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Elf3 Regulates Allergic Airway Inflammation by Controlling Dendritic Cell-Driven T Cell Differentiation

Rahul Kushwah,^{*,†,1} Jordan R. Oliver,^{*,†} Jing Wu,^{*} Zhijie Chang,[‡] and Jim Hu^{*,†}

Elf3 belongs to the Ets family of transcription factors and has been implicated in inflammation. Elf3 is highly expressed in the lungs, and *Elf3*^{-/-} mice are impaired in IL-6 production after intranasal LPS exposure. To identify the role of Elf3 in Th17-driven pulmonary inflammation, we have performed epicutaneous sensitization of *Elf3*^{-/-} mice with OVA followed by airway OVA challenge and have identified *Elf3*^{-/-} mice to be impaired in induction of Th17 response, attributable to impairment of IL-6 production by dendritic cells (DCs). However, increased serum levels of OVA-specific IgG1 and IgE were observed, pointing toward an exaggerated Th2 response. To study Th2 response, we performed i.p. sensitization of *Elf3*^{-/-} mice with OVA and confirmed loss of *Elf3* to result in an aggravated Th2 response, characterized by increased generation of IL-4-producing T cells, increased levels of OVA-specific IgE and IgG1 Ab titers, and increased serum levels of Th2 cytokines, together with extensive inflammation and mucus production in airways. *Elf3*^{-/-} DCs were impaired in priming Th1 differentiation, which, in turn, promoted Th2 differentiation. This was mediated by the ability of *Elf3*^{-/-} DCs to undergo hypermaturation but secrete significantly lower levels of IL-12 in response to inflammatory stimuli. The impairment of IL-12 production was due to impairment of IL-12p40 gene induction in *Elf3*^{-/-} DCs in response to inflammatory stimuli. Taken together, our study identifies a novel function of Elf3 in regulating allergic airway inflammation by regulating DC-driven Th1, Th2, and Th17 differentiation. *The Journal of Immunology*, 2011, 187: 000–000.

Elf3 (Ese-1) belongs to a novel subset of the Ets family of transcription factors, which also includes Ese-2 and Ese-3 (1). The Ets transcription factors comprise a gene family that has a diverse range of biological functions including cell proliferation and differentiation (1, 2). The majority of the Ets factors are expressed in hematopoietic cells and show important functions in hematopoiesis (2). Among the members of the Ets family, PU.1 and Ets1 have important functions in T cell differentiation (3). Other Ets factors such as Spi-B and Ese-3, together with PU.1, have important functions in dendritic cell (DC) development (4–8). Genetic linkage analysis of Tristan da Cunha population has implicated *ESE-2* and *ESE-3* as “asthma candidate genes” (9, 10).

Elf3 plays a role in fetal development and has also been shown to play a role in inflammation (1). Elf3 is highly expressed in epithelial-rich tissues such as lung and intestine, and proinflammatory cytokines such as IL-1 β and TNF- α are known to induce Elf3 expres-

sion in multiple cell types including monocytes via NF- κ B binding to the *Elf3* promoter (11). Our previous work has shown that ESE-1 (human homolog of *Elf3*) is upregulated in bronchial epithelial cells after cytokine stimulation, and ESE-1 subsequently upregulates *ESE-3* expression by directly binding to the promoter region (12). ESE-3 expression in DCs is induced as DCs undergo maturation (8). Elf3 has been shown to regulate genes that are involved in inflammation such as TLR11, inducible NO synthase gene, and *cox-2* (8, 13–15). Altogether, previous studies have implicated a role of Elf3 in inflammation, which is known to subsequently influence the induction of immune responses. However, it is not clear whether Elf3 affects pulmonary immune responses.

Atopic dermatitis (AD) is a common, chronic, inflammatory skin disease often associated with other systemic atopic disorders (16). Patients with AD have a high incidence of asthma (17, 18). Although initially AD was looked on as a Th2-mediated disease, recent studies have highlighted the importance of Th17 response in mediating AD pathology (16, 19). Th17 T cells are a newly identified class of helper T cells that secrete IL-17 and play a role in inflammation (20). Antigenic exposure via the skin has been shown to be associated with an induction of Th17 response. Epicutaneous sensitization of mice with a protein Ag leads to development of allergic dermatitis and airway hyperresponsiveness upon a single exposure to aerosolized Ag (21). This appears to be dependent on Th17 response, which can subsequently result in Th17-driven airway inflammatory response characterized by influx of neutrophils (22). Therefore, epicutaneous sensitization followed by airway Ag challenge has been used as a model for AD-induced asthma (22–24). Identification of genes that affect Th17 response induced upon epicutaneous sensitization is important to better understand the mechanisms involved in AD-induced asthma development. Our previous work has identified an impairment of IL-6 production in *Elf3*^{-/-} mice after intranasal exposure to LPS (12). Because IL-6 is a key cytokine involved in Th17 differentiation, we hypothesize that Elf3 may play a role in regulating Th17 response.

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Abbreviations used in this article: AD, atopic dermatitis; BALF, bronchoalveolar lavage fluid; DC, dendritic cell; MHC II, MHC class II; PAS, periodic acid–Schiff; ROR, retinoic acid-related orphan receptor; TSLP, thymic stromal lymphopoietin.

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In this study, we examined the role of Elf3 in regulating pulmonary inflammation following cutaneous Ag exposure, which is known to be dependent on Th17 response. *Elf3*^{-/-} mice were impaired in Th17 induction after epicutaneous sensitization and airway challenge. The impairment in Th17 induction was mediated by an impairment of *Elf3*^{-/-} DCs in priming Th17 response because of impairment in IL-6 induction. Although pulmonary Th17 recall response was impaired in OVA-sensitized and -challenged *Elf3*^{-/-} mice, surprisingly, the extent of airway inflammation along with serum levels of OVA-specific IgE and IgG1 titers were higher in *Elf3*^{-/-} mice compared with *Elf3*^{+/-} mice, increasing the likelihood of an exaggerated Th2 response in *Elf3*^{-/-} mice. Therefore, we sought to investigate the role of Elf3 in Th2-driven allergic airway inflammation. i.p. sensitization of *Elf3*^{-/-} mice with OVA followed by airway OVA challenge led to increased airway inflammation compared with *Elf3*^{+/-} mice, which was mediated by an exaggerated Th2 response. Further analysis revealed that, although *Elf3*^{-/-} T cells were normal, *Elf3*^{-/-} DCs underwent hypermaturation but were impaired in IL-12 production, which is a key cytokine for Th1 differentiation. Impairment of DC-mediated Th1 differentiation promoted an exaggerated Th2 response. These findings identify a key role of *Elf3* in regulating allergic airway inflammation by regulating DC-driven T cell differentiation.

Materials and Methods

Animals and mouse sensitization protocols

Elf3^{-/-} mice on C57BL/6 background were maintained in pathogen-free conditions at the Toronto Centre for Phenogenomics and all the controls were used were littermates. All the animal studies were reviewed and approved by the Toronto Centre for Phenogenomics animal care committee for human use of animals. Epicutaneous sensitization with OVA was performed as described previously (21). In brief, mice were anesthetized with isoflurane (Aerrane; Baxter, Mississauga, ON, Canada) and shaved with an electric razor. Subsequently, 100 µg OVA (grade V; Sigma, St. Louis, MO) in 100 µl normal saline was placed on a 1 × 1-cm patch of sterile gauze, which was secured to the skin with a transparent bio-occlusive dressing (Johnson and Johnson, Lake Dallas, TX). The patch remained in place for 1-wk period, after which it was removed. Two wk later, an identical patch was reapplied to the same skin site. Each mouse had a total of three 1-wk exposures to the patch separated from each other by 2-wk intervals. Inspection confirmed that the patch remained in place at the end of each sensitization period. After sensitization, mice were exposed to intranasal OVA for 3 d and subsequently sacrificed for analysis. i.p. sensitization with OVA was performed as described previously (25). In brief, mice received two i.p. injections of 100 µg OVA (grade V; Sigma) in Alum on day 1 and subsequently after 2 wk. After sensitization, mice were exposed to intranasal OVA for 3 d and then sacrificed for analysis. To label pulmonary DCs, we used OVA-FITC (Sigma) for intranasal delivery and performed analysis of pulmonary DC maturation and migration as described previously (26).

Culture of DCs

Mouse DCs were derived from mouse bone marrow cells cultured in DMEM containing recombinant mouse GM-CSF (PeproTech) as described previously (26).

Cell proliferation assay

Lymph node cells, splenocytes, or T cells were seeded in 96-well plates and stimulated with OVA (100 µg/ml) or anti-CD3 (10 µg/ml) and anti-CD28 (10 µg/ml) Ab. BrdU was added during the last 12 h of culture, after which proliferation was measured using BrdU cell proliferation, chemiluminescent ELISA, according to the manufacturer's instructions (Roche).

T cell differentiation

Naive CD4⁺CD25⁻ T cells were isolated from mouse spleens using naive CD4⁺CD62L⁺ T cell isolation kit (Miltenyi Biotec) and activated with plate-bound anti-CD3 (10 µg/ml) and soluble anti-CD28 Ab (10 µg/ml; eBioscience). For Th1 conditions, cultures were supplemented with 10 ng/ml IL-12 (PeproTech) and 20 µg/ml anti-IL-4 (eBioscience). For Th2

conditions, cultures were supplemented with 10 ng/ml IL-4 (PeproTech), 20 µg/ml anti-IFN-γ, and 20 µg/ml anti-IL-12 (eBioscience). For Th17 conditions, cultures were supplemented with 2.5 ng/ml TGF-β (PeproTech), 30 ng/ml IL-6 (PeproTech), and 50 ng/ml IL-23 (R&D Systems, Minneapolis, MN), along with 20 µg/ml anti-IL-4 and 20 µg/ml anti-IFN-γ. Four days after activation, cells were washed and stimulated as described later (see Flow Cytometry section). For Th17 differentiation in presence of DCs, bone marrow DCs were stimulated overnight with 1 µg/ml LPS and added to a culture of naive CD4⁺CD25⁻ T cells with addition of cytokines and Abs as described earlier with the exception of IL-6 addition. For Th1/Th2 differentiation in presence of DCs, bone marrow DCs were incubated overnight with 1 µg/ml LPS and added to a culture of naive CD4⁺CD25⁻ T cells without addition of any exogenous cytokines or cytokine neutralizing Abs.

Cytokine measurements

Lungs, mediastinal, axillary, and brachial lymph nodes and spleens were isolated from sensitized mice, suspended at a concentration of 2 × 10⁶ cells/ml, cultured in presence of full-length OVA (100 µg/ml) for 48–72 h, and subsequently medium was collected. DCs were stimulated with LPS or IL-1β together with TNF-α for 24 h, and subsequently the medium was collected. Cytokine measurement in the medium, serum, and bronchoalveolar lavage fluid (BALF) was carried out using mouse Th1/Th2 CBA kit (BD Biosciences) or ELISA kits (IL-12, TNF-α, IL-6, and IL-17; R&D Systems).

OVA-specific IgE and IgG1 measurements

To assess OVA-specific Ab titers, we coated 96-well plates with 100 µl OVA per well at a concentration of 20 µg/ml and incubated them overnight at 4°C. Detection of OVA-specific IgG1 was carried out using biotinylated rat anti-mouse IgG1 (BD Biosciences), and for detection of OVA-specific IgE, mouse IgE ELISA set (BD Biosciences) was used following manufacturer's instructions.

Gene expression analysis

Total RNA from cells was isolated using an RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions, and quantitative real-time RT-PCR was carried out using gene-specific primers (Table I) as described previously (12). Whole-lung RNA was isolated using TRIzol, and the expression of 84 genes involved in Th1/Th2 responses in the lungs of mice were quantified using mouse Th1-Th2-Th3 PCR-Array (SABiosciences-Qiagen).

Histological analysis

Formalin-fixed, paraffin-embedded mouse lung tissue samples were sectioned at 4 µm and stained with H&E or periodic acid-Schiff (PAS) for histological examination under a light microscope as described previously (3).

Flow cytometry

To assess cytokine production by lung and spleen cells, we stimulated lung and spleen cells from mice in vitro with OVA for 24 h. Subsequently, BD-GolgiStop was added for 4 h. Cells were harvested and surface stained with anti-CD4 (eBioscience) followed by permeabilization using BD Cytotfix/Cytoperm plus kit (BD Biosciences), followed by staining with cytokine-specific Abs (eBioscience). To assess production of cytokines by in vitro differentiated T cells, we stimulated T cells with phorbol 12-myristate 13-acetate (100 ng/ml) and ionomycin (10 ng/ml) in the presence of GolgiStop for 5 h and subsequently permeabilized/stained them as described earlier. All the Abs were purchased from eBioscience unless indicated otherwise. Flow cytometry was performed on BD-LSRII (BD Biosciences), and data were analyzed using FlowJo (TreeStar).

Statistical analysis

Student two-tailed *t* test was used for statistical analysis. A *p* value <0.05 was considered significant.

Results

Elf3^{-/-} mice mount an impaired Th17 response after epicutaneous sensitization with OVA

To study pulmonary Th17 response, we used an epicutaneous model of OVA sensitization followed by airway challenge with OVA, which has been shown to prime a Th17 response (22). Saline-

Table I. List of primer sequences used in this study

Name	Sequence
Mouse	
<i>IL-5</i>	Forward: 5'-CACCAGCTATGCATTGGAGAAATC-3' Reverse: 5'-TCTGTACTCATCACACCAAGGAAC-3'
<i>IL-6</i>	Forward: 5'-CCCAACAGACTGTCTATACC-3' Reverse: 5'-CTGCAAGTGCATCATCGTTGTTTC-3'
<i>IL-9</i>	Forward: 5'-ATGTTGGTGACATACATCCTTGC-3' Reverse: 5'-TGACGGTGGATCATCCTTCAG-3'
<i>IL-13</i>	Forward: 5'-TGACCAACATCTCCAATTGCA-3' Reverse: 5'-TTGTTTATAAGTGGGCTACTTCGATTT-3'
<i>IL-17</i>	Forward: 5'-TGTACTGTGTGATGTTGGGAC-3' Reverse: 5'-AATGCCCTGGTTTGGTTGAA-3'
<i>TSLP</i>	Forward: 5'-AGGCTACCCTGAAACTGAG-3' Reverse: 5'-GGAGATTGCATGAAGGAATACC-3'
<i>IL-25</i>	Forward: 5'-GGAGCTATGAGTTGGACAGG-3' Reverse: 5'-GGTAAAGTGGGACGGAGTTG-3'
<i>IL-33</i>	Forward: 5'-CAAAGAAGTTTGCCCATGT-3' Reverse: 5'-AAGGCAAGCACTCCACAGT-3'
<i>T-bet</i>	Forward: 5'-GTTCCTTCCTGTCCTTC-3' Reverse: 5'-CCTTGTGTGGTGAGCTT-3'
<i>RORγt</i>	Forward: 5'-AGCTTTGTGCAGATCTAAGG-3' Reverse: 5'-TGTCCCTCCTCAGTAGGGTAG-3'
<i>GATA-3</i>	Forward: 5'-GCGGGCTCTATCAGAAATGA-3' Reverse: 5'-GCTCTCCTGGCTGCAGACAGC-3'
<i>IL-12p40</i>	Forward: 5'-ACAGCACCAGCTTCTTCATCAG-3' Reverse: 5'-TCTTCTAAGGCTTTCATCTGCAA-3'
<i>IL-12p35</i>	Forward: 5'-CACCCTTGCCCTCCTAAACC-3' Reverse: 5'-CACCTGGCAGGTCCAGAGA-3'
<i>TNFα</i>	Forward: 5'-CATCTTCTCAAAATTCGAGTGACAA-3' Reverse: 5'-TGGGAGTAGACAAAGGTACAACCC-3'
<i>Elf3</i>	Forward: 5'-CTCCTGCTCCTCCGACTAC-3' Reverse: 5'-CCGCTCGCTAGTCCAGCTT-3' Probe: 5'-ACTTGGTGTGACCCTGA-3'
Human	
<i>TSLP</i>	Forward: 5'-CAGAGCCTAACCTTCAATC-3' Reverse: 5'-CTTCTTCATTGCCTGAGTAG-3'
<i>ESE-1</i>	Forward: 5'-GGCGTCTTCAAGTTCTTGCG-3' Reverse: 5'-CTCCCCTTTGTAGTAGTACCTC-3'

treated control *Elf3*^{+/+} and *Elf3*^{-/-} mice had <1% of CD4⁺ T cells as IL-17-producing cells in the spleen (Fig. 1A). However, following epicutaneous sensitization with OVA, the proportion of IL-17-producing CD4⁺ T cells increased to 6–7% in the spleens of *Elf3*^{+/+} mice. In contrast, the proportion of IL-17-producing CD4⁺ T cells was only 2–3% in the spleens of OVA-sensitized *Elf3*^{-/-} mice, indicating an impairment of Th17 induction in *Elf3*^{-/-} mice. In addition, mRNA levels of retinoic acid-related orphan receptor (ROR) γ t, which is a Th17 lineage-specific transcription factor, along with mRNA levels of IL-17, a key cytokine secreted by Th17 cells, were also reduced in the spleens of OVA-sensitized *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice, confirming Th17 impairment (Fig. 1B). Th17 response is associated with production of IL-17; therefore, we measured serum levels of IL-17 in sensitized mice (Fig. 1C). IL-17 serum levels in saline-treated *Elf3*^{+/+} and *Elf3*^{-/-} control mice were undetectable. In contrast, following OVA sensitization, although IL-17 was detected in the serum of both *Elf3*^{+/+} and *Elf3*^{-/-} mice, the levels were significantly reduced in *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice, which was consistent with the observation of reduced IL-17⁺ cells in the spleens of OVA-sensitized *Elf3*^{-/-} mice. Taken together, these findings identify that after epicutaneous sensitization with OVA, *Elf3*^{-/-} mice were impaired in priming Th17 response.

To assess the pulmonary Th17 response, we challenged OVA-sensitized mice with intranasal delivery of OVA, and subsequently the proportions of IL-17-producing CD4⁺ T cells were assessed in the lungs. Saline-treated *Elf3*^{+/+} and *Elf3*^{-/-} control mice had ~1% of CD4⁺ T cells in the lungs as IL-17-producing

cells (Fig. 1D). In contrast, following airway OVA challenge, ~8% of the pulmonary CD4⁺ T cells in OVA-sensitized *Elf3*^{+/+} mice were IL-17⁺, indicating an induction of pulmonary Th17 response, associated with OVA airway challenge after epicutaneous sensitization. However, pulmonary Th17 recall response was significantly impaired in the airways of OVA-challenged and -sensitized *Elf3*^{-/-} mice, for only 2–3% of the pulmonary CD4⁺ T cells were IL-17⁺ (Fig. 1D). In addition, mRNA levels of ROR γ t, which is a Th17 lineage-specific transcription factor, along with mRNA levels of IL-17, were also reduced in the lungs of OVA-challenged and -sensitized *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice, confirming an impaired pulmonary Th17 recall response (Fig. 1E). Pulmonary Th17 recall response is associated with production of IL-17 in the BALF. Therefore, we measured IL-17 protein levels in BALF of mice after airway challenge with OVA. In absence of epicutaneous sensitization, BALF of saline-treated *Elf3*^{+/+} and *Elf3*^{-/-} control mice had similar and extremely low levels of IL-17 (Fig. 1F). In contrast, following OVA challenge of OVA-sensitized mice, although IL-17 levels were elevated in BALF from both *Elf3*^{+/+} and *Elf3*^{-/-} mice, IL-17 levels were significantly reduced in the BALF of *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice, which was consistent with the observation of reduced IL-17-producing CD4⁺ T cells in the lungs of OVA-challenged and -sensitized *Elf3*^{-/-} mice.

To further assess pulmonary recall response, we also measured IL-17 levels released by lung cells isolated from OVA-challenged and -sensitized mice after stimulation with OVA (Fig. 1G). After OVA stimulation, lung cells from saline-treated *Elf3*^{+/+} and *Elf3*^{-/-} control mice released very low levels of IL-17. Although pulmonary cells from both OVA-challenged and -sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice secreted elevated levels of IL-17 after OVA stimulation, the levels of IL-17 released by pulmonary cells from *Elf3*^{-/-} mice were significantly reduced compared with those from *Elf3*^{+/+} mice, confirming an impaired pulmonary Th17 recall response. Next, we also looked at the proportions of IL-17-producing CD4⁺ cells in the pulmonary draining lymph nodes of *Elf3*^{+/+} and *Elf3*^{-/-} mice (Fig. 1H). Approximately 11% of CD4⁺ T cells were IL-17⁺ in the draining lymph nodes of OVA-challenged and -sensitized *Elf3*^{+/+} mice. However, the proportions of IL-17-producing CD4⁺ T cells were reduced to only 5–6% in the pulmonary draining lymph nodes of OVA-challenged and -sensitized *Elf3*^{-/-} mice, indicating an impaired recall Th17 response. Taken together, these findings indicate that after epicutaneous sensitization and challenge with OVA, Th17 response is impaired in *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice.

Elf3^{-/-} mice mount an exaggerated inflammatory response on OVA challenge after epicutaneous OVA sensitization

To better understand the pulmonary inflammatory response in OVA-sensitized *Elf3*^{-/-} mice, we measured absolute counts of infiltrating cells in BALF after airway OVA challenge (Fig. 2A). Saline-treated *Elf3*^{+/+} and *Elf3*^{-/-} control mice had few cell numbers in the BALF, as expected, because of a lack of an inflammatory response in absence of epicutaneous sensitization. In contrast, upon OVA challenge after OVA sensitization, the absolute cell counts of infiltrating cells were significantly higher in the BALF of *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice, indicating increased cellular infiltration in BALF of OVA-sensitized *Elf3*^{-/-} mice after airway OVA challenge (Fig. 2A). Next, we performed a cell composition analysis to identify proportions and absolute counts of macrophages, lymphocytes, neutrophils, and eosinophils in BALF by Giemsa–Wright staining. BALF cellular composition analysis revealed that although the absolute counts of neutrophils were similar between *Elf3*^{+/+} and *Elf3*^{-/-} mice, the proportions of

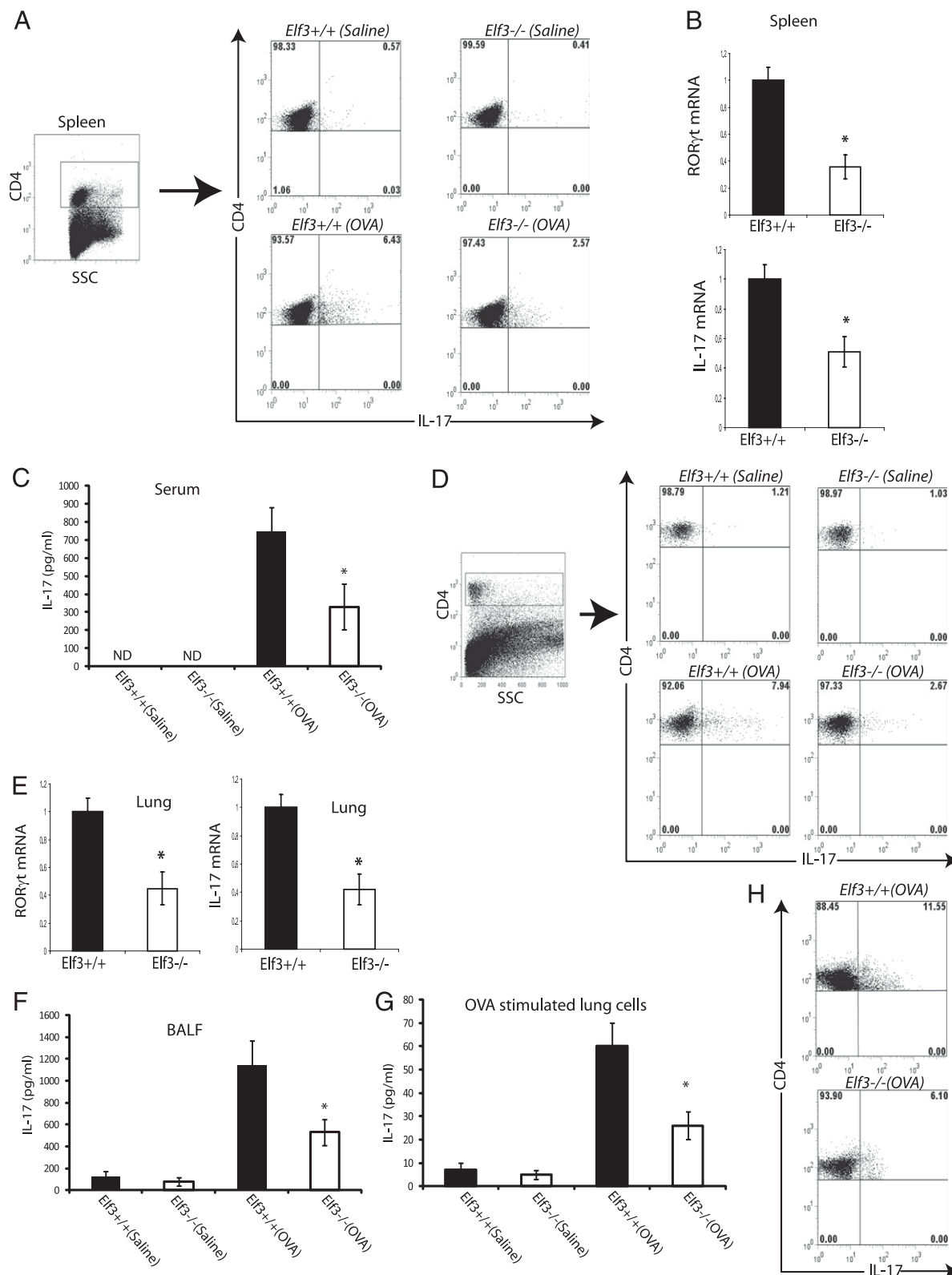


FIGURE 1. *Elf3*^{-/-} mice display an impaired Th17 response on epicutaneous sensitization with OVA. *Elf3*^{+/+} and *Elf3*^{-/-} mice were sensitized epicutaneously with OVA, and proportions of IL-17⁺ T cells were assessed in the spleens by FACS analysis. **A**, Representative FACS plots looking at IL-17 production by CD4⁺ T cells in the spleens of saline-treated or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice. **B**, Quantitative PCR analysis of RORγt and IL-17 mRNA expression in the spleens of OVA-sensitized *Elf3*^{+/+} mice compared with OVA-sensitized *Elf3*^{-/-} mice. **C**, Protein levels of IL-17 in the serum of saline-treated or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice. **D–H**, *Elf3*^{+/+} and *Elf3*^{-/-} mice were sensitized epicutaneously with OVA and then challenged intranasally with OVA. Subsequently, the proportions of IL-17⁺ T cells were assessed in the lungs by FACS analysis. **D**, Representative FACS plots looking at IL-17 production by CD4⁺ T cells in the lungs of saline-treated or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice after airway OVA challenge. **E**, Quantitative PCR analysis of RORγt and IL-17 mRNA expression in the lungs of OVA-sensitized *Elf3*^{+/+} mice compared with OVA-sensitized *Elf3*^{-/-} mice after airway OVA challenge. **F**, IL-17 protein levels in BALF of saline-treated or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice after airway OVA challenge. **G**, Levels of IL-17 released by OVA-stimulated pulmonary cells from saline-treated or OVA-sensitized *Elf3*^{-/-} mice after airway OVA challenge. **H**, Representative FACS

neutrophils were significantly reduced in the BALF of OVA-challenged and -sensitized $Elf3^{-/-}$ mice compared with $Elf3^{+/+}$ mice (Fig. 2B, 2C). Furthermore, when we looked at lymphocyte counts, lymphocyte counts in the BALF of OVA-challenged and -sensitized $Elf3^{-/-}$ mice were higher than $Elf3^{+/+}$ counterparts, which has previously been shown to be associated with Th2 response (Fig. 2B, 2C) (27). Moreover, histological analysis revealed the extent of inflammation to be more severe in the airways of OVA-sensitized $Elf3^{-/-}$ mice compared with $Elf3^{+/+}$ mice after airway OVA challenge (Fig. 2D). We also performed staining with PAS to assess mucus production in the airways. PAS staining was more prominent in the airways of OVA-sensitized $Elf3^{-/-}$ mice compared with $Elf3^{+/+}$ mice after airway OVA challenge, confirming increased inflammation in the airways of $Elf3^{-/-}$ mice compared with $Elf3^{+/+}$ mice (Fig. 2E). Altogether, these findings indicate that though neutrophilic infiltration was reduced in the airways of OVA-challenged and -sensitized $Elf3^{-/-}$ mice, consistent with an impaired Th17 pulmonary recall response, the extent of pulmonary inflammation was surprisingly higher. Furthermore, we also measured serum OVA-specific IgG1 and IgE levels, which were unexpectedly elevated several-fold in $Elf3^{-/-}$ mice compared with $Elf3^{+/+}$ mice (Fig. 2F, 2G). Because IgG1 and IgE response are largely Th2 driven, and increased airway inflammation, together with increased mucus production in the airways, is also indicative of pulmonary Th2 response, this indicates that after epicutaneous sensitization, $Elf3^{-/-}$ mice may mount an exaggerated Th2 response.

Elf3^{-/-} DCs are impaired in priming Th17 differentiation because of impairment in IL-6 production

TGF- β 1 and IL-6 are the key cytokines that play an important role in driving Th17 differentiation. TGF- β 1 mRNA levels were similar between the lungs of OVA-challenged and -sensitized $Elf3^{-/-}$ and $Elf3^{+/+}$ mice, indicating that impaired pulmonary Th17 response observed in $Elf3^{-/-}$ mice was likely not due to impaired TGF- β 1 production in $Elf3^{-/-}$ mice (data not shown). BALF from saline-treated control $Elf3^{-/-}$ and $Elf3^{+/+}$ mice had very low levels of IL-6 consistent with an absence of an inflammatory response in unsensitized mice. In contrast, after OVA challenge, IL-6 levels were significantly reduced in the BALF of OVA-sensitized $Elf3^{-/-}$ mice compared with $Elf3^{+/+}$ mice (Fig. 3A). To further confirm an impairment of IL-6 production in OVA-sensitized $Elf3^{-/-}$ mice, we also measured IL-6 levels in the serum, which again were significantly reduced in OVA-sensitized $Elf3^{-/-}$ mice compared with $Elf3^{+/+}$ mice (Fig. 3B). Cumulatively, these findings indicated an impairment of IL-6 production in OVA-challenged and -sensitized $Elf3^{-/-}$ mice, which likely was responsible for the impaired pulmonary Th17 response.

DCs are the key players in priming T cell differentiation. Because $Elf3^{-/-}$ mice had normal T cell distribution and $Elf3^{-/-}$ T cells were normal in their ability to differentiate into Th17 lineage, we shifted our focus on $Elf3^{-/-}$ DCs (Supplemental Fig. 1). To assess whether intrinsic defects in $Elf3^{-/-}$ DCs were responsible for impaired Th17 response, we tested for the ability of $Elf3^{-/-}$ DCs to prime differentiation of naive T cells into Th17 lineage. $Elf3^{-/-}$ and $Elf3^{+/+}$ bone marrow-derived DCs were stimulated with LPS and added to a culture of naive CD4⁺ T cells from $Elf3^{-/-}$ and $Elf3^{+/+}$ mice stimulated with anti-CD3 and anti-CD28 Ab. Subsequently, FACS analysis was conducted to identify proportions of IL-17-producing

CD4⁺ T cells. Among T cells cultured with $Elf3^{+/+}$ DCs, 6–7% of T cells differentiated into IL-17-producing Th17 cells, whereas among T cells cultured with $Elf3^{-/-}$ DCs, only 2–3% differentiated into IL-17-producing Th17 cells (Fig. 3C, 3D). Furthermore, the defect in Th17 differentiation was intrinsic to $Elf3^{-/-}$ DCs because culture of $Elf3^{-/-}$ or $Elf3^{+/+}$ T cells with $Elf3^{+/+}$ DCs led to similar levels of Th17 differentiation. T cells cultured with $Elf3^{-/-}$ DCs secreted significantly reduced levels of IL-17 compared with T cells cultured with $Elf3^{+/+}$ DCs, confirming an impairment of $Elf3^{-/-}$ DCs in driving Th17 differentiation (Fig. 3E). The impairment of $Elf3^{-/-}$ DCs to prime Th17 response was due to impairment in IL-6 mRNA induction and IL-6 protein production in response to LPS (Fig. 3F, 3G). In addition to $Elf3^{-/-}$ DCs, primary $Elf3^{-/-}$ airway epithelial cells were also impaired in IL-6 production, and short hairpin RNA-mediated knockdown of ESE-1 (human homolog of Elf3) in BEAS2B human bronchial epithelial cells suppressed IL-6 production, confirming a role of Elf3 in regulating IL-6 gene expression (Supplemental Fig. 2). Cumulatively, these findings indicate that impairment in Th17 differentiation observed in OVA-sensitized $Elf3^{-/-}$ mice is largely driven by $Elf3^{-/-}$ DCs, which are impaired in IL-6 production. Furthermore, impairment of IL-6 production by $Elf3^{-/-}$ airway epithelial cells may also contribute to an impairment of pulmonary recall Th17 response.

Exaggerated Th2 is observed in Elf3^{-/-} mice on i.p. sensitization followed by airway OVA challenge

To study whether $Elf3^{-/-}$ mice do, in fact, mount an exaggerated airway Th2 response, we used the i.p. model of OVA sensitization followed by OVA airway challenge, known to drive a pulmonary Th2 response. We analyzed the proportion of Th2 cells by analyzing IL-4-producing CD4⁺ T cells in the lungs of mice. In saline-treated $Elf3^{+/+}$ and $Elf3^{-/-}$ control mice, <2% of the CD4⁺ T cells in the lung were IL-4⁺, as expected in the absence of OVA sensitization (Fig. 4A). After airway OVA challenge, ~10% of the CD4⁺ T cells in the lungs of OVA-sensitized $Elf3^{+/+}$ mice were identified as IL-4⁺. However, in the lungs of OVA-sensitized $Elf3^{-/-}$ mice after airway OVA challenge, ~20% of the CD4⁺ T cells were IL-4⁺, which was approximately doubled compared with the proportions observed in OVA-challenged and -sensitized $Elf3^{+/+}$ mice, indicating an exaggerated recall Th2 response in the lungs of $Elf3^{-/-}$ mice (Fig. 4A). The hallmark of OVA-induced allergic inflammation is the extensive inflammation in the airways along with mucus production by epithelial cells, which is known to be induced by Th2 cytokines (28). Inflammation was indeed more extensive in the airways of OVA-challenged and -sensitized $Elf3^{-/-}$ mice compared with OVA-challenged and -sensitized $Elf3^{+/+}$ mice, as assessed by H&E staining of lung sections (Fig. 4B). Furthermore, PAS staining for mucus production was more prominent in the airways of OVA-challenged and -sensitized $Elf3^{-/-}$ mice compared with OVA-challenged and -sensitized $Elf3^{+/+}$ mice, indicating higher levels of Th2 cytokines being produced in the lungs that are known to drive mucus production (Fig. 4C). Serum levels of Th2 cytokines IL-4 and IL-5 were also significantly elevated in OVA-challenged and -sensitized $Elf3^{-/-}$ mice compared with OVA-challenged and -sensitized $Elf3^{+/+}$ mice, which could possibly account for increased mucus production in airways of OVA-sensitized $Elf3^{-/-}$ mice (Fig. 4D, 4E). OVA-challenged and -sensitized $Elf3^{-/-}$ mice also had higher counts of

plots looking at IL-17 production by CD4⁺ T cells from the pulmonary draining lymph nodes of saline-treated or OVA-sensitized $Elf3^{+/+}$ and $Elf3^{-/-}$ mice after airway OVA challenge. All data are presented as mean $\pm$ SD, obtained from four to five mice per group, representative of four independent experiments. * p < 0.05 compared with other groups. ND, not detectable.

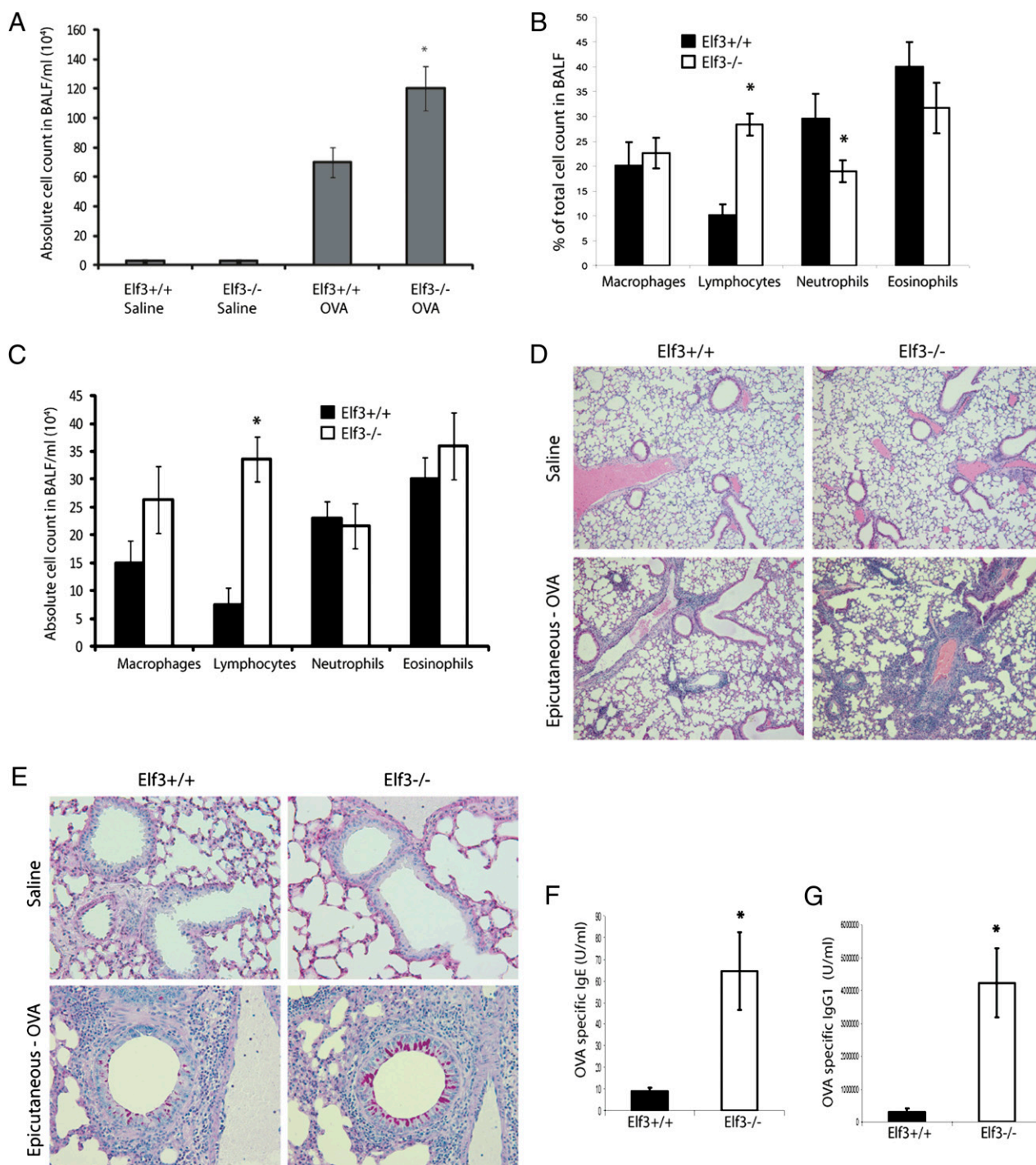


FIGURE 2. Increased inflammation is observed in the airways of epicutaneously sensitized *Elf3*^{-/-} mice with reduced proportions of neutrophils in BALF after airway OVA challenge. **A**, Histogram comparing absolute cell counts in the BALF of saline or OVA-sensitized *Elf3*^{-/-} and *Elf3*^{+/+} mice after airway OVA challenge. **B**, Histogram comparing proportions of macrophages, lymphocytes, neutrophils, and eosinophils in the BALF of saline or OVA-sensitized *Elf3*^{-/-} and *Elf3*^{+/+} mice after airway OVA challenge. **C**, Histogram comparing absolute counts of macrophages, lymphocytes, neutrophils, and eosinophils in the BALF of saline or OVA-sensitized *Elf3*^{-/-} and *Elf3*^{+/+} mice after airway OVA challenge. **D**, Histopathological analysis of H&E-stained lung sections from saline-treated or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice after airway OVA challenge (original magnification $\times 50$). **E**, Histopathological analysis of PAS-stained lung sections from saline-treated or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice after airway OVA challenge (original magnification $\times 200$). **F**, Histogram comparing serum levels of OVA-specific IgE between OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice. **G**, Histogram comparing serum levels of OVA-specific IgG1 between OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice. All data are presented as mean $\pm$ SD, obtained from six to seven mice per group, representative of four independent experiments. * $p < 0.05$ compared with other groups.

lymphocytes and eosinophils in BALF compared with OVA-challenged and -sensitized *Elf3*^{+/+} mice, consistent with an exaggerated Th2 response (Fig. 4F). Th2 response is known to drive IgE and IgG1 Ab response (29). Serum levels of OVA-

specific IgE and IgG1 titers were also significantly higher in OVA-sensitized *Elf3*^{-/-} mice compared with OVA-sensitized *Elf3*^{+/+} mice (Fig. 4G, 4H). Furthermore, OVA-stimulated lymph node cells from OVA-challenged and -sensitized *Elf3*^{-/-}

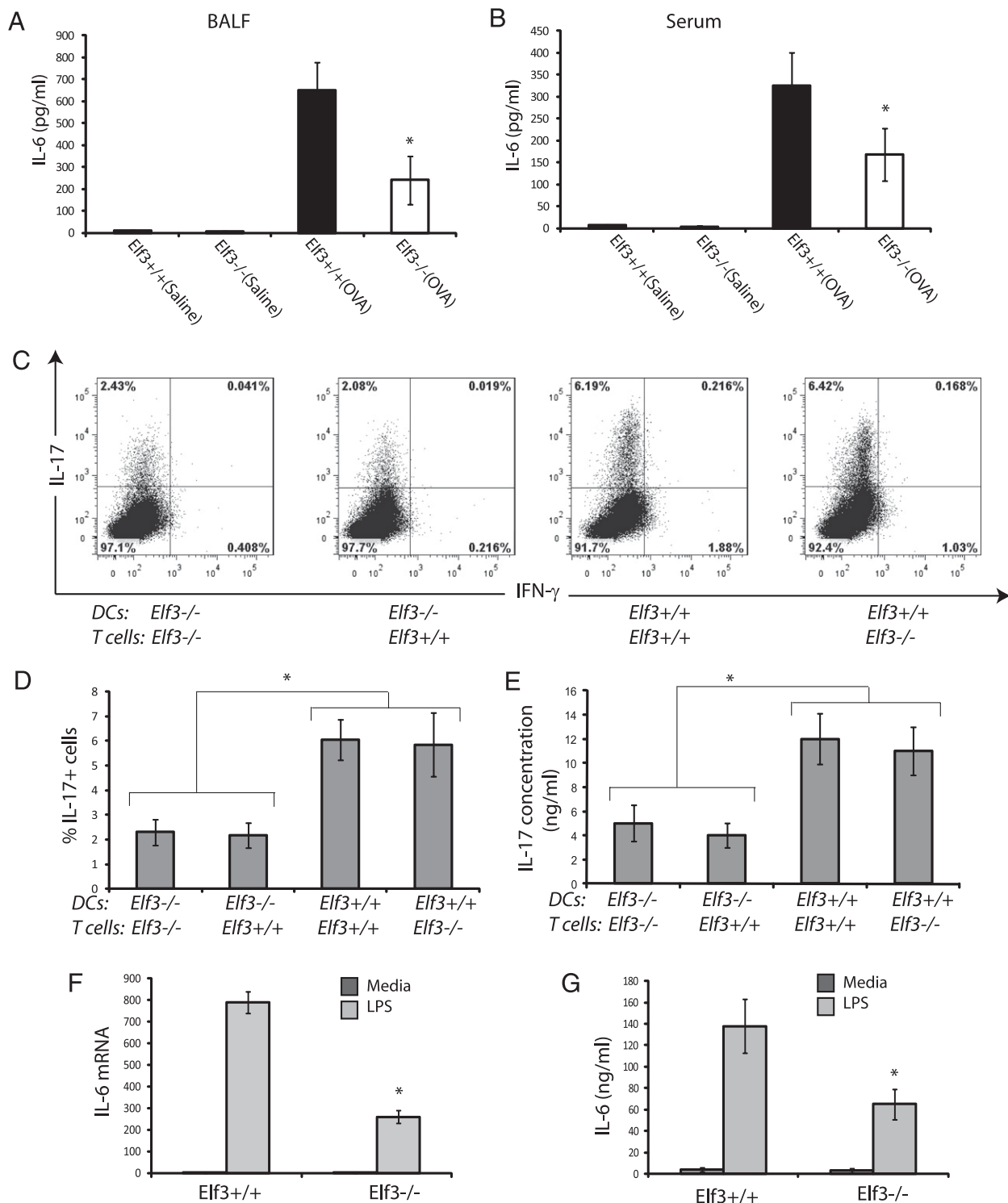


FIGURE 3. *Elf3*^{-/-} DCs are impaired in priming Th17 differentiation because of impairment in IL-6 production. **A**, IL-6 protein levels in the BALF of saline or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice after airway OVA challenge. **B**, Serum IL-6 protein levels from saline or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice after airway OVA challenge. **C–E**, Naive CD4⁺ T cells from *Elf3*^{+/+} and *Elf3*^{-/-} mice were differentiated into Th17 cells in the presence of LPS-stimulated bone marrow-derived DCs from *Elf3*^{+/+} or *Elf3*^{-/-} mice. **C**, Representative FACS plots looking at production of IFN-γ and IL-17 by *Elf3*^{-/-} or *Elf3*^{+/+} T cells cultured with *Elf3*^{-/-} or *Elf3*^{+/+} DCs. **D**, Histogram comparing proportions of IL-17⁺CD4⁺ T cells. **E**, Histogram comparing levels of IL-17 released by T cells into the media. **F**, Quantitative PCR analysis of mRNA levels of IL-6 in *Elf3*^{+/+} and *Elf3*^{-/-} DCs on stimulation with LPS. **G**, Protein levels of IL-6 released on LPS stimulation of *Elf3*^{+/+} and *Elf3*^{-/-} DCs. Results are expressed as fold induction relative to mRNA levels in lymph nodes from saline-challenged *Elf3*^{+/+} mice or unstimulated *Elf3*^{+/+} DCs. All data are presented as mean ± SD, obtained from five to six mice per group, representative of four independent experiments. **p* < 0.05 compared with other groups.

mice secreted significantly higher levels of IL-4 and IL-5 than cells from OVA-challenged and -sensitized *Elf3*^{+/+} mice (Fig. 4I, 4J). Moreover, the levels of lymph node cell proliferation in

response to OVA stimulation was significantly higher among cells from OVA-sensitized *Elf3*^{-/-} mice compared with OVA-sensitized *Elf3*^{+/+} mice (Fig. 4K). Cumulatively, these results

indicate that i.p. sensitization with OVA followed by airway OVA challenge led to aggravated allergic airway inflammatory response, mediated by an exaggerated Th2 response in *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice.

*Role of airway epithelial cells and T cells in induction of an exaggerated Th2 response in *Elf3*^{-/-} mice*

Airway epithelial cells express *Elf3* and are known to secrete cytokines that can modulate immune responses by affecting T cell differentiation (30). Therefore, we assessed the role of *Elf3* in regulating expression of cytokines that modulate Th2 response in airway epithelial cells. Thymic stromal lymphopoietin (TSLP) is a key cytokine secreted from epithelial cells that can drive a Th2 response (31, 32). However, TSLP mRNA expression was similar between cytokine-stimulated *Elf3*^{+/+} and *Elf3*^{-/-} airway epithelial cells, with no observable role of *Elf3* in regulating TSLP gene expression (Supplemental Fig. 3A, 3D, 3E). IL-25, another cytokine produced by airway epithelial cells (33) known to drive a Th2 response, had similar levels of expression in *Elf3*^{+/+} and *Elf3*^{-/-} airway epithelial cells (Supplemental Fig. 3B). However, IL-33, a known chemoattractant for Th2 cells and eosinophils, had higher levels of expression in *Elf3*^{-/-} airway epithelial cells compared with *Elf3*^{+/+} airway epithelial cells (Supplemental Fig. 3C). Furthermore, *Elf3*^{-/-} T cells were similar to *Elf3*^{+/+} T cells in their ability to differentiate into Th1 and Th2 lineages (Supplemental Fig. 1C–E). Taken together, these findings indicate that an induction of an exaggerated Th2 response observed in *Elf3*^{-/-} mice is unlikely to be dependent on *Elf3*^{-/-} airway epithelial cells or *Elf3*^{-/-} T cells.

*Increased maturation and migration of DCs in *Elf3*^{-/-} mice on OVA sensitization*

Because DCs play a central role in priming T cell responses, we assessed maturation and migration of pulmonary DCs from OVA-sensitized *Elf3*^{-/-} and *Elf3*^{+/+} mice upon challenge with FITC-labeled OVA after i.p. sensitization with OVA. To clearly identify pulmonary DCs, we first performed bronchoalveolar lavage to remove most of alveolar macrophages before isolation of cells from the lung. Moreover, gating characteristics were further used to clearly identify pulmonary DCs by excluding autofluorescent alveolar macrophages and gating on CD11c⁺CD11b⁺ DCs as described previously (26, 34). We focused on CD11c⁺CD11b⁺ DCs because this subset has been shown to be particularly critical for uptake of inhaled Ags, secretion of chemokines during OVA-induced allergic airway inflammation, and in stimulating CD4⁺ effector T cell response (35–38). The expression levels of CD86, CD80, MHC class II (MHC II), along with B7RP-1, which is known to drive a Th2 response (39, 40), were similar among pulmonary DCs from saline-treated *Elf3*^{+/+} and *Elf3*^{-/-} control mice. However, after OVA-FITC challenge of OVA-sensitized mice, the levels of DC maturation markers CD86, CD80, MHC II, as well as B7RP-1, were higher on pulmonary CD11c⁺CD11b⁺ OVA-FITC⁺ DCs in *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice (Fig. 5A). These results indicate that after airway OVA challenge, pulmonary DCs from OVA-sensitized *Elf3*^{-/-} mice underwent hypermaturation compared with pulmonary DCs from OVA-sensitized *Elf3*^{+/+} mice. Lungs of OVA-sensitized *Elf3*^{-/-} mice had significantly higher levels of CCL17, CCL22, and CCR4 mRNA expression compared with OVA-sensitized *Elf3*^{+/+} mice (Fig. 5B–D). CCL17 and CCL22 bind to chemokine receptor CCR4 on Th2 cells and recruit Th2 cells, thereby playing an important role in Th2-driven allergic inflammation (41, 42). Furthermore, CD86 mRNA levels were also elevated in the lungs of OVA-sensitized *Elf3*^{-/-} mice, which is also known to play a role

in driving a Th2 response (Fig. 5E) (43). Th2-associated markers such as CD28, CD4, GATA-3, ICOS, OX40L, and CD40Ig were also overexpressed in the lungs of OVA-sensitized *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice (Supplemental Fig. 4).

Next, we assessed migration of pulmonary DCs from the lung to the draining lymph nodes. In OVA-sensitized *Elf3*^{+/+} mice, after OVA-FITC airway challenge, ~8–10% of the CD11c⁺CD11b⁺ OVA-FITC⁺ DCs in the lungs were CCR7⁺, which increased to 20–23% in *Elf3*^{-/-} mice, demonstrating a heightened ability of *Elf3*^{-/-} pulmonary DCs to migrate to the draining lymph nodes (Fig. 5F). Basally, the proportions of CD11c⁺ DCs were similar in the draining lymph nodes of saline-treated *Elf3*^{+/+} and *Elf3*^{-/-} mice. However, following OVA sensitization and challenge, the proportion of DCs was much higher in the pulmonary draining lymph nodes of *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice (Fig. 5G). In *Elf3*^{+/+} mice, 15% of DCs that migrated from lungs to the lymph nodes were CD11c^{hi}, with the rest being CD11c^{low-int}, whereas in *Elf3*^{-/-} mice, ~29–31% of DCs that migrated from lungs to the lymph nodes were CD11c^{hi}, with the rest being CD11c^{low-int} (Fig. 5H). Most of these CD11c⁺ DCs were CD11b⁺, albeit with CD11b expression ranging from low to high. CD11c^{hi} DCs are classically the myeloid DCs that play a key role in priming T cell responses to the Ag in the draining lymph nodes (44). Furthermore, we also looked at the absolute counts of CD11c⁺ MHC II⁺ OVA-FITC⁺ DCs that migrated from the lungs to the draining lymph nodes both in control and OVA-sensitized mice (Fig. 5I). Basally, in control mice, similar numbers of DCs migrated from the lung to the draining lymph nodes. However, in OVA sensitized mice after OVA-FITC airway challenge, significantly higher numbers of DCs migrated from the lung to the pulmonary draining lymph nodes of *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice. OVA-stimulated lymph node cells from the pulmonary lymph nodes of OVA-challenged and -sensitized *Elf3*^{-/-} mice secreted significantly reduced levels of IL-12 than the cells from OVA-challenged and -sensitized *Elf3*^{+/+} mice (Fig. 5J). Together, the data indicate that following OVA sensitization and challenge, pulmonary DCs in *Elf3*^{-/-} mice undergo hypermaturation with an increased rate of migration to the lymph nodes. Furthermore, there is increased expression of Th2-inducing markers such as B7RP-1, CD86, CCL17, and CCL22, and reduced production of IL-12, which can cumulatively promote an exaggerated Th2 response.

**Elf3*^{-/-} DCs are impaired in driving Th1 differentiation, which promotes Th2 differentiation*

To assess whether intrinsic defects in *Elf3*^{-/-} DCs were responsible for exaggerated Th2 response, we tested for the ability of *Elf3*^{-/-} DCs to prime Th1 and Th2 differentiation. *Elf3*^{-/-} and *Elf3*^{+/+} bone marrow-derived DCs were stimulated with LPS and added to a culture of naive CD4⁺ T cells from *Elf3*^{-/-} and *Elf3*^{+/+} mice stimulated with CD3 and CD28 Abs. FACS analysis revealed that coculture of *Elf3*^{+/+} or *Elf3*^{-/-} T cells with *Elf3*^{+/+} DCs induced differentiation of ~8–10% of T cells into IFN-γ⁺ Th1 cells, with only 0.1–0.3% of the T cells differentiating into IL-4⁺ Th2 cells (Fig. 6A). In contrast, among T cells cultured with *Elf3*^{-/-} DCs, only 3–5% of the T cells differentiated into IFN-γ⁺ Th1 cells, but at the same time, 1–1.5% of the cells differentiated into IL-4⁺ Th2 cells. Furthermore, T cells cultured with *Elf3*^{-/-} DCs secreted significantly reduced levels of IFN-γ and significantly elevated levels of IL-4 compared with T cells cultured with *Elf3*^{+/+} DCs (Fig. 6B, 6C). Levels of Th1 or Th2 differentiation were similar for both *Elf3*^{+/+} and *Elf3*^{-/-} T cells, confirming no intrinsic T cell defect in *Elf3*^{-/-} mice. Taken together, these findings indicate that *Elf3*^{-/-} DCs displayed impairment in driving

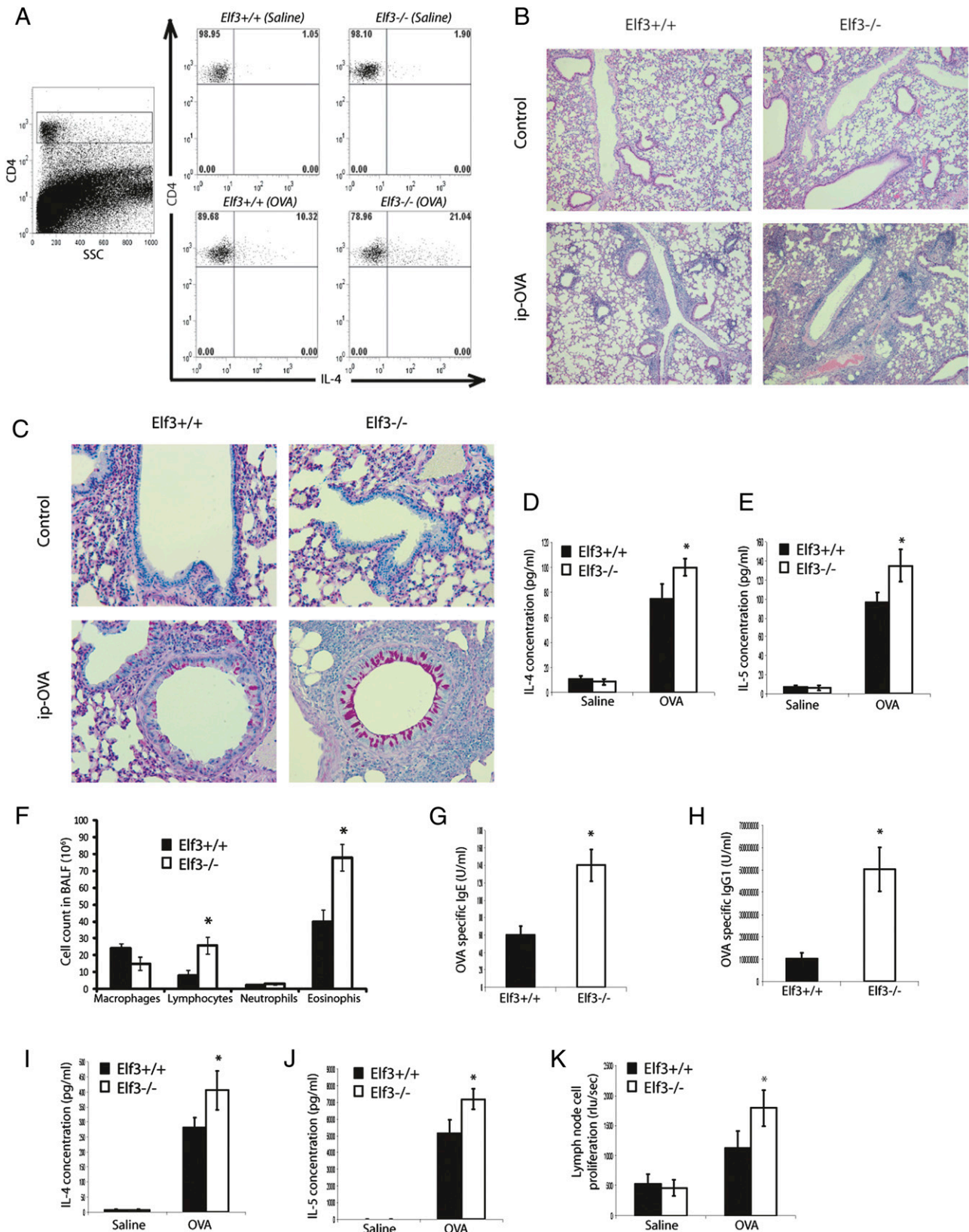


FIGURE 4. *Elf3*^{-/-} mice display an exaggerated Th2 response on OVA challenge after i.p. sensitization with OVA. *Elf3*^{+/+} and *Elf3*^{-/-} mice were sensitized i.p. with OVA and then challenged intranasally with OVA. *A*, Representative FACS plots of IL-4 production by CD4⁺ T cells from lungs of saline or OVA-sensitized mice after airway OVA challenge. *B*, Histopathological analysis of H&E-stained lung sections from saline-treated or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice after airway OVA challenge (original magnification ×50). *C*, Histological analysis of PAS-stained lung sections from saline-treated or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice after airway OVA challenge (original magnification ×200). *D* and *E*, Histogram comparing serum levels of (D) IL-4 and (E) IL-5 among saline-treated or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice after airway OVA challenge. *F*, Histogram comparing absolute counts of macrophages, lymphocytes, neutrophils, and eosinophils, identified by Giemsa-Wright staining, in the BALF of OVA-challenged and -sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice. *G*, Histogram comparing serum levels of OVA-specific IgE between OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice. *H*,

Th1 differentiation, whereas simultaneously promoting Th2 differentiation.

Elf3^{-/-} DCs undergo hypermaturation and are impaired in IL-12 production

To understand the underlying mechanism of how *Elf3^{-/-}* DCs could mediate induction of an exaggerated Th2, we focused our studies on characterizing DCs in *Elf3^{-/-}* mice during initial sensitization with OVA. Single i.p. injection of OVA-Alum led to higher numbers of DCs (identified as CD11c⁺MHC II⁺F4/80^{low} cells as described previously) (45) infiltrating the peritoneal cavity of *Elf3^{-/-}* mice compared with *Elf3^{+/+}* mice (Fig. 7A). Furthermore, after single i.p. injection of OVA-Alum, splenic DCs in *Elf3^{-/-}* mice expressed higher levels of MHC II and CD86, but not B7RP-1, compared with splenic DCs in *Elf3^{+/+}* mice (Fig. 7B). Because *Elf3^{-/-}* DCs underwent hypermaturation on a single injection of OVA-Alum, we hypothesized that *Elf3^{-/-}* DCs may have increased susceptibility to maturation compared with *Elf3^{+/+}* DCs. Consistent with our hypothesis, *Elf3^{-/-}* DCs generated from bone marrow cells showed higher expression of CD86, MHC II, and CD80 without any stimulation. Similarly, on stimulation, both with TNF- α along with IL-1 β or by LPS alone, upregulation of CD86, CD80, and MHC II was higher on *Elf3^{-/-}* DCs compared with *Elf3^{+/+}* DCs (Fig. 7C). We also looked at upregulation of mRNA for proinflammatory cytokines such as IL12p40, IL-12p35, and TNF- α (Table I), which are known to be induced in DCs following LPS stimulation (Fig. 7D–F). The induction of IL-12p35 and TNF- α was similar between LPS-stimulated *Elf3^{+/+}* and *Elf3^{-/-}* DCs. However, *Elf3^{-/-}* DCs were impaired in upregulating IL-12p40 gene expression on LPS stimulation (Fig. 7D). Next, we examined the production of IL-12 and TNF- α by *Elf3^{+/+}* and *Elf3^{-/-}* DCs upon stimulation with LPS (Figs. 7G, 7H). Although the secretion of TNF- α was similar between *Elf3^{-/-}* and *Elf3^{+/+}* DCs on LPS stimulation, secretion of IL-12 was significantly impaired from LPS-stimulated *Elf3^{-/-}* DCs compared with LPS-stimulated *Elf3^{+/+}* DCs. These findings confirm that *Elf3^{-/-}* DCs are intrinsically more susceptible to maturation but are impaired in IL-12 production, which is due to impairment of IL-12p40 mRNA induction in response to an inflammatory stimuli.

Discussion

In this report, we have identified a key function of *Elf3* in regulating airway allergic inflammation by regulating Th17 and Th2 immune response. We have shown that after epicutaneous sensitization followed by intranasal OVA challenge, *Elf3^{-/-}* mice mounted an impaired Th17 response, characterized by reduced CD4⁺IL-17⁺ cells in the lung, spleen, and the draining lymph nodes compared with sensitized *Elf3^{+/+}* mice. In accord with an impaired Th17 response, lower levels of IL-17 were identified in the serum and BALF of OVA-sensitized *Elf3^{-/-}* mice. Furthermore, the expression levels of IL-17 and Th17-specific transcription factor, ROR γ t, were also reduced in the lungs and the spleens of *Elf3^{-/-}* mice compared with *Elf3^{+/+}* mice, consistent with an impaired Th17 response. Cell composition analysis revealed that after OVA challenge, although BALF from sensitized *Elf3^{-/-}* mice had approximately twice the cell count compared

with sensitized *Elf3^{+/+}* mice, the neutrophil count was similar. In addition, as a proportion of the total cell count, the proportions of neutrophils in the BALF from sensitized *Elf3^{-/-}* mice were significantly reduced compared with sensitized *Elf3^{+/+}* mice after airway OVA challenge. These findings collectively indicated an impairment of neutrophil response in the airways of OVA-challenged and -sensitized *Elf3^{-/-}* mice, for if the response was similar, higher neutrophil counts are expected in BALF from *Elf3^{-/-}* mice compared with *Elf3^{+/+}* because of higher number of total cells in the BALF. Th17 response has previously been shown to promote neutrophil infiltration (46, 47). Therefore, it is likely that impairment of Th17 response in *Elf3^{-/-}* mice was responsible for the reduced proportion of neutrophils observed in BALF from OVA-challenged and -sensitized *Elf3^{-/-}* mice. In addition, *Elf3* has also been shown to regulate cox-2 expression in macrophages (13), and cox-2 has been shown to play a role in regulating IL-8 production from airway epithelial cells, which drives neutrophilic infiltration (48). Therefore, it is likely that lack of *Elf3* may also impair IL-8 production in the lungs, which can further limit the neutrophilic infiltration into the airways. We have identified an impairment of *Elf3^{-/-}* DCs in priming Th17 differentiation, attributable to an impairment of IL-6 production that was likely responsible for impaired Th17 response observed in *Elf3^{-/-}* mice after epicutaneous sensitization with OVA. Furthermore, impairment of IL-6 production from *Elf3^{-/-}* airway epithelial cells likely also contributed to an impairment of Th17 response.

IL-6 has previously been shown to promote Th2 differentiation by inducing IL-4 gene during CD4⁺ T cell differentiation and to suppress Th1 differentiation by inducing expression of SOCS-1 (49, 50). Therefore, impairment of IL-6 production from *Elf3^{-/-}* DCs and epithelial cells is expected to be associated with an impairment of Th2 response. Surprisingly, although pulmonary Th17 response to epicutaneous sensitization was impaired in *Elf3^{-/-}* mice, histological analyses revealed increased inflammatory response in the lungs of sensitized *Elf3^{-/-}* mice compared with sensitized *Elf3^{+/+}* mice after OVA airway challenge. In addition to the role of IL-6 in promoting Th2 differentiation, previous studies have also highlighted the anti-inflammatory role of IL-6 in regulating pulmonary inflammation. Wang et al. (51) showed that *IL-6^{-/-}* mice mounted an increased pulmonary inflammatory response compared with wild-type mice. Furthermore, transgenic mice overexpressing IL-6 in the airways under control of CC10 promoter had diminished airway inflammatory response (51). Therefore, it is likely that impaired IL-6 production from *Elf3^{-/-}* airway epithelial cells could contribute to aggravated pulmonary inflammation observed in OVA-sensitized and -challenged *Elf3^{-/-}* mice compared with *Elf3^{+/+}* mice. Furthermore, although the neutrophil proportions were reduced, the proportions of lymphocytes were elevated in BALF of epicutaneously sensitized *Elf3^{-/-}* mice compared with *Elf3^{+/+}* mice. Increased lymphocytic infiltration in BALF has previously been associated with an induction of Th2 response (27, 52). In addition, serum OVA-specific IgG1 and IgE levels were also elevated in *Elf3^{-/-}* mice, indicating an exaggerated Th2 response. Taken together, these findings indicate that after epicutaneous sensitization, although Th17 induction was impaired in *Elf3^{-/-}* mice, Th2 induction was likely exaggerated.

Histogram comparing serum levels of OVA-specific IgG1 between OVA-sensitized *Elf3^{+/+}* and *Elf3^{-/-}* mice. I–K, Pulmonary lymph node cells were harvested from saline-treated or OVA-sensitized *Elf3^{-/-}* and *Elf3^{+/+}* mice after airway OVA challenge and were subsequently stimulated in presence of OVA for 24 h. Shown are histograms comparing levels of IL-4 (I) and IL-5 (J) released into the medium. K, Histogram comparing levels of lymph node cell proliferation. All data are presented as mean $\pm$ SD, obtained from 12–14 mice per group, representative of 3 independent experiments. * $p < 0.05$, *Elf3^{-/-}* compared with *Elf3^{+/+}*.

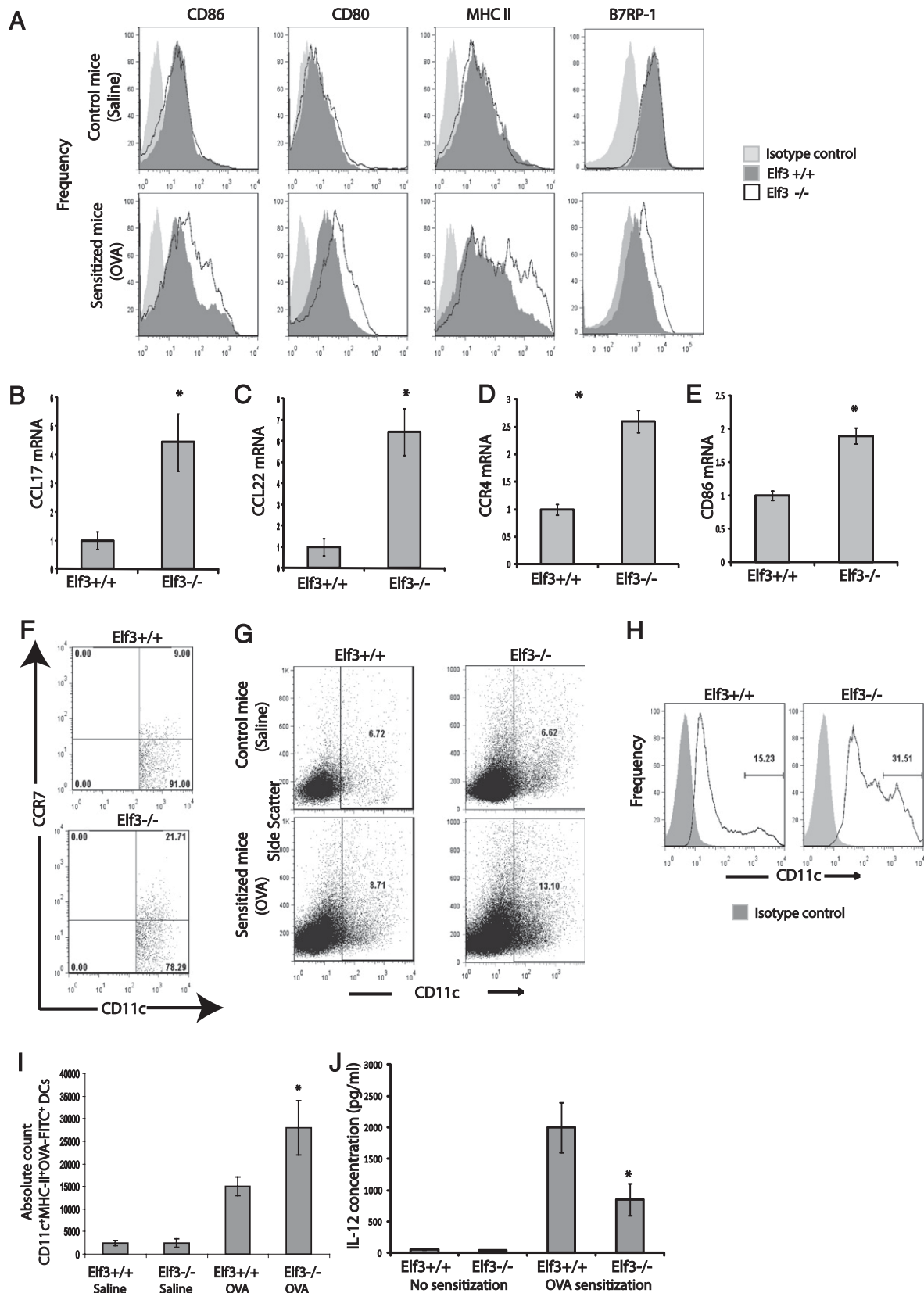


FIGURE 5. Increased DC maturation and migration is observed in *Elf3*^{-/-} mice on OVA challenge after OVA sensitization. *Elf3*^{+/+} and *Elf3*^{-/-} mice were sensitized i.p. with OVA and then challenged intranasally with OVA-FITC. **A**, Representative FACS histograms showing expression levels of CD86, CD80, MHC II, and B7RP-1 on CD11c⁺CD11b⁺OVA-FITC⁺ DCs in the lungs of OVA-sensitized or control *Elf3*^{+/+} and *Elf3*^{-/-} mice. **B–E**, Relative mRNA expression levels of CCL17 (**B**), CCL22 (**C**), CCR4 (**D**), and CD86 (**E**) in the lungs of OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice. **F**, Representative FACS plots examining expression of CCR7 on pulmonary CD11c⁺ CD11b⁺OVA-FITC⁺ DCs from OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice. **G**, Representative FACS plots of CD11c⁺ cells in the pulmonary lymph nodes of control or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice. **H**, Representative FACS histograms looking at CD11c expression on CD11c⁺MHC II⁺OVA-FITC⁺ DCs in the pulmonary lymph nodes of OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice. Indicated

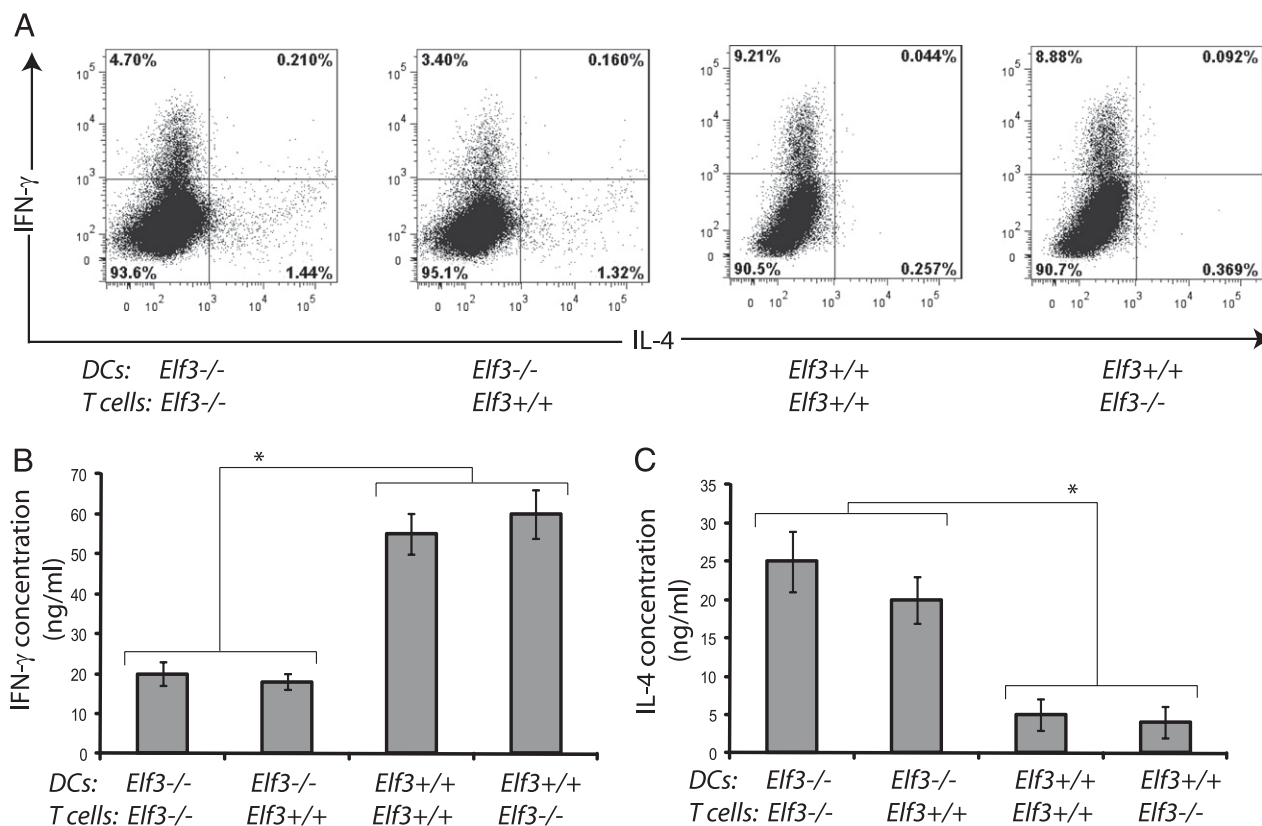


FIGURE 6. *Elf3*^{-/-} DCs are impaired in priming Th1 differentiation but promote Th2 differentiation on coculture with naive CD4⁺ T cells. *Elf3*^{+/+} and *Elf3*^{-/-} bone marrow-derived DCs were stimulated with LPS and then added to a culture of naive CD4⁺ T cells (CD4⁺CD25⁻CD62L⁺ T cells) from *Elf3*^{+/+} or *Elf3*^{-/-} mice stimulated with plate-bound anti-CD3 and soluble anti-CD28 Ab. Subsequently, T cells were harvested and stimulated with PMA/ionomycin. A, Representative FACS plots looking at production of IFN-γ and IL-4 by naive *Elf3*^{-/-} or *Elf3*^{+/+} CD4⁺ T cells cultured with *Elf3*^{-/-} or *Elf3*^{+/+} DCs stimulated with LPS. B, Histogram comparing levels of IFN-γ released by T cells into the media. C, Histogram comparing levels of IL-4 released into the media. All data are presented as mean ± SD, obtained from five independent experiments. **p* < 0.05, *Elf3*^{-/-} DCs compared with *Elf3*^{+/+} DCs.

Therefore, we used the model of i.p. OVA sensitization to explore Th2 response in *Elf3*^{-/-} mice. We have shown that upon i.p. sensitization followed by intranasal OVA challenge, *Elf3*^{-/-} mice mounted an exaggerated Th2 response, characterized by increased levels of CD4⁺IL-4⁺ cells in the lung compared with OVA-sensitized *Elf3*^{+/+} mice. Furthermore, the serum levels of OVA-specific IgG1 and IgE were much higher in sensitized *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice, along with increased inflammation and mucus production in the airways, confirming an induction of an exaggerated Th2 response in *Elf3*^{-/-} mice. IL-4 and IL-13 are Th2 cytokines that play a role in induction of allergen-induced mucus production in the airways (53, 54). Our findings identify increased IL-4 serum levels in OVA-sensitized *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice, which could mediate increased mucus production observed in the airways of OVA-sensitized and -challenged *Elf3*^{-/-} mice. In addition, mRNA levels of IL-13 were also elevated in the lungs of OVA-sensitized *Elf3*^{-/-} mice compared with *Elf3*^{+/+} mice, which could also contribute to increased mucus production (Supplemental Fig. 4).

Elf3 has previously been shown to be induced in airway epithelial cell lines during inflammation (12). Recent evidence indi-

cates that epithelial cells can play a very important role in orchestrating the differentiation of T cells (30, 33). Therefore, we hypothesized that lack of *Elf3* in airway epithelial cells may lead to increased production of cytokines that can drive an exaggerated Th2 response. However, we did not observe significant changes in expression of genes that can result in exaggerated Th2 response. TSLP, a key Th2-inducing cytokine produced from epithelial cells (31, 32), was not regulated by Elf3. However, IL-33 expression levels were higher in *Elf3*^{-/-} airway epithelial cells compared with *Elf3*^{+/+} airway epithelial cells. IL-33 is a chemoattractant for Th2 cells and can polarize naive T cells into IL-5-producing cells that do not produce IL-4 (55, 56). In our experiments, we identified exaggerated generation of IL-4-producing CD4⁺ T cells in *Elf3*^{-/-} mice. Because IL-33 cannot polarize T cells to secrete IL-4, it is likely that in our study IL-33 works as a chemoattractant. Furthermore, mRNA analysis of IL-33 expression in the lungs of OVA-challenged *Elf3*^{+/+} and *Elf3*^{-/-} mice revealed no significant difference, indicating that IL-33 likely did not play a major role in the induction of exaggerated Th2 response observed in *Elf3*^{-/-} mice (data not shown). The T cell costimulatory molecule ICOS has previously been shown to play an important role in driving a

proportions are those of CD11c^{hi} cells. I, Histogram comparing absolute count of CD11c⁺MHC II⁺OVA-FITC⁺ cells in the pulmonary lymph nodes of control or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice. J, Pulmonary lymph node cells were isolated from control or OVA-sensitized *Elf3*^{+/+} and *Elf3*^{-/-} mice and were stimulated. Shown is a histogram comparing levels of IL-12 released in the media. Data are presented as mean ± SD, obtained from 10–12 mice per group, representative of 3 independent experiments. **p* < 0.05, *Elf3*^{-/-} compared with *Elf3*^{+/+} (B–E), *Elf3*^{-/-} (OVA) compared with other groups (I, J).

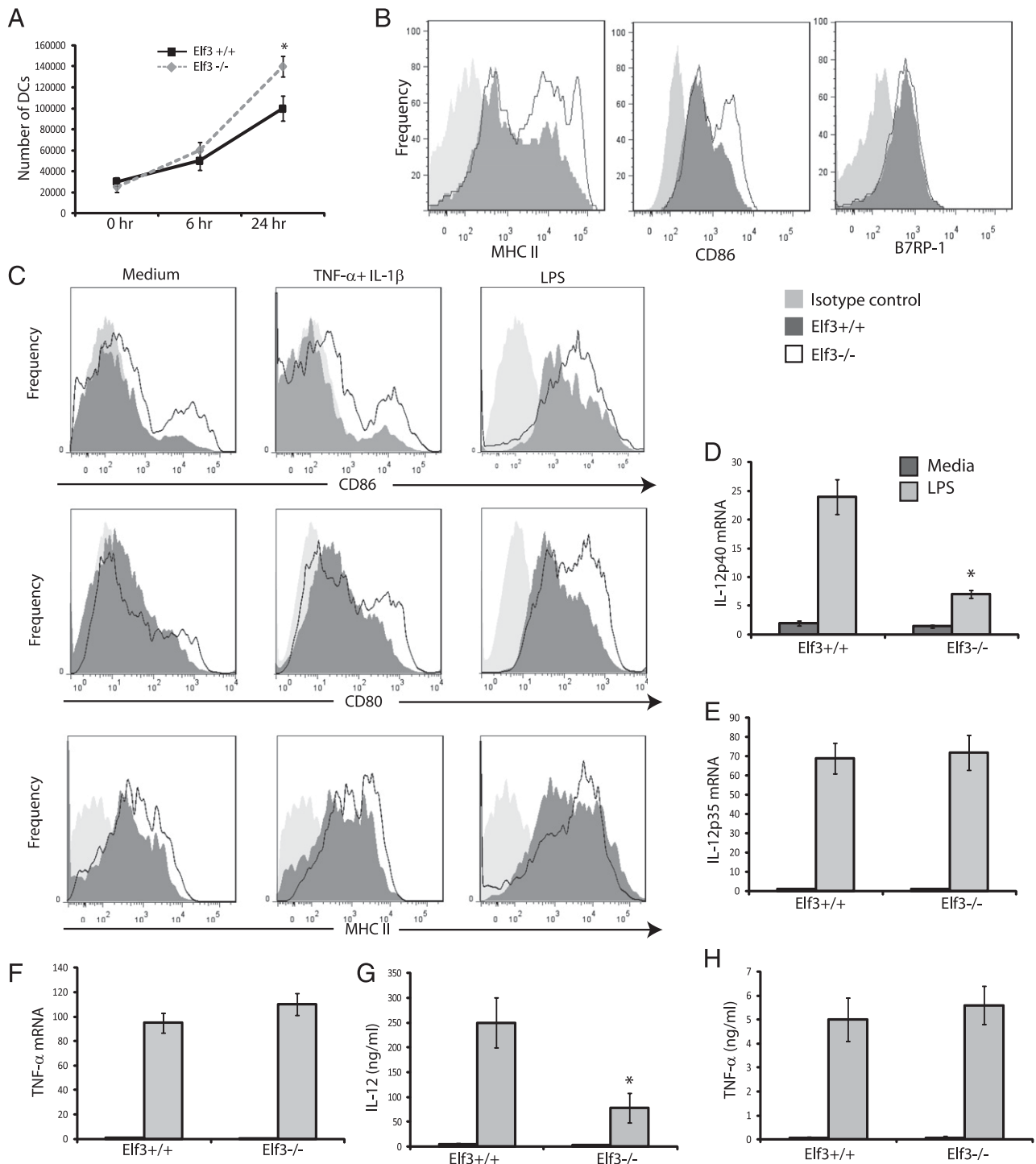


FIGURE 7. $Elf3^{-/-}$ DCs undergo hypermaturation but secrete lower amounts of IL-12 in response to inflammatory stimuli. **A**, Absolute DC count (CD11c⁺MHC II⁺F4/80^{low}) in the peritoneal lavage of $Elf3^{+/+}$ and $Elf3^{-/-}$ mice after a single i.p. injection of OVA-Alum. **B**, Representative FACS histograms looking at expression levels of MHC II, CD86, and B7RP-1 on peritoneal DCs on single i.p. injection of OVA-Alum to $Elf3^{+/+}$ and $Elf3^{-/-}$ mice. **C**, Representative FACS histograms looking at levels of CD86, CD80, and MHC II on $Elf3^{+/+}$ and $Elf3^{-/-}$ bone marrow-derived DCs at basal levels without stimulation or on stimulation with TNF- α with IL-1 β or with LPS. **D–F**, Histogram comparing mRNA expression levels of IL12p40 (**D**), IL12p35 (**E**), and TNF- α (**F**) in $Elf3^{+/+}$ and $Elf3^{-/-}$ DCs on stimulation with LPS. Levels of IL-12 (**G**) and TNF- α (**H**) released into the media on stimulation of $Elf3^{+/+}$ and $Elf3^{-/-}$ DCs with LPS. All data are presented as mean $\pm$ SD, obtained from 12–14 mice per group, representative of 5–6 independent experiments. * $p < 0.05$, $Elf3^{-/-}$ compared with $Elf3^{+/+}$.

Th2 response (40). $Elf3^{-/-}$ T cells were normal in their ability to differentiate into Th2 lineage and had similar propensity as $Elf3^{+/+}$ T cells to induce ICOS surface expression (data not shown).

The exaggerated Th2 response in $Elf3^{-/-}$ mice was characterized by increased maturation and migration of pulmonary DCs to

the draining lymph nodes. The migratory DCs were preferentially CD11c^{hi} in $Elf3^{-/-}$ mice, which have recently been shown to mediate priming of CD4⁺ T cells and subsequent induction of Th2 response (44). Moreover, higher expression of CCR7 on migratory DCs was also observed. It is likely that impairment of IL-12

production by *Elf3*^{-/-} DCs may mediate increased CCR7 expression on *Elf3*^{-/-} DCs because IL-12 blockade has been associated with upregulation of CCR7 (57). Although following OVA sensitization, B7RP-1 is downregulated on pulmonary DCs (58), the levels of B7RP-1 expression were higher on pulmonary *Elf3*^{-/-} DCs compared with *Elf3*^{+/+} DCs. We believe that this was a secondary effect of an exaggerated Th2 response and not a direct effect of lack of Elf3 in DCs, because after a single injection of OVA-Alum, although *Elf3*^{-/-} DCs underwent hypermaturation, *Elf3*^{-/-} and *Elf3*^{+/+} DCs had similar B7RP-1 levels. Moreover, upregulation of CCL17 and CCL22 observed in the lungs of OVA-sensitized *Elf3*^{-/-} mice was likely due to induction of an exaggerated Th2 response, because Th2 cytokines and Th2-dependent Ab responses are known to induce CCL17 (59) and CCL22 (60). However, following stimulation in vitro, *Elf3*^{-/-} DCs did not show elevated levels of CCL17 or CCL22 compared with *Elf3*^{+/+} DCs (data not shown). Further analysis revealed that *Elf3* plays a major role in DC function because in vitro-generated *Elf3*^{-/-} DCs were impaired in priming Th1 and instead preferentially primed Th2 differentiation. *Elf3*^{-/-} DCs underwent hypermaturation, but at the same time secreted diminished levels of IL-12, which led to an impaired Th1 response, resulting in a preferential Th2 response.

Elf3 belongs to the same subfamily as Elf5 and Ehf. Among these transcription factors, ESE-3, human homolog of Ehf, has previously been shown to regulate DC development (8). In addition to the role of Elf3 in regulating allergic airway inflammation, our findings also identify *Elf3* to play an important role in regulating DC maturation. Although in vitro-generated *Elf3*^{-/-} DCs undergo spontaneous maturation, DCs in *Elf3*^{-/-} mice have similar expression levels of maturation markers as their wild-type counterparts in absence of inflammatory stimuli. It is well accepted that DCs are highly prone to maturation and their manipulation in vitro can induce maturation (61, 62). It is possible that loss of *Elf3* results in generation of DCs that require a lower threshold of stimulation for maturation, whereby in vivo without stimulation, DCs do not undergo maturation. However, in vitro, the stress induced by culture conditions is likely to be sufficient to drive *Elf3*^{-/-} DC maturation, which is not the case with *Elf3*^{+/+} DCs.

In spite of hypermaturation, *Elf3*^{-/-} DCs are impaired in IL-12 secretion. IL-12 is a heterodimeric cytokine that induces IFN- γ -producing Th1 cells by activation of Stat4 (63). However, the impairment in IL-12 production by *Elf3*^{-/-} DCs is particularly due to impairment of IL-12p40 upregulation in *Elf3*^{-/-} DCs in response to inflammatory stimuli. In contrast with IL-12p40, the regulation of IL12p35 was normal in *Elf3*^{-/-} DCs as in *Elf3*^{+/+} DCs. IL-12 is known to suppress Th2 differentiation (64, 65), hence impairment of IL-12 production from *Elf3*^{-/-} DCs ends up driving a Th2 response. Furthermore, Th1 and Th2 differentiation balance each other and are mutually exclusive, whereby Th1 transcription factor T-bet suppresses Th2 commitment and Th2 transcription factor GATA-3 suppresses Th1 commitment (66, 67). Therefore, impairment of Th1 differentiation by *Elf3*^{-/-} DCs ends up driving Th2 differentiation.

The IL-12p40 gene product is expressed specifically in DCs and macrophages, and heterodimerizes with p19 to form IL-23. IL-23 synergizes with IL-6 to drive Th17 differentiation, and Th17 cells generated in the presence of IL-23 are more pathogenic than those generated with IL-6 and TGF- β (20, 68). Therefore, impairment of IL-12p40 upregulation in *Elf3*^{-/-} DCs may also impair IL-23 production, which coupled with IL-6 impairment could further impair the ability of *Elf3*^{-/-} DCs to induce Th17 differentiation.

Taken together, we have identified a role of Elf3 in regulating allergic airway inflammation by regulating DC-driven Th2 and

Th17 differentiation. Future studies will focus on examining the role of Elf3 in regulating lung function to identify whether Elf3 is implicated in asthma pathogenesis. Furthermore, we have also identified a role of Elf3 in regulating DC function. Although no studies have looked at the association of polymorphisms in ESE-1 (human homolog of Elf3) with autoimmune diseases, our findings indicate that ESE-1 polymorphisms may be associated with human disease. The imbalance in T cell polarization into various subsets plays a major role in human health and disease. Exaggerated Th2 response is commonly associated with asthma and allergy. Moreover, Th1 and Th17 cells play a key role in pathogenesis of a diverse group of immune-mediated diseases. Therefore, by identifying a role of Elf3 in regulating allergic airway inflammation, we are laying the framework for future studies that can further highlight the importance of this transcription factor in other immune-mediated diseases.

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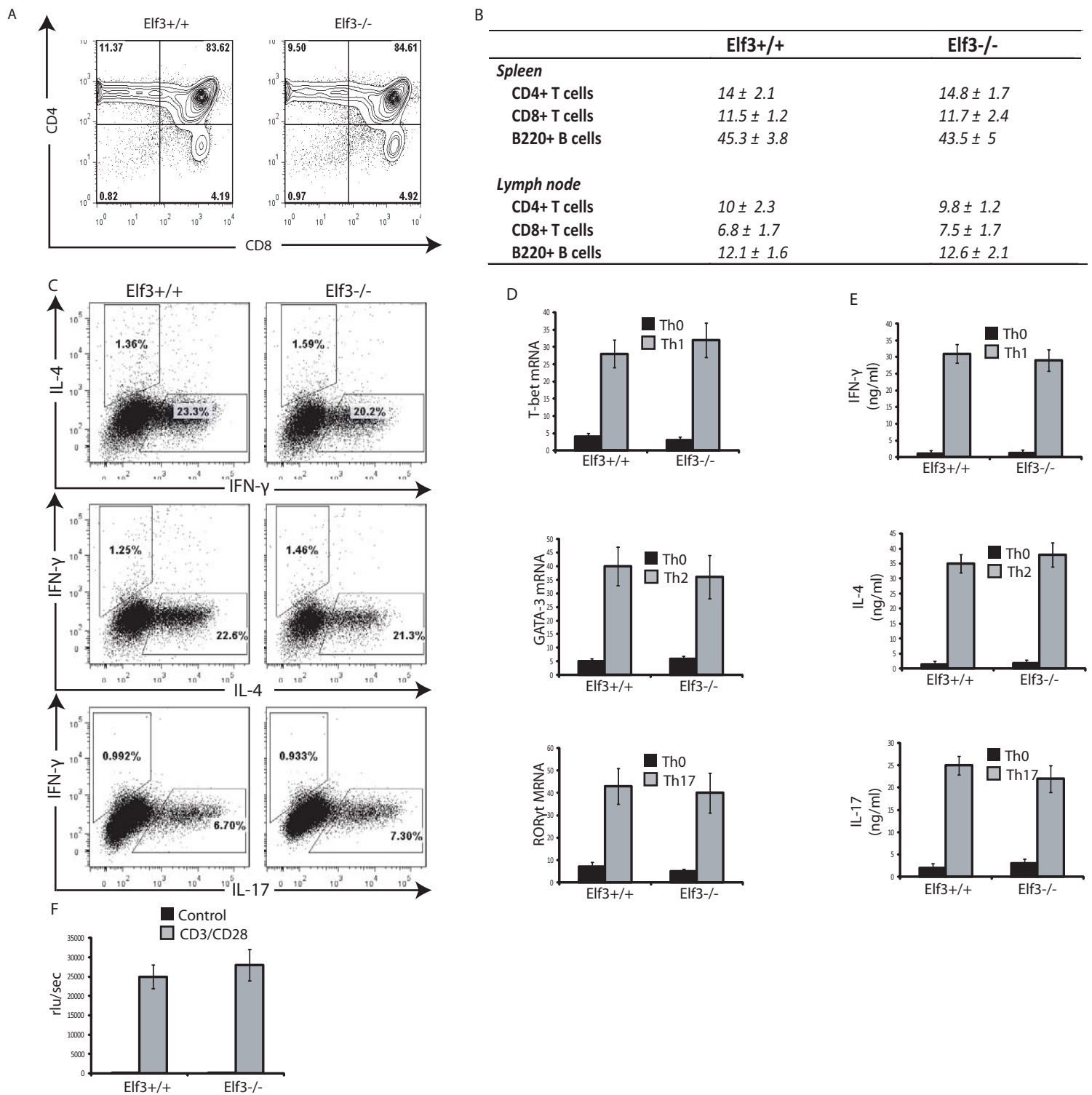
Disclosures

The authors have no financial conflicts of interest.

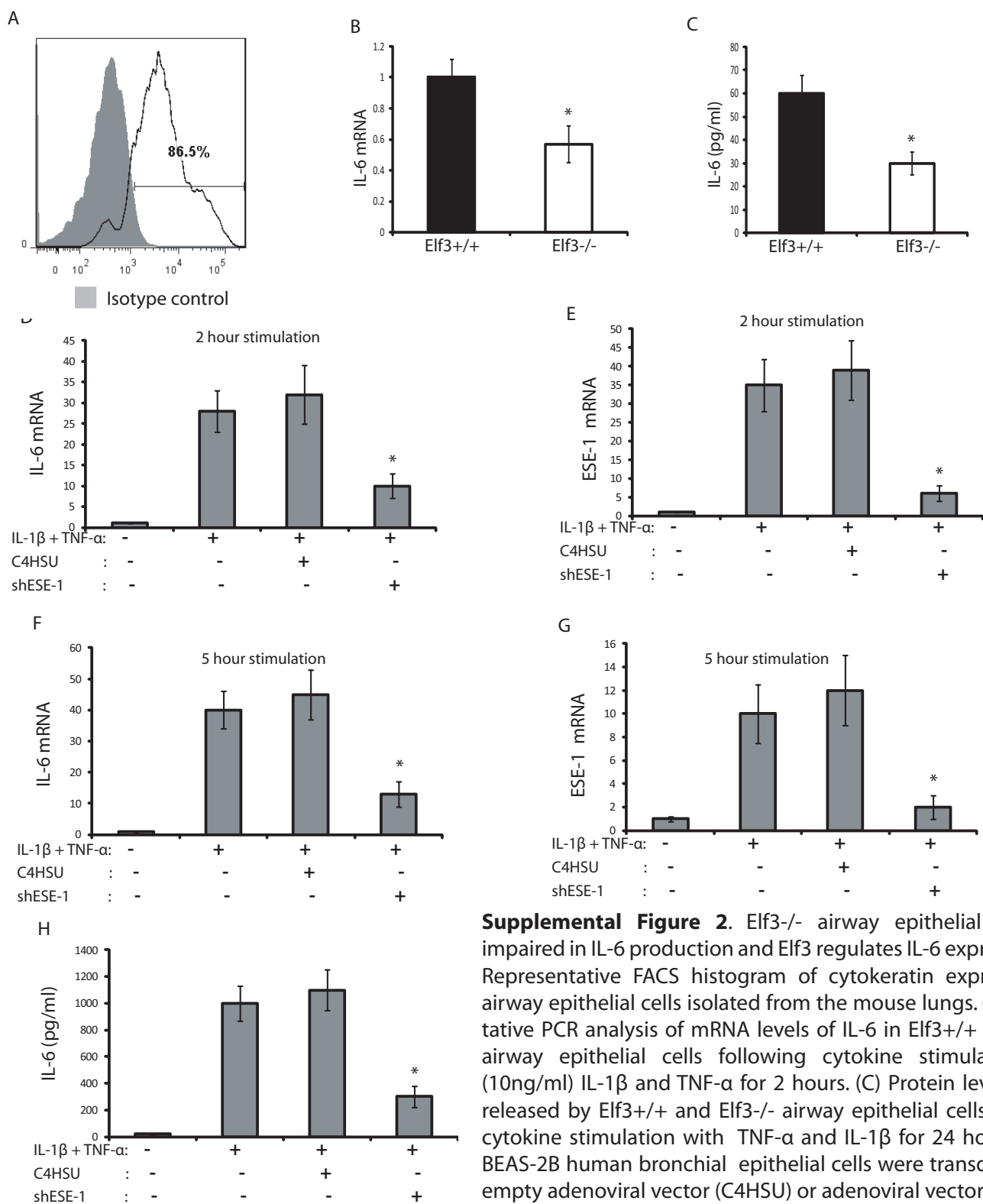
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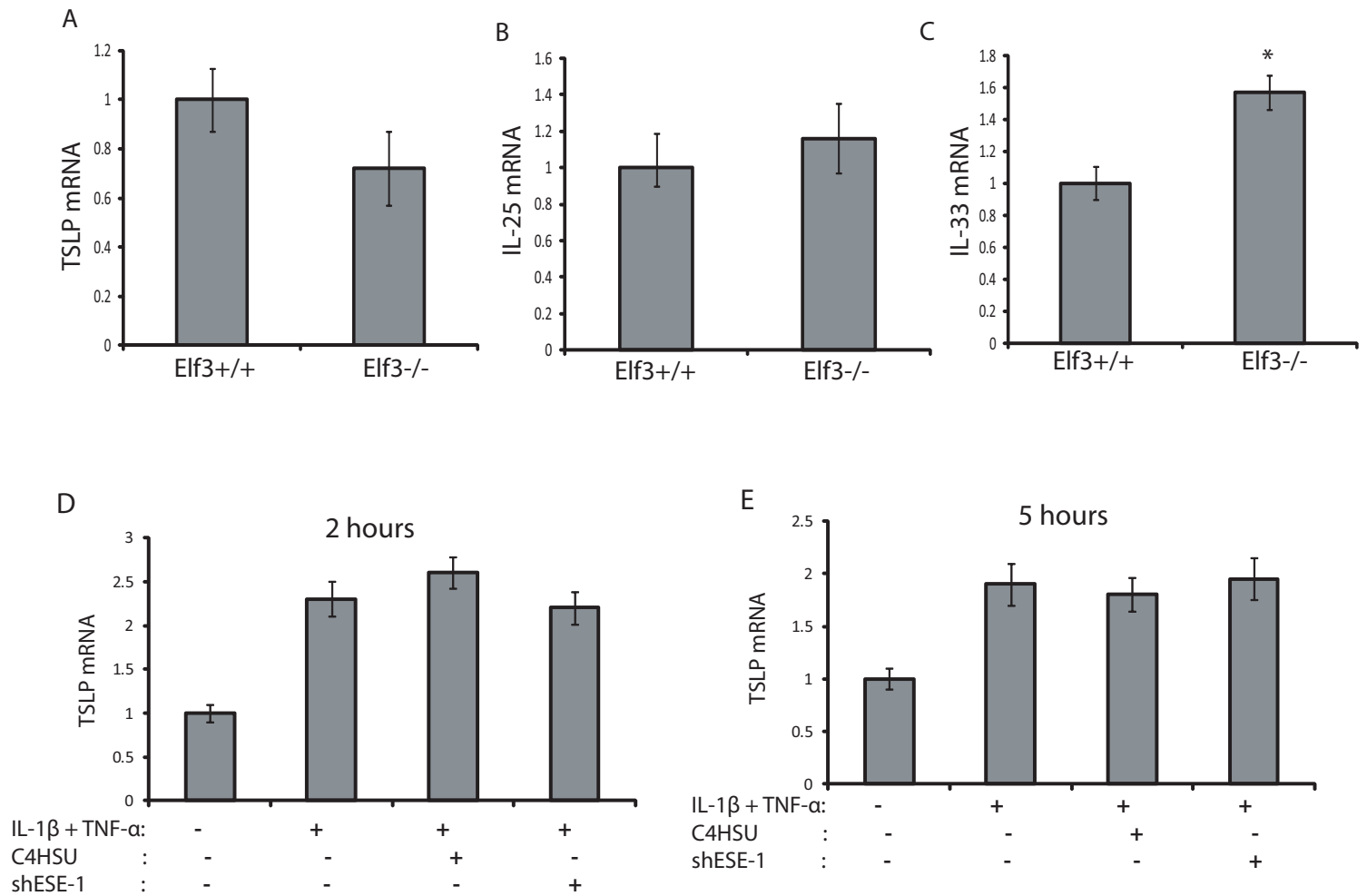
Supplemental Figure 1. T cell development, distribution and differentiation is normal in *Elf3*^{-/-} mice. (A) Representative FACS histograms showing expression of CD4 and CD8 on thymocytes isolated from *Elf3*^{+/+} and *Elf3*^{-/-} mice. (B) Distribution of CD4+ T cells, CD8+ T cells and B220+ B cells in the spleen and lymph nodes of *Elf3*^{+/+} and *Elf3*^{-/-} mice. (C) Representative FACS plots of proportions of IFN-γ+ , IL-4+ and IL-17+ cells generated upon culture of naive CD4+ T cells (CD4+CD25-CD62L+ T cells) from *Elf3*^{+/+} and *Elf3*^{-/-} mice under Th1, Th2 and Th17 polarizing conditions. (D) Real-time RT-PCR analysis of levels of T-bet, GATA-3 and RORγt mRNA expression among naive CD4+ T cells from *Elf3*^{+/+} and *Elf3*^{-/-} mice cultured under Th0, Th1, Th2 and Th17 polarizing conditions. (E) Levels of IFN-γ , IL-4 and IL-17 released by naive CD4+ T cells from *Elf3*^{+/+} and *Elf3*^{-/-} mice cultured under Th0, Th1, Th2 and Th17 polarizing conditions. (F) Histogram comparing levels of T cell proliferation upon stimulation of naive CD4+ T cells from *Elf3*^{+/+} and *Elf3*^{-/-} mice with an isotype control antibody or plate bound anti-CD3 with soluble anti-CD28 antibody. All data are presented as mean ± SD obtained from n=10 mice per group for A-B and representative of 4 independent experiments for C-F.



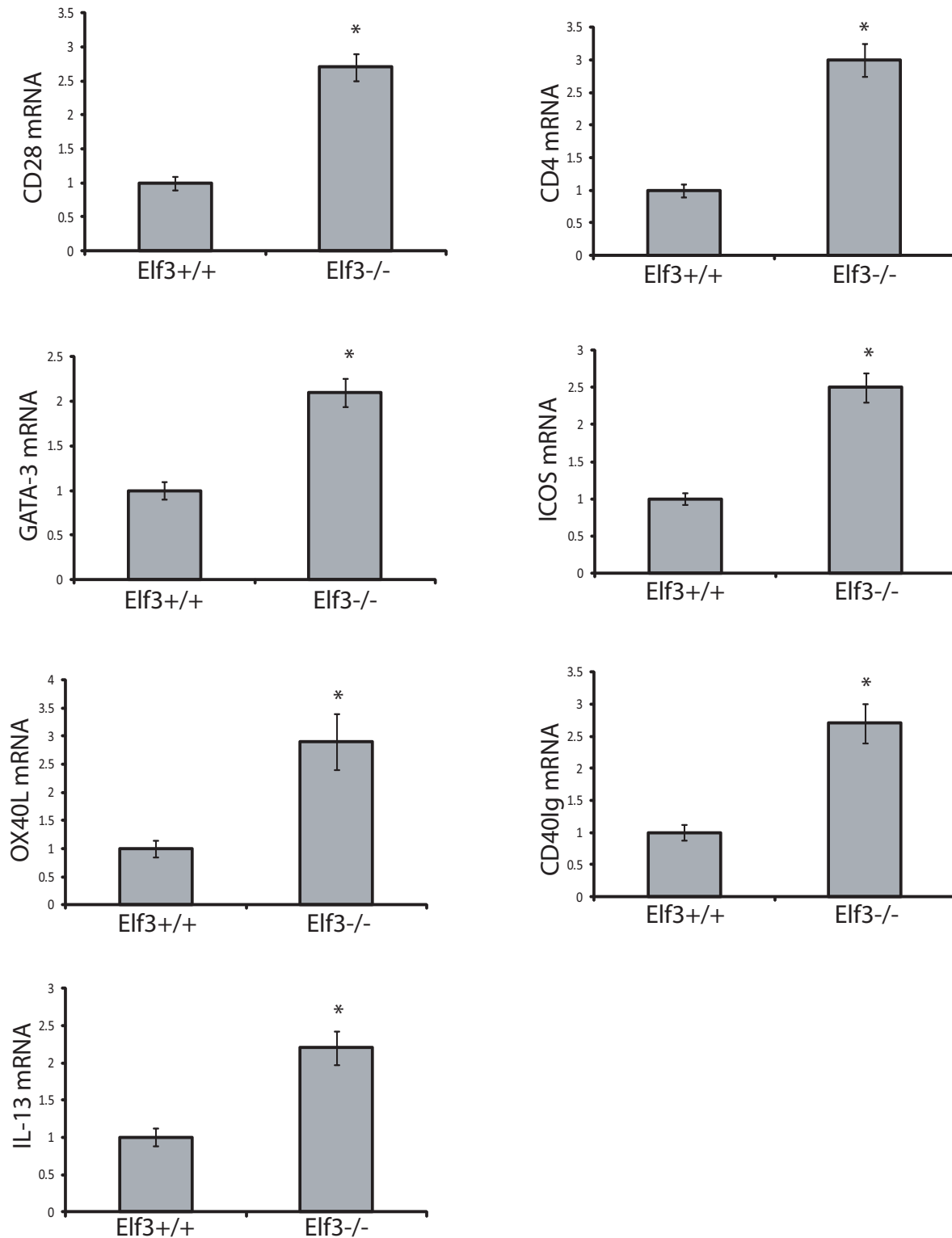
Supplemental Figure 2. Elf3^{-/-} airway epithelial cells are impaired in IL-6 production and Elf3 regulates IL-6 expression. (A) Representative FACS histogram of cytokeratin expression on airway epithelial cells isolated from the mouse lungs. (B) Quantitative PCR analysis of mRNA levels of IL-6 in Elf3^{+/+} and Elf3^{-/-} airway epithelial cells following cytokine stimulation with (10ng/ml) IL-1β and TNF-α for 2 hours. (C) Protein levels of IL-6 released by Elf3^{+/+} and Elf3^{-/-} airway epithelial cells following cytokine stimulation with TNF-α and IL-1β for 24 hours. (D-H) BEAS-2B human bronchial epithelial cells were transduced with empty adenoviral vector (C4HSU) or adenoviral vector encoding ESE-1 (human homolog of Elf3) shRNA (shESE-1) as described

(12) and were stimulated with IL-1β and TNF-α for 2 hours or 5 hours. Quantitative PCR analysis of mRNA levels of (D,F) IL-6 and ESE-1 (E,G) at 2 hours or 5 hours after stimulation. (H) Protein levels of IL-6 released 24 hours after stimulation. Results are expressed as fold induction relative to mRNA levels of indicated gene in unstimulated and untransduced cells. All data are presented as mean ± SD, representative of 3-4 independent experiments. *P < .05, compared to all other groups.

Supplemental method: Airway epithelial cell isolation and culture. Mouse lungs were perfused with 30 ml of 0.9% saline. Dispase (Promega) was instilled through the trachea, followed by instillation of agarose (Sigma). Lungs were immediately covered with ice. Subsequently, lungs were removed and incubated in 1 ml dispase for 45 minutes. Next, lungs were transferred to a 100mm culture dish containing DMEM with Dnase I, where the tissue was teased from the bronchi. The cell suspension was filtered progressively through 100 μm and then 40 μm cell strainer and then through nylon gauze. Cells were then plated on 100 mm culture plates coated with CD45 and CD32 antibodies (eBioscience) and incubated for 2-3 hours at 37 °C. Next, the non-adherent cells were cultured in plates coated with 80% Matrigel in DMEM (BD Biosciences). BEAS-2B, a cell line established from normal human bronchial epithelial cells (ATCC) was maintained in DMEM.



Supplemental Figure 3. Regulation of Th2 polarizing cytokines in Elf3^{-/-} airway epithelial cells. (A-C) Primary mouse airway epithelial cells were stimulated with IL-1 β and TNF- α , and mRNA levels of (A) TSLP, (B) IL-25 and (C) IL-33 were assessed by real-time RT-PCR. (D-E) ESE-1 (human homolog of Elf3) does not regulate TSLP expression in human airway epithelial cells. BEAS-2B human bronchial epithelial cells were transduced with empty adenoviral vector (C4HSU) or adenoviral vector encoding ESE-1 shRNA (shESE-1) and then were stimulated with IL-1 β and TNF- α for (D) 2 hours or (E) 5 hours. TSLP expression was quantified using real-time RT-PCR. Shown are relative expression levels of TSLP upon indicated treatments. All data are presented as mean $\pm$ SD, obtained from 8-9 mice per group for A-C or from 4 independent experiments for D-E. *P < .05, compared to other groups.



Supplemental Figure 4. Th2 associated genes are overexpressed in the lungs of Elf3^{-/-} mice compared to Elf3^{+/+} mice. Elf3^{+/+} and Elf3^{-/-} mice were sensitized intraperitoneally with OVA as described in materials and methods and were subsequently challenged intranasally with OVA. Following, OVA challenge, lungs were harvested and real-time RT-PCR was used for PCR array as described in materials in methods. Shown are histograms comparing expression levels of the indicated genes between Elf3^{+/+} and Elf3^{-/-} mice. All data are presented as mean +/- SD obtained from n=4-5 mice per group. *P<0.05, Elf3^{-/-} versus Elf3^{+/+}.