

Lung CD11c⁺ cells from mice deficient in Epstein-Barr virus-induced gene 3 (EBI-3) prevent airway hyper-responsiveness in experimental asthma

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Epstein-Barr virus-induced gene (EBI)-3 codes for a soluble type 1 cytokine receptor homologous to the p40 subunit of IL-12 that is expressed by antigen-presenting cells following activation. Here, we analyzed the functional role of EBI-3 in a murine model of asthma associated with airway hyper-responsiveness (AHR) in ovalbumin-sensitized mice. Upon allergen challenge, EBI-3^{-/-} mice showed less severe AHR, decreased numbers and degranulation of eosinophils and a significantly reduced number of VCAM-1⁺ cells in the lungs as compared to wild-type littermates. We thus analyzed lung CD11c⁺ cells before and after allergen challenge in these mice and found that before allergen challenge, lung CD11c⁺ cells isolated from EBI-3^{-/-} mice express markers of a more plasmacytoid phenotype without releasing IFN- α as compared to those from wild-type littermates. Moreover, allergen challenge induced the development of myeloid CD11c⁺ cells in the lungs of EBI-3^{-/-} mice, which released increased amounts of IL-10 and IL-12 while not expressing IFN- α . Finally, inhibition of EBI-3 expression in lung DC could prevent AHR in adoptive transfer studies by suppressing mediator release of effector cells into the airways. These results indicate a novel role for EBI-3 in controlling local immune responses in the lungs in experimental asthma.

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Abbreviations: **AHR:** airway hyper-responsiveness · **BAL:** bronchoalveolar lavage · **CBA:** cytometric beads array · **EBI:** Epstein-Barr virus-induced gene · **MBP:** major basic protein · **MCh:** methacholine · **mDC:** myeloid dendritic cell · **pDC:** plasmacytoid dendritic cell · **RI:** airway resistance

Introduction

Epstein-Barr virus (EBV) infection results in the expression of various genes by the host cell such as Epstein-Barr virus-induced gene (EBI)-3 on infected B cells. The EBI-3 gene encodes for a soluble type 1 cytokine receptor, homologous to the p40 subunit of interleukin-12 (IL-12) which is also expressed by antigen-presenting cells following activation. Recently,

EBI-3 has been found to be associated with a new IL-12p35-related subunit, denoted p28, to form a non-covalently bound linked heterodimeric cytokine (EBI-3/p28) called IL-27 [1]. IL-27 (EBI-3/p28) is known to be an early product of activated antigen-presenting cells that is produced upon TLR ligation. It drives a rapid clonal expansion of naive but not memory CD4⁺ T cells, and synergizes with IL-12 to trigger IFN- γ production via T-bet from naive CD4⁺ T cells [1–4]. Although the presence and biological functions of EBI-3 *per se* or of the EBI-3/EBI-3 homodimer remain still unclear, a distinct receptor for IL-27 is the orphan receptor WSX-1/TCCR that is associated with gp130 [5]. WSX-1/TCCR deficiency leads to impaired IFN- γ production and Th1 differentiation and increased susceptibility to infections with intracellular pathogens [6, 7]. WSX-1 is a novel class I cytokine receptor with homology to the IL-12 receptors, and it is highly expressed in lymphoid tissue [7]. It has been suggested that STAT-1 is activated through interaction with the tyrosine residue in the cytoplasmic domain of WSX-1. Furthermore, IL-27 induces expression of T-bet and IL-12R β 2 through WSX-1 signaling in wild-type naive CD4⁺ T cells, indicating that IL-27/WSX-1 signaling is important for the initial commitment of the Th1 responses [8, 9]. Although IL-27 has been shown to positively regulate Th1 pathways, EBI-3 could have a distinct function that is not yet understood [10]. These data indicate that EBI-3 can signal independently from p28 and WSX-1.

Asthma is a disease that is notable for augmented Th2 and impaired Th1 cytokine responses [11, 12]. A possible explanation for the altered Th1 responses in asthma may relate to altered IL-12 levels and IL-12 signal transduction [11]. Release of IL-12 (p40/p35) from antigen-presenting cells directs the differentiation of T cells into Th1 cells with up-regulation of IFN- γ transcription and secretion [12, 13].

EBI-3 is expressed by tissue macrophages and dendritic cells (DC) which play an important functional role in asthma [14, 15]. Furthermore, EBI-3 deficiency, in contrast to IL-27 inactivation, is associated with a diminished production of Th2 cytokines [16] which are known to regulate allergic airway inflammation in asthma [17]. Therefore, we sought to better understand the role of EBI-3 in asthma through analysis of EBI-3-deficient mice. We observed that targeted deletion of EBI-3 protects from airway hyper-responsiveness (AHR) in experimental asthma. Subsequent studies demonstrated that DC from the lungs of EBI-3^{-/-} mice, although expressing surface markers of a more plasmacytoid DC (pDC) phenotype, completely lack IFN- α . In addition, we discovered that allergen challenge leads to the development of a population of EBI-3^{-/-} myeloid DC (mDC) which released increased amounts of IL-10 and IL-12 while not expressing INF- α , thus inhibiting AHR in

the absence of EBI-3. This effect was sustained by a defect in the adhesion molecule VCAM-1 in lung cells and inhibition of the production of inflammatory mediators by local effector cells. Consistently, intravenous injection of ovalbumin (OVA)-primed lung EBI-3^{-/-} CD11c⁺ cells into wild-type mice ameliorated OVA-induced AHR by increasing IL-12, IFN- γ and IL-10 levels in the lungs of recipient mice. These results define a novel multifaceted role for EBI-3 in controlling local immune responses in the lungs in experimental asthma.

Results

Reduced AHR and decreased activated eosinophils in the airways of EBI-3^{-/-} mice in experimental asthma

Allergic asthma is a syndrome characterized by reversible AHR and inflammation [17]. In an initial series of studies aiming at the characterization of the functional role of EBI-3 in asthma, we determined whether EBI-3 deficiency would protect from the development of experimental airway inflammation and hyper-responsiveness. Accordingly, wild-type and EBI-3-deficient mice were subjected to a murine model of asthma using OVA sensitization and challenge (OVA/OVA). Control mice instead received PBS aerosol as challenge (OVA/PBS). AHR was measured by using invasive airway plethysmography in anesthetized mice (Fig. 1A). It was found that, upon allergen challenge, EBI-3^{-/-} mice developed less severe AHR as compared to wild-type littermates (Fig. 1A and Supporting Information Fig. I).

Airway inflammation in asthma is characterized by an accumulation of activated eosinophils and lymphocytes in the airways [17, 18]. Mice lacking EBI-3 had decreased degranulation (Fig. 1B) and numbers (Fig. 1C and Supporting Information Fig. II) of eosinophils in the bronchoalveolar lavage fluid (BALF) as compared to wild-type mice, as determined by immunohistochemical staining for the major basic protein (MBP) and Diff Quick differential cell staining, respectively. The decrease of eosinophils was replaced by an increase in macrophages/DC in the BALF of EBI-3^{-/-} mice (Supporting Information Fig. II). We thus asked whether the reduced number of eosinophils would be dependent on a defect in the migration of eosinophils. To answer this question, we analyzed VCAM-1 expression on total lung cells and discovered a reduced expression of VCAM-1 (or CD106) on the non-lymphocyte gated cell population in the lungs of EBI-3^{-/-} mice as compared to the wild-type littermates (Fig. 1D). These results imply that inflammatory EBI-3^{-/-} effector cells may have an impaired ability to adhere and to home to the lungs as compared to the wild-type cells.

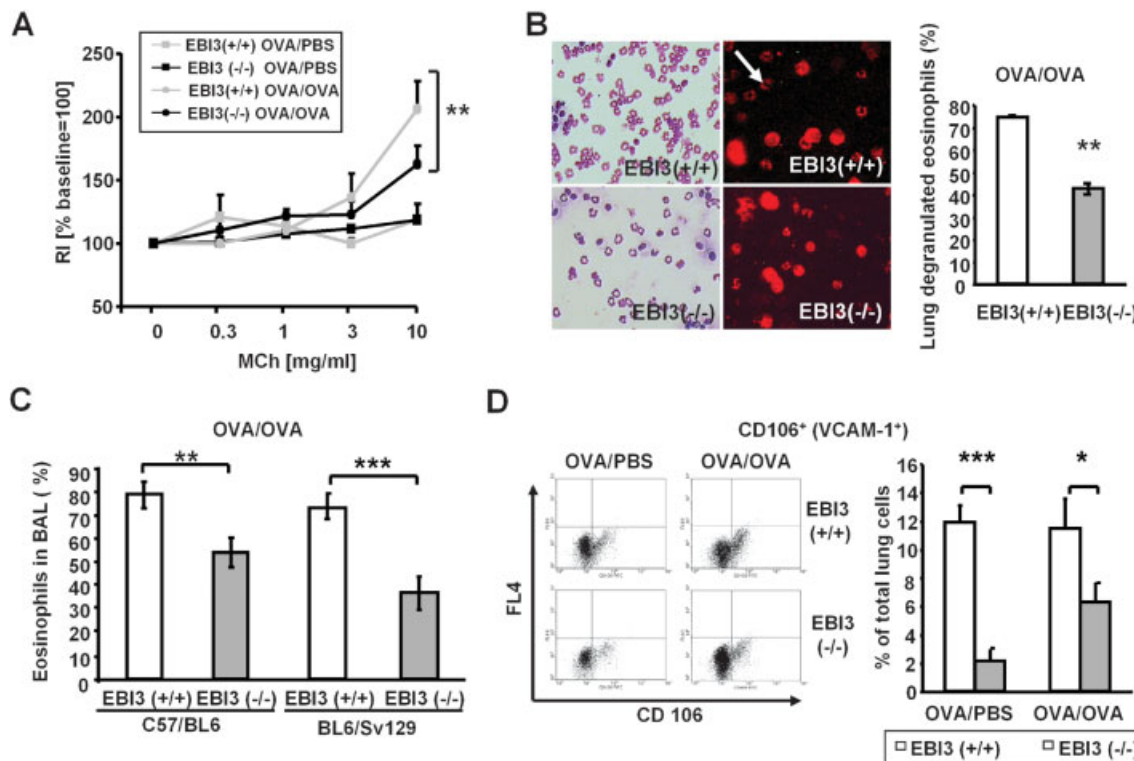


Figure 1. Targeted deletion of EBI-3 leads to amelioration of AHR and airway inflammation in a murine model of asthma after OVA sensitization and challenge. Mice were sensitized to OVA followed by either PBS aerosol (OVA/PBS) or OVA aerosol challenge (OVA/OVA). To assess lung functions, invasive airway plethysmography (A) was performed 24 h after the last allergen challenge. This method showed a protection in AHR development in EBI-3-deficient mice on a C57BL/6 genetic background after OVA sensitization and OVA challenge ($n = 5$ –6 per group). A representative experiment out of three is shown. These AHR results were confirmed after using head-out airway plethysmography (Supporting Information Fig. 1) in unrestrained awake mice after OVA sensitization and challenge ($n = 5$). (B) Decreased degranulation of eosinophils in the lungs of EBI-3^{-/-} mice as measured after immunohistochemistry with an antibody directed against the eosinophilic MBP ($n = 3$; $p = 0.008$). (C) Decreased eosinophil counts in BALF of EBI-3^{-/-} mice on both a pure C57BL/6 and on a mixed Sv129/C57BL/6 genetic background compared to wild-type littermates ($n = 4$ –5 per group). (D) Decreased VCAM-1 (CD106) expression in the lungs of EBI-3^{-/-} mice as shown by FACS analysis gated on CD3-negative cells ($n = 5$; $p = 0.003$) on a C57BL/6 genetic background. The y-axis (FL-4) is an empty channel not occupied by antibody. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; mean values $\pm$ SEM are shown.

Down-regulation of GATA-3 and IL-4 and up-regulation of T-bet and IFN- γ in EBI-3^{-/-} lungs

To understand the effect of EBI-3 deficiency on cytokine production in the airways, we analyzed IL-4 levels in the BALF of EBI-3-deficient mice and observed a defect in IL-4 production in comparison to wild-type littermates after OVA sensitization and challenge (Fig. 2A). As activation of IL-4 signal transduction is known to induce GATA-3 for polarization of naive CD4⁺ T cells into Th2-type cells [19–21], we next determined the levels of GATA-3 in the lungs of EBI-3-deficient mice. Compared to the wild-type littermates, EBI-3^{-/-} mice exhibited reduced lung levels of GATA-3 protein (Fig. 2A, lower panel) after OVA sensitization and challenge. Although not significantly, also IL-5 production was decreased in the airways, consistent with a decreased airway eosinophilia (Fig. 2A). In contrast, in the absence of EBI-3 there was an increased production of the Th1

cytokine IFN- γ in the BALF (Fig. 2B), a cytokine known to play a protective role in the development of asthma [22]. Furthermore, EBI-3 deficiency led to increased levels of T-bet, the master transcription factor of Th1 cells [23–26], after OVA sensitization and challenge (Fig. 2B, lower left panel). Finally, a significant increase in the activated form of STAT-4 (phospho-STAT-4) was observed after OVA sensitization and challenge, consistent with an activation of IL-12 signal transduction [27] or STAT-4-dependent signaling in EBI-3-deficient mice (Fig. 2B, lower right panel).

We then asked whether the IFN- γ production would induce lung injury in EBI-3-deficient mice. To this aim, we analyzed TNF- α in the airways. This cytokine is in fact involved both in asthma and other lung disorders such as acute lung injury [28]. As shown in Fig. 2B (upper right panel), TNF- α was decreased in the airways of EBI-3^{-/-} mice after allergen challenge, suggesting that augmented IFN- γ levels do not induce lung injury in this

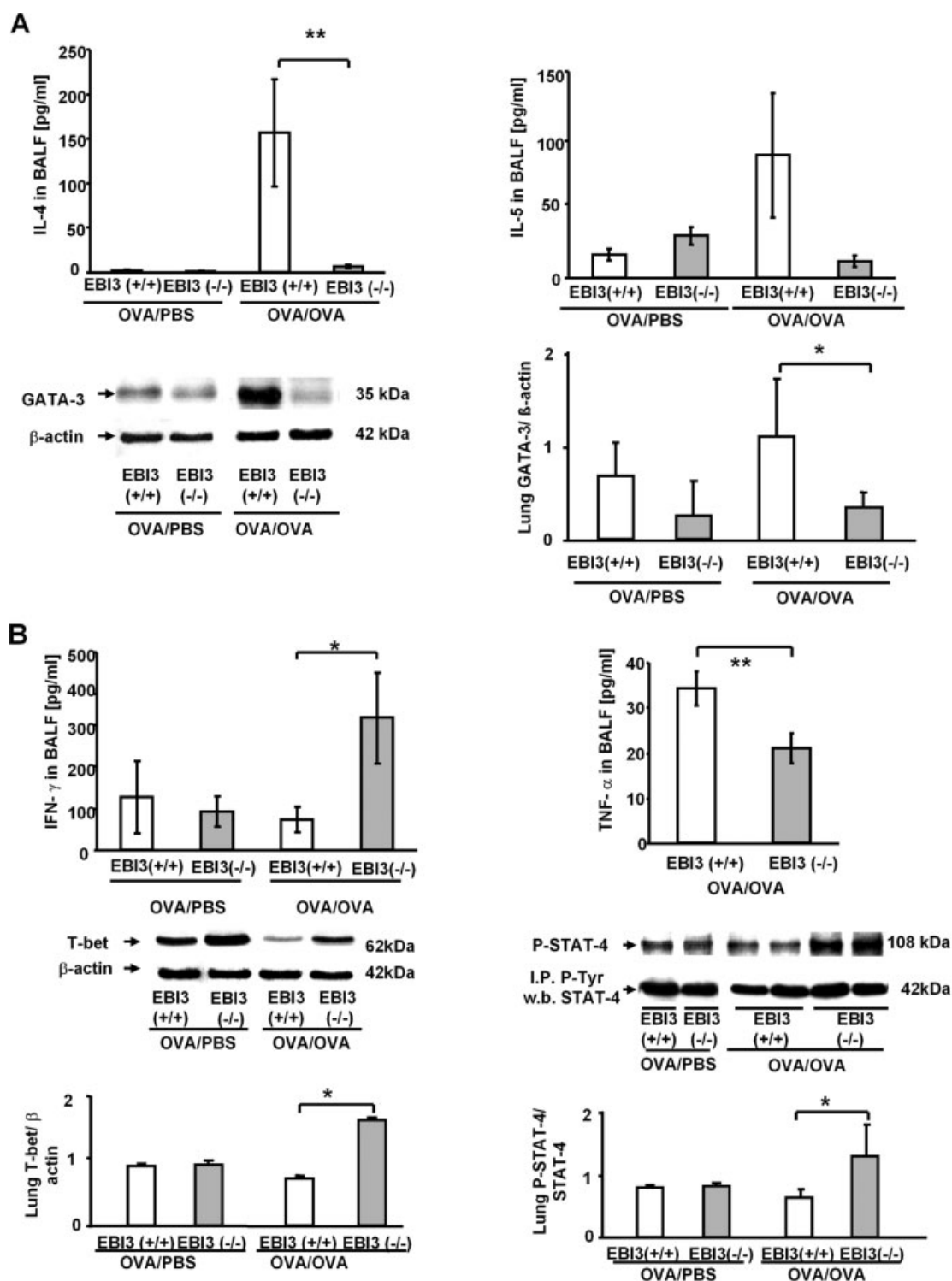


Figure 2. Decreased GATA-3 and IL-4 and increased T-bet and IFN- γ in the airways of EBI-3-deficient mice. (A) Analysis of IL-4 levels in the BALF of EBI-3-deficient mice after OVA sensitization and challenge. A statistically significant reduction of the IL-4 levels in BALF of OVA-challenged EBI-3-deficient mice was found as compared to wild-type mice ($n = 3$ –4 per group, Sv129/C57BL/6, $p = 0.06$). Although not significant, also IL-5 was down-regulated in the lungs of EBI-3 $^{-/-}$ mice (right upper panel) ($n = 3$). GATA-3 is the main transcription factor controlling the IL-4 locus. We found decreased expression of GATA-3 (lower left panel) protein in the airways of EBI-3-deficient mice as compared to wild-type mice on an Sv129/C57BL/6 genetic background. (B) By contrast, the Th1 signature cytokine IFN- γ was up-regulated in these mice after OVA sensitization and challenge, as was its major inducing transcription factor T-bet (C57BL/6 genetic background) ($n = 4$). Moreover, STAT-4 phosphorylation downstream of IL-12 signal transduction was activated (phosphorylated) in EBI-3-deficient lungs on a mixed Sv129/C57BL/6 genetic background. A representative experiment out of two is shown ($n = 3$ –4 per group). Quantitative densitometric analysis was performed by using BioDocAnalyze version 2.0 (Biometra, Göttingen, Germany). * $p < 0.05$, ** $p < 0.01$; mean values $\pm$ SEM are shown.

model. Although not necessarily associated with allergic asthma, the level of specific IgE is also elevated in this pathology. Analysis of OVA-specific immunoglobulins showed no significant increase in IgE and IgG1 as well as a non-significant decrease in IgG2a production in the serum, indicating that the defect in IL-4 levels was not affecting B cells (Supporting Information Fig. III). It is also possible that the effect of EBI-3 on B cells is related to IL-27, as WSX-1 (IL-27R)-deficient mice have increased IgE levels in the serum [8].

Impairment of IL-4 and IL-5 production by EBI-3^{-/-} lung CD4⁺ T cells

We then asked the question whether the defect in AHR in EBI-3^{-/-} mice would be due to the lack of *in situ* proliferation or migration of CD4⁺ T cells after allergen challenge. To this aim, we isolated lung CD4⁺ T cells both from EBI-3^{-/-} and wild-type mice, labeled them with CFSE and measured their proliferation after either PBS or OVA challenge *in vivo*. As shown in Fig. 3 and Supporting Information Table I, both lung CD4⁺ T cell populations isolated from the lungs of EBI-3 wild-type and EBI-3^{-/-} mice proliferated similarly before allergen challenge. Furthermore, allergen challenge led to a similarly increased proliferation rate in both wild-type and EBI-3^{-/-} lung CD4⁺ T cells during mitosis 6 (M6), indicating that both wild-type and EBI-3^{-/-} mice were sensitized properly and able to generate proliferating CD4⁺ T cells in the airways upon antigen challenge (Fig. 3, upper panels, and Supporting Information Table I). Although the lymphocytes (B and T cells) in the BALF of EBI-3^{-/-} mice were decreased, as assessed by morphological analysis (EBI-3^{+/+}: $0.052 \pm 0.017 \times 10^5$ cells/BAL, EBI-3^{-/-}: $0.020 \pm 0.003 \times 10^5$ cells/BAL), the total amount of CD4⁺ T cells, as assessed by FACS analysis in isolated cells, was increased in the lungs of EBI-3^{-/-} mice, both in the absence or presence of allergen challenge and as compared to those isolated from the wild-type littermates (Fig. 3, lower panel). Taken together, these results suggested that the reduction of AHR in EBI-3-deficient mice does not relate to impaired numbers and *in situ* CD4⁺ T cell proliferation.

We next investigated whether the decreased AHR in EBI-3^{-/-} mice was due to an intrinsic T cell defect in the Th2 type of cytokine production known to play a role in the pathogenesis of allergic asthma. Thus, we examined cytokine levels in the supernatants of CD4⁺ T cells isolated from the lungs of PBS- or OVA-challenged wild-type or EBI-3^{-/-} mice (OVA/PBS or OVA/OVA, respectively). The cytokine content of the supernatants collected from CD4⁺ T cells 24 h after stimulation with anti-CD3 and anti-CD28 antibodies was measured by CBA. Figure 4A shows IL-4, IL-5 (in dark and light blue, respectively) and IFN- γ (in red) production by lung

CD4⁺ T cells in a dot blot, measured after FACS analysis. The most important changes in cytokine levels are highlighted in the upper right panels, showing a significant decrease in IL-4 and IL-5 release (Fig. 4A and Supporting Information Table II) from lung CD4⁺ T cells derived from EBI-3-deficient mice sensitized and challenged with OVA. Importantly, IFN- γ release was not changed in the supernatants of CD4⁺ T cells isolated from EBI-3-deficient mice compared to those purified from wild-type littermates, indicating that the increased IFN- γ observed in the BALF of EBI-3-deficient mice was not derived from CD4⁺ T cells. The decrease in IL-4 production by lung CD4⁺ T cells from EBI-3^{-/-} mice was confirmed by ELISA in the supernatants of lung CD4⁺ T cells that were stimulated with anti-CD3/anti-CD28 antibodies (Fig. 4B). We then wanted to see whether IL-4 would correct the Th2 defect in CD4⁺ T cells isolated from the lungs of mice lacking EBI-3. Accordingly, we cultured highly purified lung CD4⁺ T cells from wild-type and EBI-3^{-/-} mice under Th2-skewing conditions by adding IL-4. *In vitro* Th2-skewed lung EBI-3^{-/-} CD4⁺ T cells exerted no defect in IL-13 production, suggesting the existence of a selective defect in IL-4 and IL-5 production in EBI-3^{-/-} mice. In addition, exogenous IL-4 led to an increase in IL-13 production in the supernatant of EBI-3^{-/-} lung CD4⁺ T cells 6 days after the beginning of cell culture, compared to the levels observed in wild-type mice (Fig. 4B), suggesting that IL-4 can redirect those cells to develop into Th2 cells producing even more elevated levels of IL-13 in the absence of EBI-3. In addition, it is possible that the Th2 cells do not home properly to the lungs of EBI-3^{-/-} mice due to a defect in the adhesion molecule VCAM-1, which is also reduced in the lungs of EBI-3^{-/-} mice (Fig. 1D).

Lung CD11c⁺ cells from EBI-3-deficient mice produce IFN- γ after OVA challenge

We then investigated the effect of repeated *in vivo* OVA challenges on lung EBI-3^{-/-} CD11c⁺ cells. Antigen given both systemically and locally during OVA sensitization and challenge led to enhanced production of IFN- γ (Fig. 5B, left panel) by lung CD11c⁺ cells. In a subsequent series of studies, we wanted to investigate whether IFN- γ was indeed secreted by lung DC and not by the small contaminating CD3⁺ (CD4⁺ or CD8⁺) T cell population. Therefore, we sorted first CD11c⁺ cells by magnetic beads and then separated these cells by FACS sorter into CD11c⁺CD3⁻ and CD11c⁺CD3⁺ subsets (Fig. 5A). After isolation, we cultured separately both cell populations from wild-type and EBI-3-deficient mice. It was found that lung CD3⁻ EBI-3^{-/-} CD11c⁺ cells released significantly increased amounts of IFN- γ as compared to those isolated from wild-type mice (Fig. 5B, left and middle panels). By contrast, the CD11c⁺CD3⁺

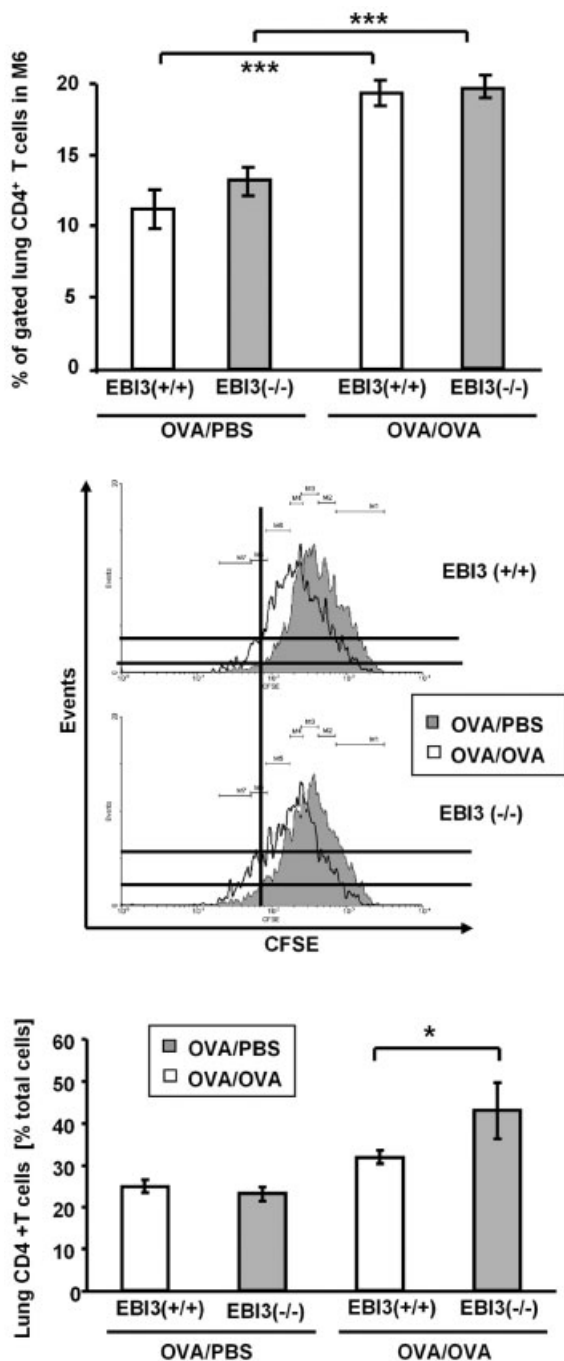


Figure 3. OVA sensitization and challenge induced normal proliferation of EBI-3^{-/-} lung CD4⁺ T cells. To understand whether the lack of AHR and inflammation in the lungs of EBI-3^{-/-} mice was due to a defect in CD4⁺ T cell proliferation, we isolated CD4⁺ T cells from the lungs (C57BL/6), labeled them with CFSE and measured the cell cycle during 4 days. As can be seen, both wild-type and EBI-3^{-/-} lung CD4⁺ T cells increased their proliferation rate as they were exposed to OVA challenge. In fact, a significantly higher number of CD4⁺ T cells both from wild-type and EBI-3^{-/-} mice advanced to M5 (mitosis number 5) and M6 (mitosis number 6) 4 days after the beginning of the cell culture at M1, as compared to the respective CD4⁺ T cell cultures that were obtained from mice not exposed to OVA challenge (OVA/PBS) ($n = 6-8$ per group; $p < 0.001$). Shown on the left-hand-side is M6 (Supporting Information Table I). To see whether there was a defect in CD4⁺ T cells in the lungs of EBI-3^{-/-} mice, we isolated and counted the total number of lung CD4⁺ T cells by FACS analysis. However, the total number of CD4⁺ T cells was not decreased but significantly increased in the lungs of EBI-3^{-/-} mice after allergen challenge (lower right panel), indicating that the lower level of GATA-3 was not due to a defect in CD4⁺ T cells. This also indicates that the decrease in total lymphocytes seen in the BALF (Supporting Information Fig. II) is either a finding that does not relate to the CD4⁺ T cells or is present only in the BALF and not in the total cells of the lung. * $p < 0.05$, *** $p < 0.001$; mean values $\pm$ SEM are shown.

Before allergen challenge, lung EBI-3^{-/-} CD11c⁺ cells express increased surface plasmacytoid and lymphoid DC activation markers

We then investigated phenotypic differences in lung antigen-presenting cells in the absence of EBI-3 [29]. One possibility would have been the presence of different kinds of lung DC populations in the absence of EBI-3 expression. As can be seen in Fig. 6A, B and Table 1, lung CD11c⁺ cells isolated from PBS-treated EBI-3^{-/-} mice, upon depletion of draining lymph nodes, showed increased expression of CD8 α , indicating a more activated phenotype in the absence of allergen challenge (Fig. 6A and Table 1) [30–32]. However, after allergen challenge, the lung CD11c⁺ cells from EBI-3^{-/-} mice contained an increased number of mature DC, as they are CD11c⁺ MHC class II⁺ cells (Fig. 6A, the most right panels). This population was not contaminated with macrophages as we gated on the granulocytes population, excluding highly fluorescent cells before different surface markers were analyzed (Fig. 6A, very left-hand-side panels) [33, 34]. Moreover, PDCA-1, a distinct surface marker for pDC, was increased on the surface of DC (CD11c⁺) isolated from the lungs of EBI-3^{-/-} mice as compared to those isolated from the lungs of wild-type littermates (Fig. 6A, lower right panels, compared to Fig. 6A, upper right panels, and Table 1). However, other markers such as CD40, CD80 and CD86 were not significantly changed in EBI-3^{-/-} CD11c⁺ cells from OVA-challenged mice as compared to control wild-type

cell population from EBI-3^{-/-} mice released little or no IFN- γ . Moreover, allergen challenge led to a decreased IFN- α production by lung CD11c⁺CD3⁻ cells as compared to the wild-type littermates (Fig. 5C). Taken together, these data suggested that EBI-3^{-/-} CD11c⁺ DC, and not other contaminating CD11c⁺CD3⁺ cells, release increased amounts of IFN- γ and less IFN- α as compared to CD11c⁺ DC isolated from wild-type littermates. Moreover, these results indicate Th2-repressing properties by lung DC isolated from the lungs of EBI-3-deficient mice.

mice (Table 1). We then wanted to further characterize the phenotype of DC from EBI-3^{-/-} mice before allergen challenge. It has been demonstrated that pDC express

CD11c in low amounts and do not express CD123 (IL-3 α chain) [35]. Consistently, as shown in Fig. 6B, before allergen challenge (OVA/PBS), both EBI-3^{-/-} CD11c^{high}

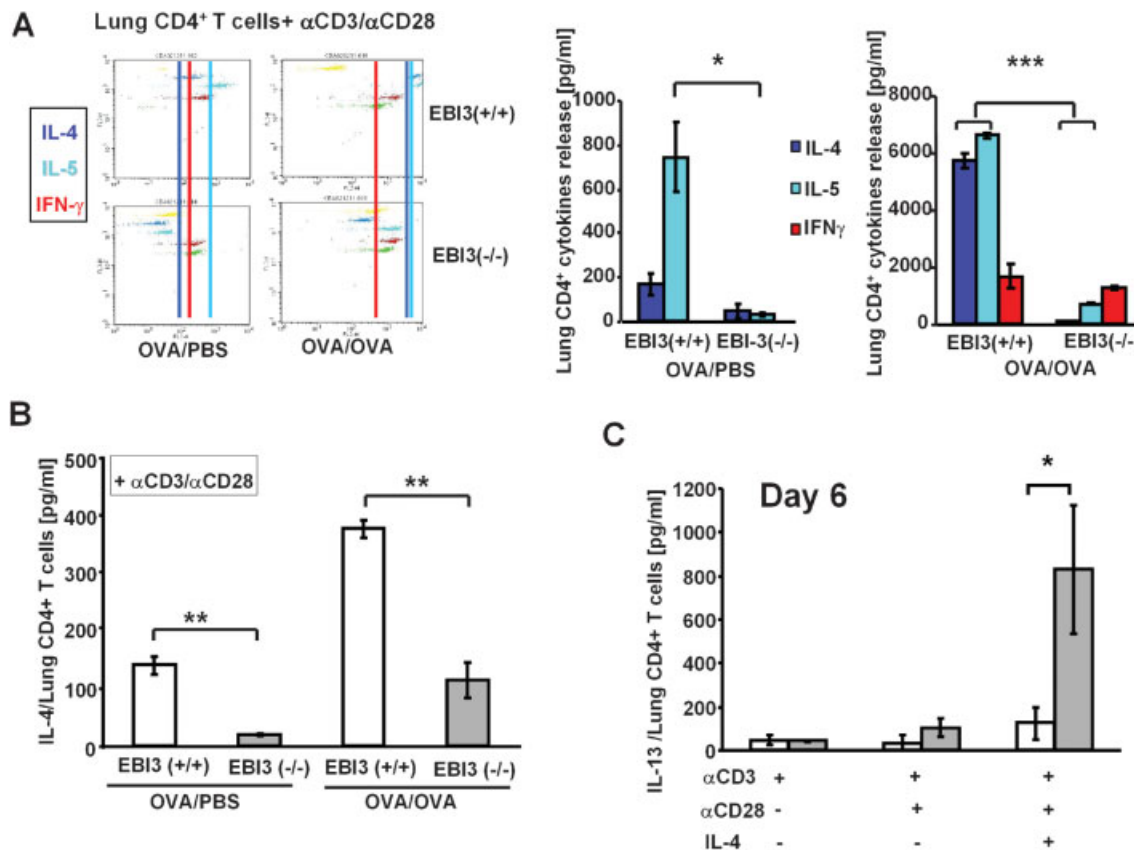


Figure 4. Decreased IL-4 and IL-5 production by lung EBI-3^{-/-} CD4⁺ T cells in a murine model of asthma. To understand whether the defect in IL-4 and IL-5 found in the BALF could also relate to a defect in Th2 cells in the lungs of EBI-3^{-/-} mice, we isolated lung CD4⁺ T cells and analyzed by CBA the amount of different cytokines at once released by a constant number of CD4⁺ T cells. CBA was performed on the isolated purified lung CD4⁺ T cell supernatants. CD4⁺ T cells isolated from the lungs of EBI-3^{-/-} mice secreted decreased amounts of IL-4 and IL-5 as compared to those isolated from the wild-type mice that were sensitized with OVA and challenged with PBS (OVA/PBS) (left panels) or OVA (OVA/OVA) (right panels). On the upper right-hand-side the results are depicted ($n = 2$) (see also Supporting Information Table II). (B) The same results were obtained using an ELISA for IL-4 after anti-CD3/anti-CD28 antibody challenge of lung T cells overnight (Sv129/C57BL/6 genetic background; $n = 4-5$). (C) Exogenous addition of IL-4 to the lung CD4⁺ T cell culture significantly induced IL-13 in EBI-3-deficient CD4⁺ T cells (dark grey bars) as compared to wild-type cells (light grey bars; $n = 3$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Mean values $\pm$ SEM are shown.

Table 1. Characterization of lung CD11c⁺ DC

PBS/PBS							
% of CD11c ⁺ cells	CD80	CD40	CD86	CD8 α	MHC II	PDCA-1	B220
EBI-3 ^{+/+}	77.56 $\pm$ 1.86	13.37 $\pm$ 3.37	31.70 $\pm$ 7.93	0.59 $\pm$ 0.14	n.d.	32.32 $\pm$ 1.57	0.558 $\pm$ 0.43
EBI-3 ^{-/-}	82.99 $\pm$ 4.37	11.62 $\pm$ 4.97	33.24 $\pm$ 4.25	15.52 $\pm$ 1.3***	n.d.	43.70 $\pm$ 4.64*	0.92 $\pm$ 0.31
OVA/OVA							
% of CD11c ⁺ cells	CD80	CD40	CD86	CD8 α	MHC II	PDCA-1	B220
EBI-3 ^{+/+}	96.98 $\pm$ 0.28	95.00 $\pm$ 0.54	14.53 $\pm$ 0.0	54.82 $\pm$ 1.21	3.04 $\pm$ 0.39	26.76 $\pm$ 1.01	1.592 $\pm$ 1.4
EBI-3 ^{-/-}	97.17 $\pm$ 0.45	94.98 $\pm$ 1.65	8.06 $\pm$ 0.0	59.7 $\pm$ 1.98*	5.34 $\pm$ 0.49**	40.51 $\pm$ 3.98*	1.895 $\pm$ 0.39

n.d., not determined

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; significance values from EBI-3^{+/+} versus EBI-3^{-/-}

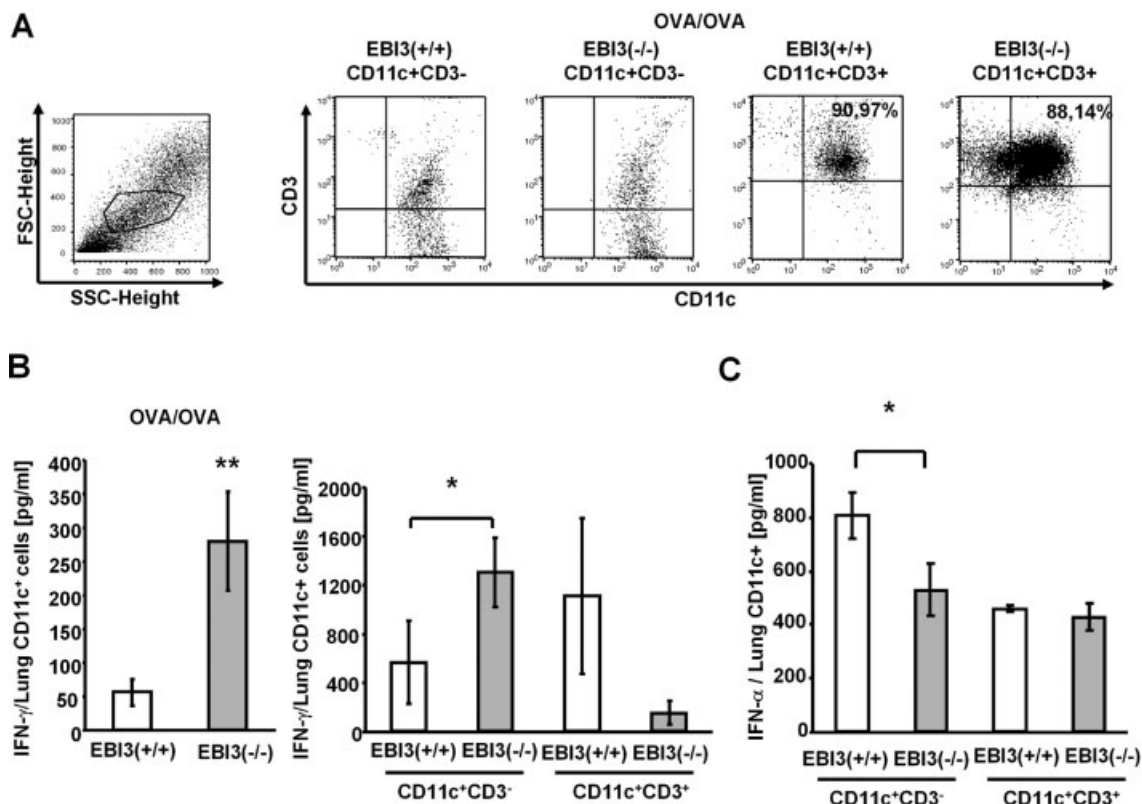


Figure 5. CD11c⁺ cells (CD11c⁺CD3⁻) isolated from the lungs of EBI-3-deficient mice release increased amounts of IFN-γ and decreased amounts of α-IFN *ex vivo*. We then wanted to investigate the cellular sources of IFN-γ found in the BALF of EBI-3^{-/-} mice. (A) To rule out the possibility that the lung CD11c⁺ cell population would contain either CD4⁺ or CD8⁺ T cells known to produce IFN-γ, we separated by FACS sorter the CD11c⁺CD3⁺ (purity 88.14–90.97%) from the CD11c⁺CD3⁻ (purity 87.18–90.96%) cell populations. CD11c⁺CD3⁻ and CD11c⁺CD3⁺ cell populations both from wild-type and EBI-3^{-/-} mice were cultured overnight and IFN-γ release was analyzed by ELISA. (B) Significantly increased IFN-γ levels were found in the supernatant of lung CD11c⁺CD3⁻ DC isolated from OVA-sensitized and -challenged EBI-3^{-/-} mice as compared to those isolated from wild-type C57BL/6 littermates. In contrast, the CD11c⁺CD3⁺ cell population from the lungs of EBI-3^{-/-} mice released lower amounts of IFN-γ (*n* = 5–6). (C) The CD11c⁺CD3⁻ population from EBI-3^{-/-} mice released decreased amounts of IFN-α (*n* = 3) as compared to those isolated from the wild-type littermates. **p* < 0.05; ***p* < 0.01. Mean values ± SEM are shown.

and CD11c^{low} cells express less CD123 as compared to those purified from the wild-type littermates.

Upon allergen challenge, EBI-3^{-/-} lung CD11c⁺ cells become specialized in producing IL-12 but not IFN-α

pDC are known to release IFN-α, a multifunctional immunomodulatory cytokine with profound anti-inflammatory properties. In fact, it favors both the induction of Th1 cytokines as well as the suppression of Th2 cytokines, consistent with the phenotype observed by us in EBI-3^{-/-} mice [30, 36]. Surprisingly, we could not find IFN-α in the supernatants of EBI-3^{-/-} DC before allergen challenge (Fig. 7A, left panels). IFN-α is known to be produced by pDC upon viral infection. It is therefore possible that EBI-3 is a protein responsible for the modulation of IFN-α production upon EBV infection. Moreover, pDC are known to have limited ability to produce IL-12. However, after allergen

challenge, beside the presence of the CD11c^{low} CD123^{low} DC, we noticed the increased presence of DC with a CD11c^{high} CD123^{high} phenotype in the lungs of EBI-3^{-/-} mice (Fig. 7B, right panels). These results indicate the presence of a mixed DC population in the lungs of EBI-3-deficient mice after allergen challenge, with a more myeloid phenotype as they express increased amounts of the IL-3R, and as compared to the DC isolated from the lungs of wild-type littermates. Consistently, before allergen challenge, DC from EBI-3^{-/-} mice as well as those from the wild-type littermates produce limited amounts of IL-12, indicating a more plasmacytoid or lymphoid activated phenotype. However, we found that CD11c⁺ lung cells from EBI-3^{-/-} mice produced large amounts of IL-10 (Fig. 7A), a cytokine released by mDC. We then analyzed DC from EBI-3^{-/-} mice after allergen challenge, as they had shown increased expression of myeloid markers (Fig. 7B). As shown in Fig. 7B, CD11c⁺ cells from OVA-challenged EBI-3^{-/-} mice released increased amounts of IL-12p70, IL-10 and IFN-γ while

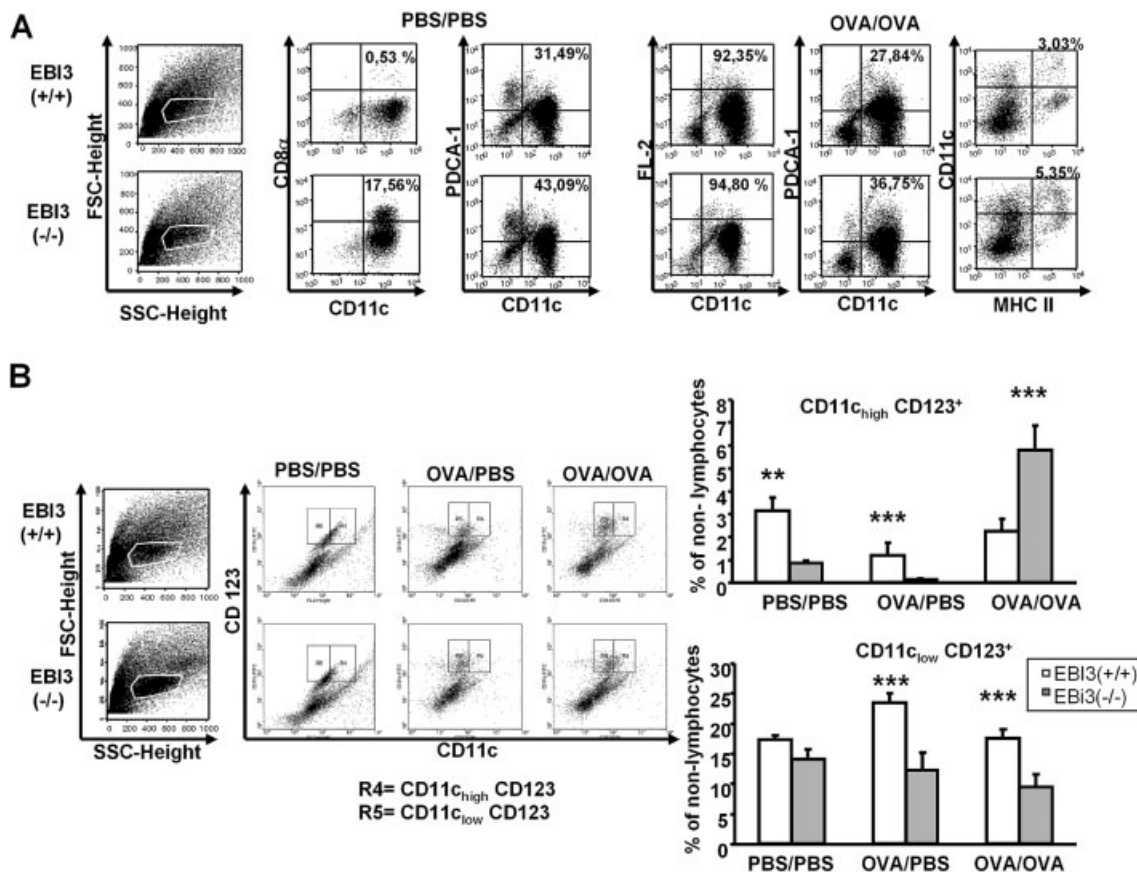


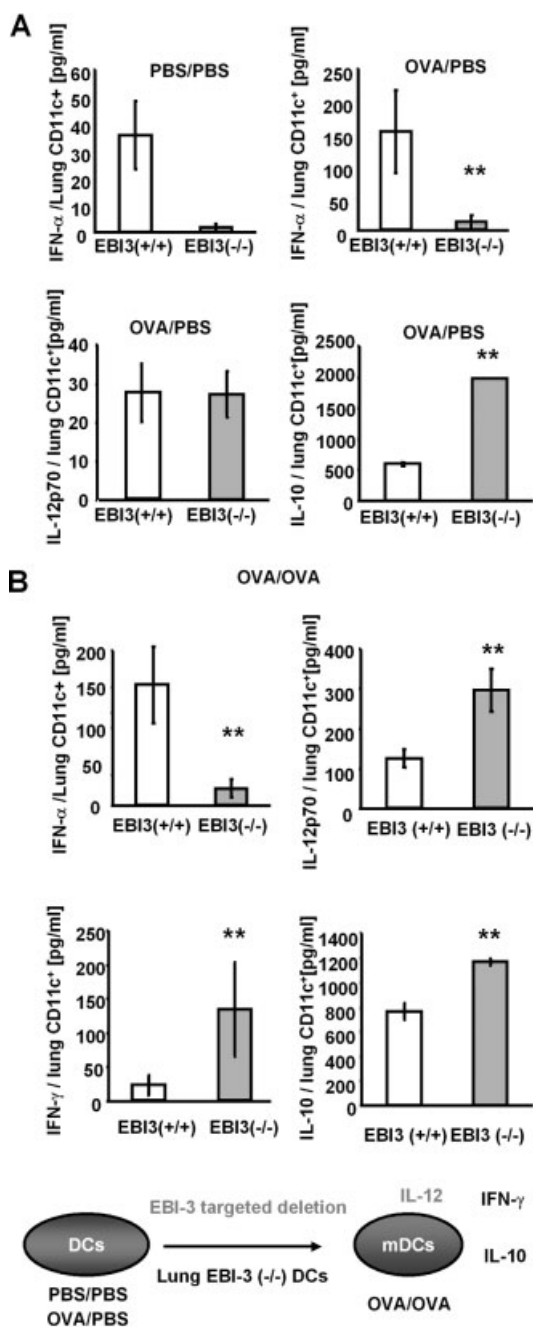
Figure 6. Before allergen challenge, CD11c⁺ cells isolated from EBI-3-deficient mice are of more plasmacytoid phenotype. Based on IFN- γ release by lung DC from EBI-3^{-/-} mice, we investigated the possibility of the presence of a DC population of plasmacytoid phenotype in the lungs of EBI-3^{-/-} mice. To better define the characteristics of lung CD11c⁺ cells gated on the non-autofluorescent cells, we performed FACS analysis with different surface markers for plasmacytoid and myeloid phenotype. Lung CD11c⁺ cells, both from EBI-3^{-/-} and wild-type mice, were purified by using Miltenyi magnetic beads as described in Material and methods. The purity of this population was over 92%, as shown by FSC/SSC and anti-CD11c antibody staining (A) [two first OVA/OVA right panels; the y axis (FL-2) here is an empty channel not occupied by antibody binding]. The expression of defined markers for DC maturation such as CD80, CD86 was comparable in DC from the lungs of PBS-treated EBI-3^{-/-} mice and from wild-type C57BL/6 littermates (Table 1). In contrast, the expression of CD8 α and PDCA-1 as specific markers for activated and plasmacytoid DC populations, respectively, was more elevated on the surface of lung EBI-3^{-/-} DC resulting in a cell population comprising a more activated plasmacytoid subset as compared to wild-type DC (A) (and Table 1). Moreover mature DC are defined as CD11c⁺ MHC class II⁺ double-positive cells. We then analyzed this cell population and found that it is increased in the lungs of EBI-3^{-/-} mice as compared to wild-type littermates (right-hand-side panels and Table 1). A representative dot plot is shown ($n = 4$). (B) Plasmacytoid DC express low levels of CD11c as well as the IL-3R α chain (CD123). We thus analyzed separately CD11c^{high} and CD11c^{low} cells for CD123. We found that before and after allergen challenge, the CD11c^{low} cells from EBI-3^{-/-} mice expressed lower levels of CD123, consistent with a plasmacytoid phenotype. However, allergen challenge led to the increase of a CD11c^{high} CD123^{high} population in the lungs of EBI-3^{-/-} mice, consistent with a myeloid phenotype. ** $p < 0.01$; *** $p < 0.001$. Mean values $\pm$ SEM are shown.

continuing not to produce IFN- α . Taken together, these data show the development of DC with a more myeloid phenotype as far as cytokine release is concerned in the lungs of EBI-3^{-/-} mice after allergen challenge.

OVA-primed lung DC from EBI-3-deficient mice inhibit AHR in recipient wild-type mice

To further understand the function of EBI-3-deficient lung OVA/OVA CD11c⁺ cells in a murine model of asthma, we injected these purified cells (CD11c⁺ cells

>90%) intravenously into OVA-sensitized and -challenged wild-type mice. Under these conditions, CD11c⁺ cells from EBI-3^{-/-} OVA-sensitized and -challenged mice were able to significantly suppress AHR (Fig. 8A) and to induce IL-12 production in the airways of wild-type recipient mice as compared to lung EBI-3^{+/+} CD11c⁺ cells from OVA-sensitized and -challenged mice (Fig. 8B). Moreover, these cells led to an increase of IL-10, while IFN- α was down-regulated. Taken together, these data suggested that EBI-3-deficient lung DC from OVA/OVA mice significantly contribute to the suppres-



sion of AHR because they produce high amounts of IL-12, IL-10 and IFN- γ and suppressed IFN- α production in reconstituted mice (Fig. 8C).

Discussion

In this study, we demonstrate an important regulatory role of EBI-3 in lung DC. In fact, EBI-3 deficiency in lung DC significantly inhibited allergic AHR in a murine model of experimental asthma. Furthermore, we shed light onto the immunopathological mechanism responsible for this effect. In fact, EBI-3 deficiency induced the

Figure 7. Upon allergen challenge EBI-3^{-/-} lung CD11c⁺ cells are of myeloid phenotype as they are specialized in producing IL-12 but not IFN- α . CD11c⁺ cells can also be classified in accordance to their cytokine release as plasmacytoid, as they do not release IL-12 and much IFN- α whereas myeloid DC release increased IL-12 and IL-10 and reduce the amounts of type I interferons released. We therefore isolated lung CD11c⁺ cells and looked for release of these cytokines in their supernatants. As shown in (A), before allergen challenge, DC from EBI-3^{-/-} mice released low amounts of IL-12 compared with those isolated from wild-type mice, indicating the absence of a myeloid phenotype. However, IFN- α was also absent in the supernatants of EBI-3^{-/-} DC. Moreover, before allergen challenge, EBI-3^{-/-} DC released huge amounts of IL-10. (B) This part of the figure shows the cytokine release of EBI-3^{-/-} DC after allergen challenge. As shown, after allergen challenge, the latter released increased IL-12 as compared to those isolated from the wild-type littermates. Moreover, they continue to produce more IL-10 and, as also shown in Fig. 5B, they up-regulated IFN- γ . These data are consistent with a more myeloid phenotype of lung DC upon allergen challenge of EBI-3^{-/-} mice, consistent with the finding described in Fig. 4B for the surface marker CD123 on CD11c^{high} DC. ** $p < 0.01$. Mean values $\pm$ SEM are shown.

development of a lung DC population with increased numbers of both activated (CD8 α ⁺) and plasmacytoid cells (PDCA-1⁺) before allergen challenge while antigen challenge resulted in the development of an mDC population inducing IFN- γ and IL-12 responses. pDC are known to release IFN- α , a cytokine with profound anti-inflammatory properties favoring the induction of Th1 cytokines as well as the suppression of Th2 cytokines [35, 36]. However, unlike pDC, EBI-3^{-/-} CD11c⁺ cells did not produce IFN- α and low amounts of IL-12 before allergen challenge. Similarly to the pDC, however, EBI-3^{-/-} DC had low expression of CD123, the IL-3R α chain on the cytoplasmic membrane of CD11c^{low} DC. Upon allergen challenge, there was a shift in the phenotype of CD11c⁺ cells in the lungs of EBI-3^{-/-} mice. In fact, they up-regulated the IL-3R α chain (CD123) as well as CD11c on their surface while releasing more IFN- γ , IL-10 and IL-12 without expressing IFN- α , as compared to DC isolated from the airways of wild-type littermates. These cells resembled now the myeloid-type DC. However, a clear subdivision of these cells into canonic groups is difficult due to the presence of different types of DC simultaneously in the lung. Moreover, we cannot rule out the possibility that our isolated CD11c⁺ cells contain macrophage-like cells, since the latter have been described in the BALF and more recently in the lung [33, 34, 37]. Adoptive intravenous transfer of OVA-challenged lung CD11c⁺ cells from OVA-sensitized EBI-3^{-/-} mice into wild-type littermates significantly improved the AHR in recipient mice, while inducing IL-12, IL-10 levels and suppressing IFN- α levels as compared to adoptive transfer with wild-

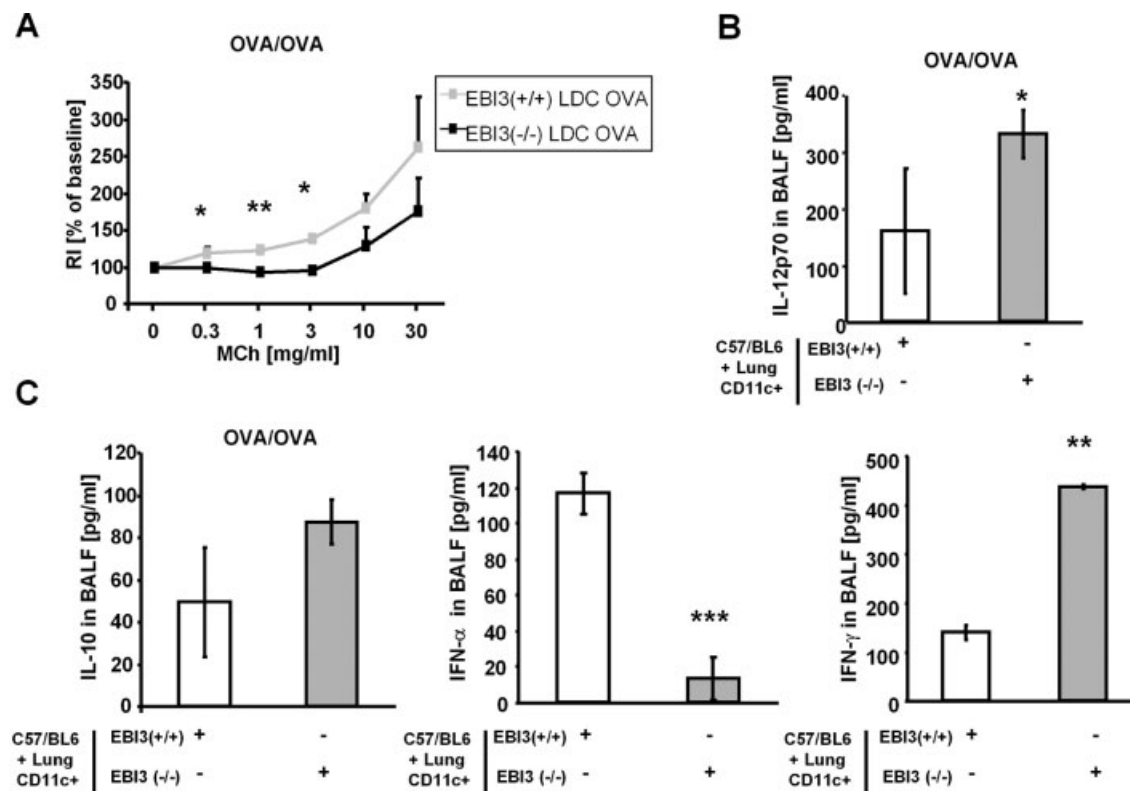


Figure 8. Intravenous injection of IFN- γ - and IL-12-producing lung CD11c⁺ cells from EBI-3^{-/-} mice protects recipient wild-type mice from AHR. (A) Lung CD11c⁺ cells were isolated from OVA-sensitized and -challenged (OVA/OVA) wild-type and EBI-3^{-/-} mice, and cultured overnight after being loaded with OVA. OVA-sensitized and -challenged wild-type C57BL/6 mice were then injected intravenously with CD11c⁺ cells derived from either the lungs of EBI-3^{+/+} or EBI-3^{-/-} mice (both on a C57BL/6 genetic background), and after three additional days, in which the recipient mice were challenged with OVA, mice were analyzed for AHR (5–6 mice per group). Mice receiving EBI-3^{-/-} lung CD11c⁺ cells were protected from AHR as compared to those receiving CD11c⁺ cells from EBI-3^{+/+} mice. (B) Moreover, the levels of the Th1-induced cytokines IL-12 and IFN- γ itself were significantly increased in the airways of mice adoptively transferred with CD11c⁺ lung cells lacking EBI-3 as compared to wild-type controls. Consistent with a myeloid phenotype, also IL-10 was increased while IFN- α (C) was down-regulated in the lungs of wild-type mice reconstituted with CD11c⁺ cells from EBI-3^{-/-} mice. By contrast, TNF- α levels did not differ in wild-type and EBI-3^{-/-} lung CD11c⁺ cell-reconstituted mice (Supporting Information Fig. IV). A representative experiment out of three is shown ($n = 5-6$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Mean values $\pm$ SEM are shown.

type CD11c⁺ cells. This indicates a direct relationship between the transferred CD11c⁺ cells and the amelioration of AHR. EBI-3-deficient CD11c⁺ cells induced a proliferative CD4⁺ T cell response with markedly reduced Th2 cytokines, thereby inhibiting eosinophilic airway inflammation upon OVA aerosol challenge as compared to wild-type cells (Fig. 4) [16, 29]. Consistently, we could not find an increase in IFN- γ in the supernatant of CD4⁺ T cells isolated from the EBI-3^{-/-} mice, probably as a consequence of the down-regulation of IFN- α . Moreover, EBI-3 deficiency led to the down-regulation of the expression of the adhesion molecule VCAM-1 (CD106) [38]. These data correlated well with the decreased number of eosinophils and Th2 cytokines in the airways of EBI-3^{-/-} mice, suggesting a defect in adhesion and therefore migration and survival of the CD4⁺ Th2 effector cells as well as eosinophils in the lungs of these mice. Moreover, the eosinophils that were

able to develop or to migrate to the lung contained more MBP, indicating a decreased degranulation of eosinophils in the lungs of EBI-3^{-/-} mice, resulting in decreased damage to the bronchial epithelium. In this context, a slight increase of the IgE levels in the serum of EBI-3^{-/-} mice was noted. This effect could be related to the defective IL-27 expression in EBI-3^{-/-} mice since also WSX-1 (IL-27R)-deficient mice have increased IgE levels [8]. Interestingly, adoptive transfer of DC from OVA-sensitized and -challenged mice reduced AHR and induced IL-12 levels in the airways of recipient mice without changing eosinophilia, suggesting that eosinophilia is controlled by EBI-3-deficient T cells rather than DC [32].

Although the biologic functions of the EBI-3 monomer and the EBI-3/EBI-3 or IL-12p35/EBI-3 dimers are still poorly understood, IL-27 (EBI-3/p28) is known to be an early gene expressed by activated antigen-

presenting cells, produced upon TLR ligation that synergizes with IL-12 to trigger IFN- γ production via T-bet from naive CD4⁺ T cells [2–4]. As TLR ligation directly induces EBI-3 gene transcription [29], EBI-3 expression is induced by the same stimuli that favor IL-27 production, and this could be the reason of assimilating their function.

In the present manuscript, we have determined the functional role of EBI-3 in lung CD4⁺ T cells and CD11c⁺ DC using EBI-3-deficient mice. Our findings demonstrate that targeted deletion of EBI-3 in lung DC induces a shift in the phenotype and cytokines released by these cells, influencing the phenotype of the primed CD4⁺ T cells in the lung. Moreover, this cytokine profile seems to be dependent on the type and amount of antigen presented to the DC. In fact, we found, similarly to data in experimental colitis [29] and in contrast to the *L. major* model [39], reduced production of two Th2 cytokines in EBI-3-deficient mice, as EBI-3-deficient lung CD4⁺ T cells produced less IL-4 and IL-5 as compared to wild-type CD4⁺ T cells in a murine model of asthma after OVA sensitization and challenge. Moreover, repeated antigen challenge in lung EBI-3^{-/-} CD11c⁺ cells led to increased release of IFN- γ or IL-12 while IFN- α continued to be down-regulated as compared to wild-type lung CD11c⁺ cells. This could be consistent with the findings in the *L. major* model in which targeted deletion of EBI-3 led to increased production of IFN- γ by total cells isolated from lymph nodes [39]. Importantly, we defined here for the first time that the cells overproducing IFN- γ in EBI-3-deficient mice are CD11c⁺ and not CD4⁺ T cells, as they are CD3⁻. Moreover, we demonstrated that the CD4⁺ T cells isolated from the lungs of EBI-3^{-/-} mice do not overproduce IFN- γ ; rather, they released reduced amounts of Th2 cytokines. Taken together, these data indicate that targeted deletion of EBI-3 does not lead to a shift in Th1 cells, rather to an inhibition of the Th2 pathway without influencing the Th1 cell subset. This is consistent with the activation of STAT-4 and up-regulation of T-bet in the lungs of EBI-3-deficient mice. In fact, T-bet is known to enhance the expression of the IL-12R β 2 chain, thereby favoring IL-12R signaling via STAT-4 in T cells [23].

Analysis of lung biopsy specimens from patients with allergic rhinitis has shown a reduced expression of the IL-12R β 2 chain, suggesting a protective role of IL-12 in allergic diseases [11–13]. The expression of the IL-12R β 2 chain subunit in naive T cells is induced by IL-27 production together with IL-12 in a T-bet-dependent fashion, which triggers the growth of naive but not memory T cells [1, 9]. These findings indicate a defect extrinsic to the CD4⁺ T cell compartment in EBI-3-deficient mice. In fact, these cells maintain a high plasticity, indicating a blockade in the absence of EBI-3 at an early stage of development where directional

decisions can still take place. Our results suggest that EBI-3 is an essential protein secreted by antigen-presenting cells that is necessary for naive T cells to differentiate into Th2 cells by inducing IL-4 production in this murine model of asthma. In addition, our studies further suggest that DC-derived EBI-3 can interact with conventional CD4⁺ T cells directly or through dimerization with p28, IL-12p35, or another yet to be defined heterodimeric binding partner [10].

Our data identify EBI-3 as a key regulator of lung CD11c⁺ cells, a cell population that plays an important role in regulating lung immune responses [35, 40] also by influencing their adhesion (via down-regulation of VCAM-1) and migration in the target tissue. The important functional role of EBI-3 was highlighted by the finding that intravenous injection of lung EBI-3^{-/-} CD11c⁺ cells into wild-type mice ameliorated OVA-induced AHR in recipient mice by inducing IL-12 and reducing IFN- α . Moreover, in this short-term experiment, the number of neutrophils was negligible and TNF- α was not increased (Supporting Information Fig. IV), indicating no induction of acute lung injury. Moreover, the number of eosinophils was not affected, indicating that in the EBI-3-deficient mice there is a primary defect in the migration of these cells into the lung. These results indicate a novel role for EBI-3 in controlling local T cell-dependent immune responses in the lung. In particular, inhibition of EBI-3 expression in lung CD11c⁺ cells can prevent AHR upon OVA sensitization and challenge in experimental asthma. These findings have important implications for the design of new therapies for allergic diseases such as asthma.

Materials and methods

Mice, cytokines and antibodies

Wild-type as well as EBI-3^{-/-} mice were both on an Sv129/C57BL/6 and C57BL/6 genetic background and were maintained as previously described [16, 29]. Purified recombinant mouse-specific IL-4 (10 ng/mL) was from Peprotech (Rocky Hill, NJ); anti-CD3 (5 μ g/mL) and anti-CD28 antibodies (2 μ g/mL) were from BD PharMingen (Heidelberg, Germany). Monoclonal mouse anti-mouse GATA-3, monoclonal mouse anti-mouse phospho-tyrosine, polyclonal rabbit anti-mouse STAT-4, polyclonal goat anti-mouse β -actin (Santa Cruz Biotechnology, Heidelberg, Germany) and monoclonal anti-mouse T-bet antibodies (kind gift from Prof. L. H. Glimcher, Harvard Medical School, Boston, MA) were used for Western blot analysis. For the analysis of murine primary cells from spleen and lung, the following fluorochrome-labeled antibodies for FACS analysis and FACS sorting were used: FITC anti-mouse CD4 (clone RM4-5), FITC hamster anti-mouse CD11c (clone HL3) (BD Pharmingen), PE anti-mouse CD80 (B7.1) (clone 16-1011), PE anti-mouse CD86 (B7.2) (clone

P03.1), PE anti-mouse CD3e (clone 145-2C11), PE anti-mouse CD40 (clone 1C10), PE anti-mouse CD8 α (clone LY-2), PE-Cy5.5 anti-mouse B220 (CD45R, clone RA3-6B2), FITC anti-mouse CD106 (VCAM-1, clone 429), PE anti-mouse CD123 (IL-3R, clone 5B11) (all above-mentioned antibodies purchased from eBioscience, San Diego, CA), PE anti-mouse CD11c, FITC anti-mouse MHC class II, and PE anti-mPDCA-1 (last three antibodies all purchased from Miltenyi Biotech, Bergisch-Gladbach, Germany).

Allergen sensitization and challenge protocol in mice

Mice at 6–8 wk of age received an intraperitoneal injection of 100 μ g OVA (Calbiochem, San Diego, CA) complexed with alum on days 0 and 7. On days 18, 19, and 20, the animals received OVA in PBS per aerosol (10 mg OVA/mL PBS in aerosol solution) (OVA/OVA) or PBS aerosol alone (OVA/PBS). At day 21, mice were measured by invasive body plethysmography and then sacrificed to isolate spleen/lung cells as described below.

Assessment of airway reactivity by invasive body plethysmography

AHR was measured invasively by using a body plethysmograph 48 h after the last aerosol exposure (Buxco Electronics, Inc., Wilmington, NC) as described [25]. Mechanical airway flow at 160 strokes/min ensured the intratracheal delivery of methacholine (MCh) applied through an aerosol delivery unit. The concentration of MCh ranged from 0.3 to 30 mg MCh per mL and the airway resistance was measured for 5 min after delivery of 10 μ L of each concentration for different groups of mice ($n = 5$ per group). Data are reported as airway resistance (RI) above the baseline value taken before receiving MCh. Data are expressed as mean values of RI $\pm$ SEM.

Collection and analysis of the BALF and subsequent isolation and purification of lung CD4 $^{+}$ T cells

At 24 h after the last aerosol challenge with either OVA or saline, BAL of the right lung was performed with 0.75 mL saline for four times. BALF was collected and an aliquot was stained with Trypan blue solution and cells counted for viability determination using a Neubauer chamber. Samples were centrifuged at 1500 rpm for 5 min and cell pellets were resuspended in 1 mL PBS. Cytospins were made by centrifugation at 500 rpm for 5 min. Eosinophils and macrophages/DC were detected by staining according to Diff Quick (Dade Behring, Marburg, Germany) (Supporting Information Fig. II). Activated lung eosinophils were measured by histological staining by eosinophilic MBP to determine the degranulation status of eosinophils as previously described [41]. The cytopins were analyzed with a Zeiss microscope using a 40 $\times$ objective. The supernatants were frozen and subsequently analyzed by ELISA.

Proliferation of lung CD4 $^{+}$ cells after OVA sensitization

Lung CD4 $^{+}$ cells were isolated by immunomagnetic separation as described [42]. They were counted and the percentage of

the total lung cells is depicted in Fig. 2C (lower panel). Lung CD4 $^{+}$ cells (10^5 /well) were then labeled with CFSE (Molecular Probes, Eugene, OR) in the presence of soluble anti-CD3 (2.5 μ g/mL) and anti-CD28 antibodies (1 μ g/mL) overnight and as previously described [42]. After 24 h, the starting incorporated fluorescence was recorded (M1). The proliferation of the CFSE $^{+}$ lung CD4 $^{+}$ T cells was then taken from the percentage of daughter cells that would reach generation (or mitosis = M) M4, M5 or M6. Up to six lungs were analyzed per each experimental group.

FACS analysis and CBA

To assure the purity of CD4 $^{+}$ T cell isolation, routinely, 5×10^5 cells were washed with 1 mL PBS, then incubated for 30 min in 100 μ L PBS containing 5 μ g/mL anti-CD4-FITC antibody (BD PharMingen). Cells were washed with 1 mL PBS and subsequently fixed in 1 mL 2% PFA/PBS (Sigma, Deisenhofen, Germany) solution and analyzed. The resulting cell suspensions were measured by FACSCalibur and analyzed by using CellQuest Pro version 4.02 (BD PharMingen).

CD4 $^{+}$ T cells isolated from the lungs of OVA-sensitized and -challenged mice were incubated overnight in the presence of plate-bound anti-CD3 and soluble anti-CD28 antibodies. The supernatants were then analyzed by FACS by using a CBA (mouse Th1/Th2 kit obtained from BD Bioscience Pharmingen, San Diego, CA) in accordance with the manufacturer's instructions and as previously described [38, 39]. Following flow cytometric acquisition, the sample results were generated in graphical and tabular formats using the BD CBA Analysis Software (BD PharMingen).

Lung CD11c $^{+}$ cells characterization and isolation of CD11c $^{+}$ CD3 $^{-}$ lung cells

The cells positively selected for CD11c from the lungs of mice (with magnetic beads from Miltenyi as previously described) were stained for DC characteristic maturation markers like CD11c, CD80, CD86, IL-3R α (CD123) and CD40, and additionally, the cells were stained for determination of their lymphoid (CD8 α^{+}) or plasmacytoid character (mPDCA-1 $^{+}$) (diluted to 60–120 ng/100 μ L in PBS per 2×10^5 CD11c $^{+}$ cells). The CD11c $^{+}$ population then was stained with an antibody against CD11c (FITC) and CD3 (PE). Afterwards, these cells were sorted for both markers, and the obtained CD11c $^{+}$ CD3 $^{-}$ and CD11c $^{+}$ CD3 $^{+}$ populations were separately cultured for 24 h with OVA (500 μ g/mL) (Fig. 3). The supernatants were collected and the IFN- α and IFN- γ content measured by ELISA.

Reconstitution of C57BL/6 wild-type mice with lung CD11c $^{+}$ cells

CD11c $^{+}$ cells were isolated from the lungs of OVA-sensitized and -challenged mice by the use of specific CD11c positive selection beads (CD11c MicroBeads; Miltenyi Biotech). These cells were used for either the reconstitution of wild-type mice or for cell culture and FACS analysis to determine the purity and the character of the isolated cells. For *in vivo* reconstitution experiments, lung CD11c $^{+}$ cells (1×10^5 /200 μ L RPMI/

mouse) were injected intravenously into C57BL/6 mice previously aerosolized once with 1% OVA in PBS for 30 min. The next 2 days after reconstitution, the mice were aerosolized again with OVA and 24 h after the last aerosol, invasive airway plethysmography and BAL was performed to analyze cytokines by ELISA. The purity of the injected CD11c⁺ cells was analyzed by FACS staining and reached more than 90%.

ELISA

Mouse IL-4, IL-5, IFN- γ , TNF- α , IL-10, and IL-12p70 were detected using a specific sandwich ELISA (OptEIATM; all purchased from BD PharMingen), and IL-13 release of CD4⁺ T cells was analyzed by using a mouse-specific ELISA kit (Duo set-IL-13; R&D Systems, Wiesbaden, Germany). IFN- α was analyzed by using a monoclonal capture rat anti-mouse primary antibody (PBL, Piscataway, NJ) and for detection a goat anti-rabbit IFN- α antibody and a goat anti-rabbit HRP antibody were used in a TMB-based detection system. Levels of OVA-specific IgG1, IgG2a and IgE antibody titers were determined by ELISA technique as described [43].

Protein extraction, immunoprecipitation and Western blot analysis

Tissue proteins were extracted and protein concentration was determined as described [25, 41].

For immunoprecipitation, 120–300 μ g total lung proteins were precleared with 1 μ g of appropriate IgG (Santa Cruz Biotechnology) according to the primary antibody and 20 μ L A/G plus Agarose (Santa Cruz Biotechnology) for 30 min at 4°C. After centrifugation at 2500 rpm for 5 min, the supernatant was collected and incubated with 2 μ g primary antibody against STAT-4 or phospho-tyrosine (described above) for 1 h at 4°C, followed by addition of 20 μ L A/G plus Agarose. Immunoprecipitation was completed by incubation at 4°C overnight under rotation. Next day, the pellet was washed four times with PBS and finally resuspended in 50 μ L PBS. Western blots were performed as described before [25, 42] with loaded 15 μ L of STAT-4 immunoprecipitate and staining for phospho-tyrosine and the phospho-tyrosine immunoprecipitate for STAT-4 as described above.

Statistical analysis

Differences were evaluated for significance ($p < 0.05$) by Student's two-tailed t -test for independent events (Excel, PC). Data are given as mean values $\pm$ SEM.

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