supernatants were determined by Luminex (a–d). There was significant difference in the cytokine responses of cells from EBI$^{-/-}$ mice and BALB/c mice. Data are reported as mean ± SEM, n = 5, cytokine levels from three separate experiments. Statistical significance was determined by unpaired Student's t-test; *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.

with WT mice. These results were consistent with the results of the BAL fluid analysis as shown in Fig. 1.

EBI3 deficiency results in augmented IgE and decreased IgG2a responses

It is well documented that levels of serum IgE are strongly associated with the severity of asthma.[37] To confirm the findings of augmented Th2 responses in EBI3$^{-/-}$ mice we measured anti-OVA IgE levels in WT and EBI3$^{-/-}$ mice (Fig. 3). In previous studies, we have shown that OVA + LPS^{low}-sensitized WT mice produce high levels of anti-OVA IgE as a marker of Th2 generation.[35] In contrast, no anti-OVA IgE was detected in OVA + LPS^{high}-treated WT mice. The data presented here show that EBI3$^{-/-}$ mice produced significantly higher levels of anti-OVA IgE, compared with WT mice, following OVA + LPS^{low} sensitization. Furthermore, OVA + LPS^{high}-sensitized EBI3$^{-/-}$ mice also produced remarkable levels of anti-OVA IgE. In contrast, the levels of anti-OVA IgG2a, a Th-1-associated cytokine, were significantly decreased in OVA + LPS^{high}-sensitized EBI3$^{-/-}$ mice, compared with WT mice. Taken together, these data, along with the airway inflammation data presented above, indicate that EBI3$^{-/-}$ mice are somewhat Th2 prone, even when sensitized with the normally Th-1-inducing OVA + LPS^{high} regimen. Likewise, Th1 responses appear defective in EBI3$^{-/-}$ mice.

Enhanced production of Th2 cytokines and reduced levels of IFN-γ in EBI3$^{-/-}$ CD4$^+$ T cells stimulated *in vitro*

For additional confirmation of Th2 cytokine overproduction by EBI3$^{-/-}$ T cells, we investigated the effects of EBI3 deficiency *in vitro*. CD4$^+$ naive T cells (CD44^{low} CD66L^{high}) were purified from the spleens of WT and EBI3$^{-/-}$ mice and cultured for 4 days with syngeneic APCs from the spleens of WT or EBI3$^{-/-}$ mice in the presence of anti-CD3 and IL-2. On day 4, cells were washed and restimulated *in vitro* with plate-bound anti-CD3 for a further 2 days. Cytokine levels in culture supernatants were then analysed using a multiplex-based bead technology. As shown in Fig. 4, CD4$^+$ T cells from EBI3$^{-/-}$ mice showed augmented production of Th2 cytokines following *in vitro* stimulation, while IFN-γ production was decreased. Interestingly, an even more marked increase was observed if both CD4$^+$ T cells and APCs were from EBI3$^{-/-}$ mice. The absence of EBI3 led to potent production of Th2 cytokines with reduced IFN-γ production. These *in vitro* results were consistent with the findings reported above of increased Th2 and decreased Th1 generation *in vivo* in the EBI3$^{-/-}$ mice.

IFN-γ production is enhanced in EBI3$^{-/-}$ T-cell cultures by IL-12

We sought to determine if we could bypass the inhibitory effect of EBI3 protein deficiency on generation of Th1 responses by addition of exogenous IL-12. Naive CD4$^+$ T cells (CD44^{low} CD66L^{high}) were stimulated *in vitro* for

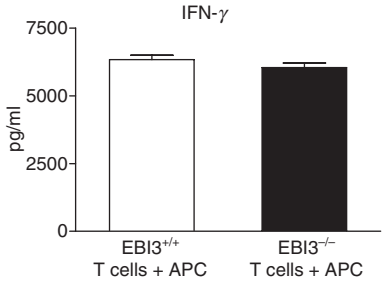

Figure 5. Sorted mouse $CD4^+$ $CD44^{low}$ $CD66L^{high}$ (naive) T cells from EBI3-deficient ($EBI^{-/-}$) and wild-type (WT) mice were cultured with antigen-presenting cells (APC) from $EBI^{-/-}$ and WT mice in the presence of anti-CD3 monoclonal antibody, interleukin-2 (IL-2) and IL-12 for 4 days. Levels of interferon-γ (IFN-γ) were measured and comparison was made between WT and EBI3-deficient cells. There was no difference in IFN-γ responses of cells from $EBI3^{-/-}$ mice and BALB/c mice once IL-12 was added to the culture media. Data are means ± SEM, are representative of three independent experiments.

4 days with anti-CD3 monoclonal antibody, IL-2 and saturating amounts of IL-12. In the presence of IL-12, naive $CD4^+$ T cells from $EBI3^{-/-}$ mice were capable of producing substantial amounts of IFN-γ (Fig. 5). This suggested that in the absence of EBI3 protein, exogenous IL-12 itself would be sufficient to generate Th1 responses *in vitro*.

Epicutaneously induced Th2 responses are similar in $EBI3^{-/-}$ and WT mice

From the results described above, it was difficult to distinguish whether the heightened Th2 responses seen in $EBI3^{-/-}$ mice were a consequence of a lack of Th1-mediated inhibition or of a direct inhibitory effect of EBI3 protein on Th2 responses. To better dissect which effect was predominant, we evaluated Th2 responses induced by epicutaneous OVA exposure, a model that produces highly skewed Th2 responses, with little evidence of Th1 generation. In this model; WT and $EBI3^{-/-}$ mice were exposed to epicutaneous OVA on day 0, and skin-draining LNs were harvested on day 4.[36] The LN cells were then restimulated *in vitro* by culture with OVA and APCs, and levels of Th2 cytokines and IFN-γ were measured in culture supernatants. As shown in Fig. 6, equally high levels of the Th2 cytokines IL-5 and IL-13 were present in cultures of LN cells from either WT or $EBI3^{-/-}$ mice. Therefore, there was no enhancement of the Th2 response generated in epicutaneously sensitized mice, as had been seen in our inhalational model. The data suggest that EBI3 deficiency leads primarily to a defect in Th1 generation, and that the enhanced Th2 responses seen in $EBI3^{-/-}$ mice are probably the result of an indirect release from Th1-mediated suppression.

Discussion

Allergic airway disease remains an important public health problem associated with significant morbidity and mortality in all ages. Understanding the risk factors and natural history of this disease is important for the development of new targets to control airway inflammation. Therapeutic intervention has focused on the appreciation that airway obstruction in asthma is composed of both bronchial smooth muscle spasm and variable degrees of airway inflammation, with prominent infiltration of lymphocytes and eosinophils. This inflammatory response is believed to be the result of aberrant Th2 immune responses to commonly inhaled antigens and defective

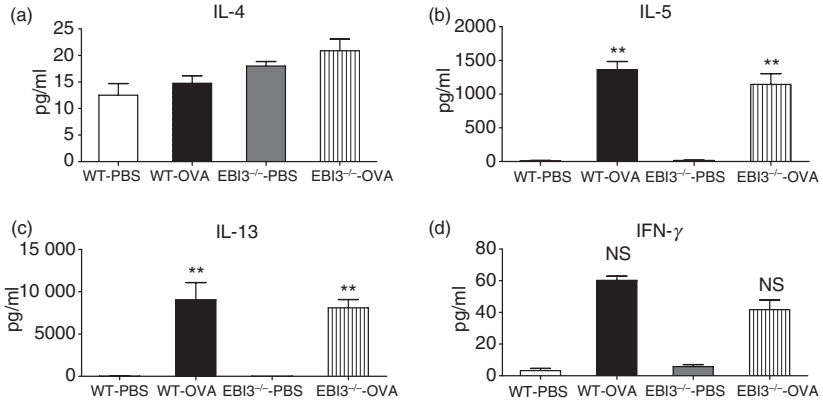

Figure 6. Cytokine responses in draining axillary lymph nodes after epicutaneous ovalbumin (OVA) sensitization. Wild-type (WT) BALB/c and EBI3-deficient ($EBI3^{-/-}$) mice were sensitized epicutaneously with OVA or PBS and on day 4 axillary lymph node cells were isolated and stimulated *in vitro* with OVA and antigen-presenting cells (APC) from naive BALB/c spleen. Cytokines in culture supernatants measured at 48 hr (a–d). There was no difference in cytokine responses of cells from $EBI3^{-/-}$ mice and BALB/c mice after OVA sensitization. Data are reported as mean ± SEM cytokine levels from three separate experiments. Statistical significance was determined by unpaired Student's *t*-test; $^{**}P < 0{\cdot}01$ and N.S., not significant.

Th1 responses. Despite extensive investigations to elucidate the signals required for initiation and development of Th1 and Th2 immunity, these mechanisms are still not well-defined.

In this study we investigated the role of EBI3 in Th1-mediated or Th2-mediated airway inflammatory responses using well established mouse models. EBI3 has been proposed to play a critical role in the regulation of T-cell functions and various studies have revealed that EBI3 receptor signalling influences a variety of immune cell types, resulting in inhibition of either Th1 or Th2 type responses. The studies presented here were performed using our previously developed mouse models of Th1-mediated and Th2-mediated airway inflammation generated by adjusting the LPS dose used during initial antigen inhalation.[35,36] In these models, exposure to a high level of LPS during the initial inhalational exposure to OVA results in antigen-specific Th1 responses, whereas a low dose of LPS results in Th2 responses. By using these well-established models we were able to analyse the role of EBI3 in the induction of Th1-mediated and Th2-mediated airway inflammation. As evidenced by our airway inflammation data, EBI3-deficient mice showed enhanced Th2 responses, with increased numbers of eosinophls in the airways following inhaled antigen challenge. This increase in Th2-type airway inflammation was seen following a normally Th1-inducing sensitization protocol (high-dose LPS), as well as after the normally Th2-inducing sensitization protocol (low-dose LPS). To our knowledge, this is the first study showing the role of EBI3 in Th1-mediated airway inflammation and comparing the results with Th2-mediated airway inflammation.

Enhanced Th2 generation in EBI3$^{-/-}$ mice was also indicated by analysis of the cytokine profiles in lung-draining lymph nodes. Levels of IFN-γ were extremely low in the OVA + LPS^{high}-sensitized EBI3$^{-/-}$ mice, whereas Th2 cytokines were elevated. In addition, naive CD4$^+$ T cells isolated from EBI3$^{-/-}$ mice also produced more Th2 cytokines and less IFN-γ following *in vitro* stimulation. Furthermore, addition of IL-12, a Th1 skewing cytokine, into the T-cell cultures, overpowered the deficiency of Th1 development by cells from EBI3$^{-/-}$ mice. In the presence of IL-12 EBI3-deficient cells produced levels of IFN-γ that were comparable to those produced by cells from WT mice. This also indicates that EBI3 is probably a contributor to IFN-γ production from Th1 cells but it is not absolutely required. Finally, the data from epicutaneously sensitized mice provide further evidence that the increased Th2 responses seen in the EB13$^{-/-}$ mice were probably the result of a lack of EBI3-stimulated Th1 responses, and hence of a release of Th2 responses from Th1-mediated inhibition, rather than a direct suppression of Th2 inflammation by EBI3.

WSX-1 is a class I cytokine receptor with homology to gp130 of the IL-6R and IL-12R families.[38] EBI3 has been identified as a ligand for WSX-1, inducing the proliferation of naive CD4$^+$ T cells and production of IFN-γ in synergy with IL-12.[4] Experiments using WSX-1-deficient mice revealed that WSX-1 plays an important role in Th1 differentiation during infection with some intracellular pathogens. Namely, WSX-1-deficient mice showed increased susceptibility to infection with *Listeria monocytogenes* and *Leishmania major* because of impaired IFN-γ production.[9,13] It was also demonstrated that WSX-1 deficiency gave rise to augmentation of allergen-induced airway hyper-responsiveness and lung inflammation. Antigen-induced lung inflammation involving goblet cell hyperplasia and eosinophilic infiltration was significantly augmented in OVA-challenged WSX$^{-/-}$ mice. Also, GATA-3, a Th2 transcription factor, did not seem to be involved in the overproduction of Th2 cytokines by WSX-1-deficient lymphocytes, because the expression of *GATA3* was comparable between WT and WSX-deficient mice. Similarly, it was demonstrated that IL-27/WSX-1 interaction induces T-bet and IL-12Rβ2 expression through activation of signal transducer and activator of transcription 1.[9] These studies support the data presented here that the overproduction of Th2 cytokines in EBI3$^{-/-}$ mice is not the result of a direct inhibitory effect of EBI3 on Th2 development, but rather is secondary to a lack of Th1 stimulating effect of EBI3.

In conclusion, we have demonstrated that EBI3 deficiency is associated with enhanced development of Th2-mediated airway inflammatory responses, and that EBI3 probably plays a critical role in the generation of Th1-mediated airway inflammation, thereby protecting against Th2-mediated allergic airway inflammation.

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Disclosures

The authors have no financial conflict of interest.

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