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Dynamic control of Th2 cell responses by STAT3 during allergic lung inflammation in mice

Hoyong Lim¹, Minkyong Cho¹, Garam Choi, Hyeongjin Na, Yeonseok Chung^{*}

Laboratory of Immune Regulation, Research Institute of Pharmaceutical Science, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea

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ABSTRACT

Signal transducer and activator of transcription (STAT) family molecules play essential roles during the differentiation of helper T cells from naïve precursors. Although the role of STAT3 in driving Th17 cell polarization has been well established, its role on Th2 responses to allergens remains incompletely understood. By employing T cell-specific STAT3 deficient mice, we demonstrate that STAT3 in T cells plays diverse role on Th2 cells depending on their locations in an animal model of allergic asthma. In the bronchial lymph nodes, STAT3-deficient T cells produced significantly reduced levels of Th2 cytokines. The frequencies of Th2 cells among CD4⁺ T cells in the lung were comparable between STAT3-sufficient and STAT3-deficient T cells. By contrast, STAT3-deficient T cells in the airway exhibited significantly enhanced production of Th2 cell cytokines compared to STAT3-sufficient T cells. Interestingly, a major population of IL-4/5 producers among STAT3-deficient T cells in the airway co-produced IFN γ . The frequency of Th17 cells was significantly diminished whereas that of Th1 cells was increased in all the lung-associated tissues. Our results demonstrate the dynamic and opposing roles of STAT3 during the development of Th2 cells from bronchial lymph nodes to the airway and propose the need of careful consideration on STAT3-targeting approaches for the treatment of lung diseases.

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1. Introduction

Allergic asthma is a heterogeneous inflammatory disease of the lung characterized by airway hyper-reactivity, mucus hyperplasia, remodeling of airway, and symptoms of recurrent wheezing and shortness of breath resulting from intermittent airway obstruction [1–5]. These pathological features are commonly attributed to activities of allergen specific helper T cell responses via the recruitment of inflammatory cells into the airway including eosinophils and neutrophils [1,2,6,7].

It has been well documented that Th2 cells mediate eosinophilic asthma while Th17 cells drive neutrophilic asthma by producing IL-5 and IL-17, respectively [6,8–14]. One of the most commonly used medications for asthma in humans is steroid due to its anti-inflammatory and immunosuppressive effects [15–18]. In particular, inhaled corticoids are found to be effective in controlling Th2 cell-driven eosinophilic asthma [19–21]. However, long term use of steroid can result in a number of side effects including fungal infection in the airway. Moreover, recent studies revealed that neutrophilic asthma is resistant to corticosteroid [21–25]. Therefore there is an urgent need to develop a therapeutic intervention for the treatment of asthma. In this regard, a number of approaches have been performed in order to inhibit Th2

pathway by blocking Th2 cytokines or their receptors in experimental animals and in humans [26–29]. So far, however, targeting Th2 immunity in clinical setting has been disappointing [25,30]. More recently, monoclonal antibodies against IL-17 or IL-17RA have been clinically tested in asthmatic patients with corticoid-resistant asthma, and found to have limited effects in controlling clinical symptoms [31,32]. Notably, these antibodies were proven to be effective in controlling other Th17 cell-mediated immune disorders such as psoriasis [21,33]. Hence blockade of either Th2 or Th17 cell response is likely not sufficient to suppress ongoing asthma.

A novel subset of helper T cells producing both IL-17 and IL-4 has been found in patients with chronic severe asthma [5,34]. Memory and effector T cells co-expressing the transcription factors GATA3 and ROR γ t and co-producing Th17 and Th2 cytokines have been reported in mice [35]. It will be important to test whether targeting Th2 and Th17 cell pathways simultaneously can ameliorate the symptoms of T cell-mediated allergic asthma.

After stimulation with their cognate antigens, CD4⁺ T cells can be differentiated into diverse effector subsets. Activation of STAT molecules by innate cytokines is essential for the differentiation of helper T cell subsets; STAT4, 6, 3 and 5 are indispensable for the polarization of Th1, 2, 17 and regulatory T cells from naïve T cells, respectively [1, 36–46]. Interestingly, a few recent studies proposed that STAT3 is also required for optimal Th2 cell differentiation through its binding to Th2 cell associated gene loci as well as via enhancing STAT6 activity in T cells [37,47].

^{*} Corresponding author.E-mail address: yeonseok@snu.ac.kr (Y. Chung).¹ These authors contributed equally to this work.

Based on these findings, one can surmise that STAT3 delivers intracellular signals crucial for both Th2 and Th17 cells. Accordingly, targeting STAT3 might be an effective therapeutic approach for inhibiting both helper T cell responses and therefore for the treatment of allergic asthma. In this study, we performed a series of experiments to define the role of STAT3 on pulmonary helper T cell responses against intranasal fungal allergens.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from Orient (Gyeonggi-do, Republic of Korea). OT-II and B6.SJL mice were purchased from Jackson Laboratory (Maine, USA). STAT3^{flox/flox}CD4-Cre mice were kindly provided by Drs. C. Dong (MD Anderson Cancer Center, USA) and S. Akira (Osaka University, Japan) and were crossed with OT-II to generate T cell-specific STAT3-deficient OT-II mice. All mice were maintained in the specific pathogen free facility at the vivarium of Seoul National University. All animal experiments were performed using a protocol approved by Institutional Animal Care and Use Committee Seoul National University (SNU-140602-2-2).

2.2. Allergen-induced lung inflammation

Mice were anesthetized with isoflurane and were intranasally administered with a mixture of 7 µg of proteinase extracted from *Aspergillus melleus* (Sigma, St Louis, MO) and 20 µg of OVA (Grade V, Sigma) (Asp/Ova) in 50 µl PBS every other day for a total of four times (days 0, 2, 4, and 6). Sixteen hours after the last challenge, all mice were euthanized and bronchial lymph nodes, lungs and bronchoalveolar lavage fluid were obtained for further analysis.

2.3. Adoptive transfer study

To examine the role of STAT3 on CD4⁺ T cells, naïve CD4⁺ T cells isolated from either STAT3^{flox/flox}CD4-Cre(+)OT-II or STAT3^{flox/flox}CD4-Cre(−)OT-II mice by CD4⁺ T cell isolation kit (Miltenyi Biotec, Germany) were adoptively transferred into B6.SJL mice. In some experiment, naïve CD4⁺ T cells from STAT3^{flox/flox}CD4-Cre(+)OT-II mice (CD45.2^{+/+}) and B6.SJL×OT-II mice (CD45.2⁺/CD45.1⁺) were mixed at 1:1 ratio before transferring them into B6.SJL mice (CD45.1^{+/+}). On the following day, the recipient mice were intranasally challenged with Asp/Ova and were analyzed as described above. Staining with anti-CD45.1 and anti-CD45.2 was used to distinguish each donor T cell population during flow cytometric analysis.

2.4. Flow cytometry

Cells obtained from mice were incubated for 4 h with PMA (100 ng/ml, Sigma-Aldrich) and ionomycin (1 µM, Sigma-Aldrich) in the presence of Brefeldin A and Monensin (all from eBioscience). Cells were washed with PBS containing 1.5% FBS (Invitrogen, Grand Island, NY), and stained with Pacific Blue-conjugated anti-CD45.1 mAb (A20, BioLegend), Pacific Blue or PerCP-Cy5.5-conjugated anti-CD45.2 (104, BioLegend) and PerCP-Cy5.5 or APC-Cy7-conjugated CD4 mAb (104 or GK1.5, BioLegend) for gating of CD4⁺ T cells. Cells were incubated in fixation buffer (eBioscience) for 20 min at 4 °C and washed with intracellular staining buffer (eBioscience). Intracellular cytokine staining was performed by using Alexa488 or brilliant 711-conjugated anti-IFNγ mAb (XMG1.2, eBioscience and BioLegend), PE or FITC-conjugated anti-IL-17A mAb (TC11-18H10.1, BioLegend), Alexa647-conjugated anti-IL-4 mAb (11B11, BioLegend) and APC-conjugated anti-IL-5 mAb (TRFK3, BioLegend), PE-conjugated anti-IL-13 mAb (eBio13A, eBioscience). Cells were analyzed by FACS Aria II flow cytometer (BD

Bioscience, San Jose, CA) and data were analyzed using FlowJo software (TreeStar, Ashland, OR).

2.5. Isolation of lymphoid cells from the lungs

We surgically removed the lungs and teased into small pieces by using gentleMACS™ dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and then digested them with RPMI 1640 medium containing 10% of FBS, 0.5 mg/ml of collagenase (Roche, Switzerland), 2 mg/ml of Dispase (Invitrogen), and 30 µg/ml of DNase I (Sigma-Aldrich) for 40 min at 37 °C. The lung cells were filtered (100 µm) then washed with PBS containing 1.5% FBS. Lymphoid cells were separated by density separation with lymphocyte separation medium (MP Biomedicals, Solon, OH) and were harvested from interphase.

2.6. Analysis of bronchoalveolar lavage (BAL) fluid

To obtain the BAL fluid from the mouse, the air lumen was washed twice with 0.7 ml of PBS containing proteinase inhibitor cocktail (GenDepot, Houston, TX) following trachea was cannulated. Obtained BAL fluid was centrifuged, supernatant was collected and IFNγ, IL-5, and IL-17 concentrations were determined by ELISA (all from BioLegend). Cells in the pellet were resuspended with PBS and attached 3000 of cells onto slide glass by cytospin, and were stained with Diff-Quik (Siemens, Newark, DE) for differential cell counts. We calculated absolute numbers of each cell population based on total cell counts.

2.7. Histology

Lungs were incubated in formalin for 12 h at 4 °C for fixation then embedded in paraffin. Thick (5 µm) sections were cut from paraffin embedded lung tissue using a microtome and stained with hematoxylin and eosin or with periodic acid Schiff (PAS) reagent according to the manufacturer's instruction, and cells were visualized by light microscopy (×20) (Carl Zeiss, Thornwood, NY) equipped with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

2.8. ELISA

To determine antigen-specific cytokine production from bLN cells, 1 × 10⁶ of cells were cultured in the presence of 0, 5, 25 µg/ml OVA for 3 days. The amounts of IFNγ, IL-4, IL-5, and IL-17 in cultured supernatant were measured by ELISA kit (all from BioLegend). All assays were done according to the manufacturer's instructions.

2.9. Th2 and Th17 cell differentiation in vitro

CD4⁺ T cells were isolated from either STAT3^{flox/flox}CD4-Cre(+)OT-II or STAT3^{flox/flox}CD4-Cre(−)OT-II mice by CD4⁺ isolation kit (Miltenyi Biotec, Germany) and then CD62L⁺CD25[−]CD44[−] cells were obtained by Aria III cell sorter (BD BioScience, USA). These naïve CD4⁺ T cells (1 × 10⁵/well) were co-cultured with BMDC (1 × 10⁴/well) in RPMI-1640 supplemented with 10% FBS and soluble anti-CD3 (0.5 µg/ml) (BD BioScience, USA) for 4 days. For Th2 or Th17 CD4⁺ T cell induction, cells were stimulated with either IL-4 (10 ng/ml) (PeproTech Inc., USA) plus IL-2 (10 ng/ml) (PeproTech Inc., USA) or LPS (100 ng/ml) (Sigma, USA) plus TGF-β (2 ng/ml) (PeproTech Inc., USA), respectively.

2.10. Quantitative real-time PCR

We obtained total RNA from the lung cells of allergen challenged mice or CD4⁺ T cells after in vitro differentiation using TRIzol (Invitrogen) and synthesized complementary DNA using amfiRivert reverse transcriptase (GenDepot). Levels of mRNA transcript were quantified with iTaq-SYBR Green Supermix (Bio-Rad Laboratories, Hercules,

CA) and the ABI-PRISM 7900 detection system (Applied Biosystems, Foster City, CA). Data were normalized to expression of the *Actb*. The primer pairs are listed in Supplementary Table 1.

2.11. Statistics

Data were analyzed with GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Statistics were calculated with the two-tailed Student's t-test or Mann–Whitney U test. *p* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Reduced expression of type 2 cytokines in STAT3-deficient T cells stimulated under Th2 cell differentiation condition

To investigate the role of STAT3 on helper T cell responses in asthma, we first utilized in vitro differentiation of Th2 and Th17 cells since they are known as pathogenic helper T cell subsets in allergic asthma. Naïve CD4⁺ T cells from CD4Cre(+)STAT3-floxed (referred as CD4^{STAT3}^{-/-}) mice, in which *Stat3* was selectively deleted in T cells, or STAT3-sufficient (CD4^{STAT3}^{+/+}) mice were co-cultured with dendritic cells in the presence of IL-4 or LPS plus TGF- β to induce Th2 or Th17 cell differentiation, respectively. As expected, STAT3-deficient T cells failed to become IL-17 producers (Fig. 1A). Similarly, STAT3-deficient T cells stimulated in a Th2 differentiation condition exhibited significantly lower frequency of IL-13 producers compared to STAT3-sufficient T

cells (Fig. 1B). This decrease in IL-13-producing T cells among STAT3-deficient T cells was likely not due to increased IFN γ since the frequency of IFN γ -producing T cells remained comparable between two groups (Fig. 1B), although they showed increased levels of *Ifng* and *Tbx21* transcript. Quantitative real-time PCR analysis revealed that all Th2 cell signature genes including *Gata3*, *Il4*, *Il5* and *Il13* were down-regulated in the absence of STAT3 in T cells (Fig. 1C), indicating that Th2 cell lineage program was hampered by STAT3-deficiency in T cells during in vitro differentiation.

3.2. Decreased Th2 cell responses in STAT3-deficient T cells in the bronchial lymph nodes upon intranasal allergens

To explore the role of STAT3 on pulmonary Th2 and Th17 responses in vivo, we employed an animal model of allergic asthma driven by repeated intranasal challenges of fungal proteinase allergen and ovalbumin (Asp/Ova). Our previous works showed that the intranasal challenges induce not only Th2 cell responses but also Th17 and Th1 cell responses [48,49]. We intranasally challenged CD4^{STAT3}^{-/-} mice and CD4^{STAT3}^{+/+} mice with Asp/Ova every other day for a total of four times. One day after the last challenge, we obtained lymphoid cells from the bronchial lymph nodes (bLN), stimulated them with ovalbumin ex vivo. Consistent with the well-known role of STAT3 on Th17 cell differentiation [43], we observed a significantly decreased amount of IL-17 in the supernatant of CD4^{STAT3}^{-/-} lymphoid cells compared with control (Fig. 2). Conversely, the amount of IFN γ was significantly higher in the former than the latter group, presumably due to the

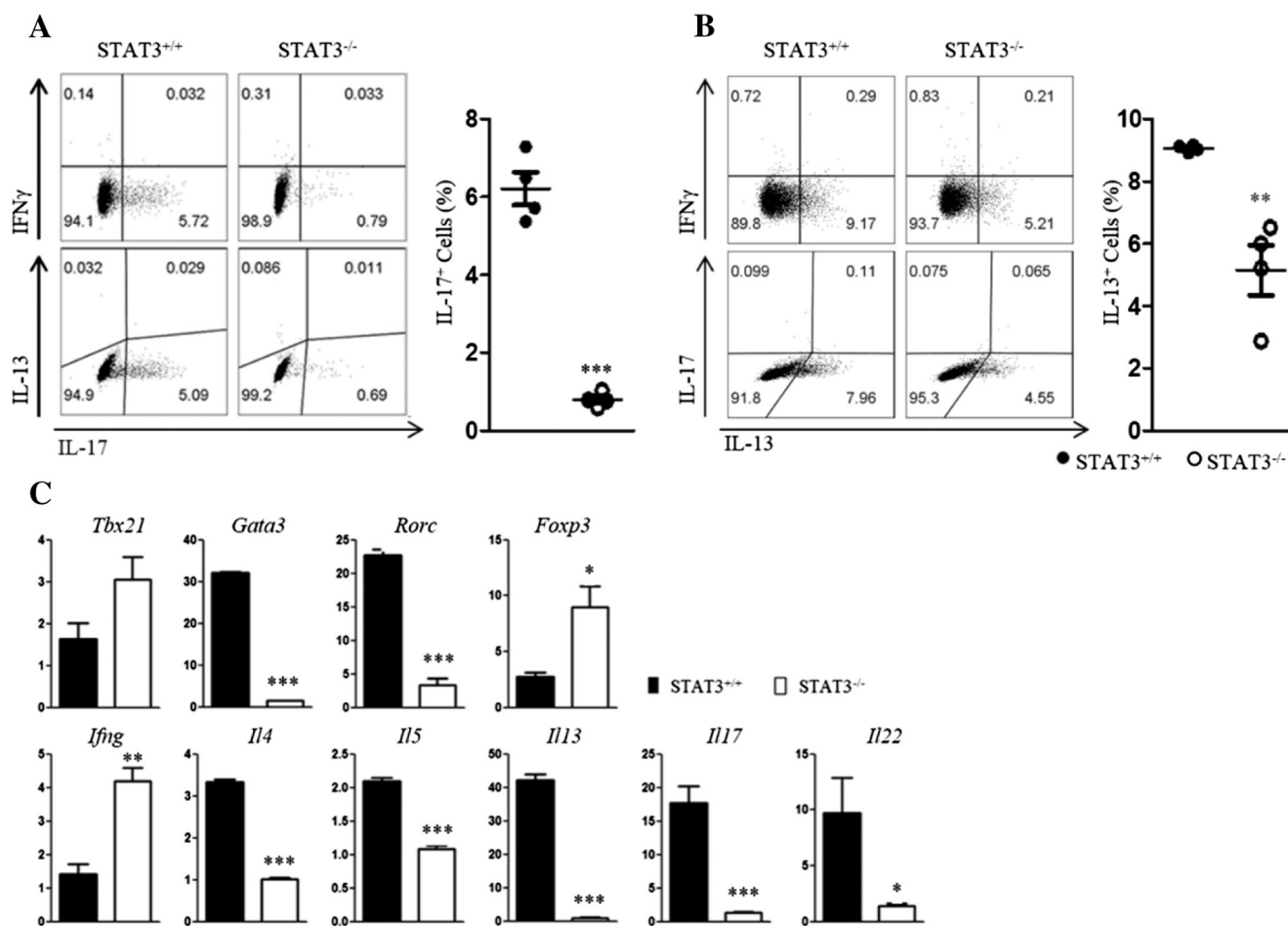


Fig. 1. Effects of STAT3-deficiency on the in vitro differentiation of Th17 cell and Th2 cell. FACS-sorted naïve CD4⁺ T cells from either CD4^{STAT3}^{+/+} or CD4^{STAT3}^{-/-} mice were co-cultured with bone marrow-derived dendritic cells for 4 days under Th17 (A) or Th2 (B & C) differentiation condition. Expression of IL-13, IFN γ and IL-17 by the cultured CD4⁺ T cells was measured by intracellular staining (A & B). mRNA levels of the indicated genes were measured by quantitative RT-PCR (C). **, *p* < 0.01 or ***, *p* < 0.001 in comparison with CD4^{STAT3}^{+/+} group.

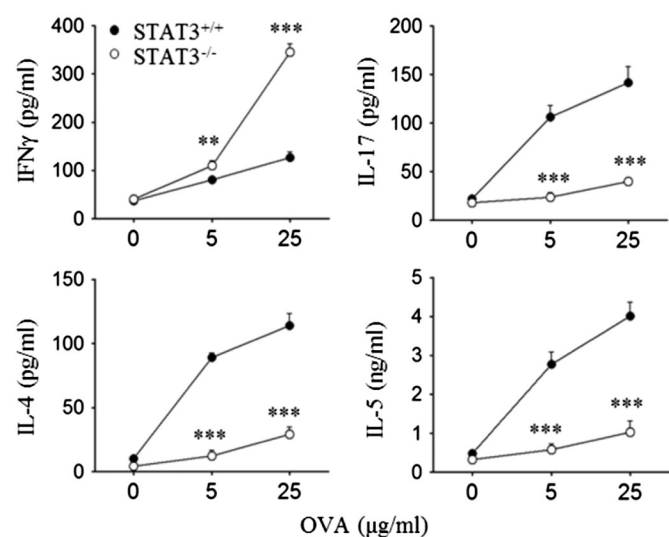


Fig. 2. Effects of STAT3-deficiency on cytokine production by T cells from the bronchial lymph nodes of mice challenged with intranasal allergens. Groups of CD4^{STAT3+/+} and CD4^{STAT3-/-} mice (n = 4–7) were intranasally injected with Asp/OVA every other days for four times. One day after the last challenge, lymphoid cells from the bronchial lymph nodes were restimulated with OVA for 3 days, and the amounts of indicated cytokines in the supernatant were measured by ELISA. Data are representative of three independent experiments. **, p < 0.01 or ***, p < 0.001 in comparison with CD4^{STAT3+/+} group.

inhibitory role of STAT3 on Th1 cell [38,43]. Consistent with the data in Fig. 1, the production of type 2 cytokines IL-4 and IL-5 was almost completely abolished in the CD4^{STAT3-/-} lymphoid cells (Fig. 2, lower panels). The observed decreased Th2 cell responses in the bLNs were, at least in part, consistent with a recent study [37,47]. Collectively, the

bLNs of CD4^{STAT3-/-} mice showed an almost complete decrease of both Th17 and Th2 cell responses whereas they exhibited increased Th1 cell responses to intranasal allergens.

3.3. Normal Th2 cell responses in STAT3-deficient T cells in the lung

We next comparatively analyzed helper T cell responses in the lung of CD4^{STAT3-/-} and CD4^{STAT3+/+} mice. As shown in Fig. 3, we observed a significantly increased IFNγ-producing CD4⁺ T cells among total CD4⁺ T cells (CD4^{STAT3+/+} vs CD4^{STAT3-/-}; 4.18 ± 0.468 vs 12.51 ± 1.086 , $p < 0.0001$) and decreased IL-17-producing CD4⁺ T cells (CD4^{STAT3+/+} vs CD4^{STAT3-/-}; 12.40 ± 0.757 vs 1.88 ± 0.194 , $p < 0.0001$), which correlated well with Th1- and Th17-cytokine profiles of the dLN in Fig. 2. Interestingly, although the Th2 responses of the dLN of CD4^{STAT3-/-} mice were significantly diminished, the frequency of IL-4 and/or IL-5-producing Th2 cell in the lungs was comparable between the two groups (CD4^{STAT3+/+} vs CD4^{STAT3-/-}; 4.65 ± 0.341 vs 3.75 ± 0.3640 , $p = 0.0832$) (Fig. 3A & B). In addition, a unique population of IFNγ and IL-4/5 co-producing CD4⁺ T cell was significantly increased (CD4^{STAT3+/+} vs CD4^{STAT3-/-}; 0.334 ± 0.045 vs 1.400 ± 0.141 , $p < 0.0001$) while that of IL-4/5 and IL-17 co-producing CD4⁺ T cells was decreased (CD4^{STAT3-/-} vs CD4^{STAT3+/+}; 1.017 ± 0.060 vs 0.281 ± 0.031 , $p < 0.0001$) in the lung of the former group (Fig. 3A & B).

To further characterize diverse immune aspects of the lungs, we measured the levels of mRNA transcript of genes related to helper T cell responses and lung inflammation. Consistent with increased frequency of Th1 cell and decreased frequency of Th17 cells, the mRNA level of *Ifng* was significantly increased while that of *Il17* was decreased in the lung of CD4^{STAT3-/-} mice. Interestingly, the levels of *Il4*, *Il5*, *Il10*, *Il13* and *Ccl11* (encoding eotaxin-1) were all remarkably decreased in the lung of CD4^{STAT3-/-} mice compared with CD4^{STAT3+/+} control. We also observed decreased levels of *Muc5ac* and *Clca3* in the former group, although *Clca3* did not reach statistical significance. The levels

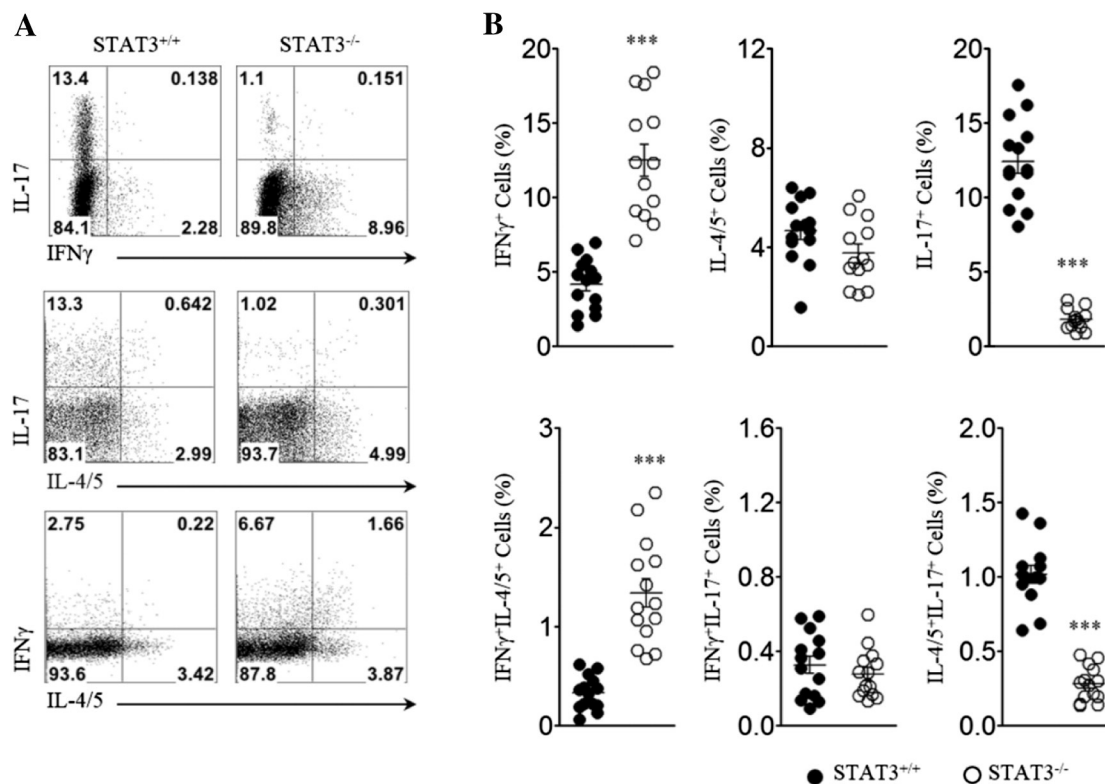


Fig. 3. Effects of STAT3-deficiency on cytokine production by T cells from the lungs of allergen challenged mice. Groups of CD4^{STAT3+/+} and CD4^{STAT3-/-} mice were intranasally injected with Asp/OVA for four times. One day after the last challenge, the expression of indicated cytokines by CD4⁺ T cells from the lungs was analyzed by intracellular staining after restimulation with PMA and ionomycin. Representative FACS plots were shown in A. Data are mean $\pm$ SD (B). Data are pooled from three independent experiments. ***, p < 0.001 in comparison with CD4^{STAT3+/+} group.

of cellular infiltration into the lung were comparable while those of mucus production from epithelial cells seemed slightly reduced in the $CD4^{STAT3-/-}$ mice (Fig. 4B). Thus, although the decrease in Th17 cell responses in the lung of $CD4^{STAT3-/-}$ mice was consistent with those seen in the dLN, the Th2 cell responses in the same mice were not defective in the lungs.

3.4. Enhanced Th2 cell responses in STAT3-deficient T cells in the bronchoalveolar lavage

The observed differences in Th2 cell responses between the bLNs and the lung prompted us to analyze helper T cell subsets in the bronchoalveolar lavage (BAL). Consistent with those in the bLNs and the lung, the frequency of IFN γ -producers among $CD4^+$ T cells in the BAL fluid was significantly increased in the $CD4^{STAT3-/-}$ mice ($CD4^{STAT3+/+}$ vs $CD4^{STAT3-/-}$; 14.17 ± 1.234 vs 53.41 ± 2.117 (%), $p < 0.0001$) while that of IL-17-producers was significantly decreased in the same mice ($CD4^{STAT3+/+}$ vs $CD4^{STAT3-/-}$; 19.35 ± 1.509 vs 3.75 ± 0.178 (%), $p < 0.0001$) (Fig. 5A & B). Importantly, we observed a significantly increased frequency of IL-4/5-producers among $CD4^+$ T cells in the BAL fluid of $CD4^{STAT3-/-}$ mice compared with that of $CD4^{STAT3+/+}$ mice ($CD4^{STAT3+/+}$ vs $CD4^{STAT3-/-}$; 21.26 ± 1.414 vs 36.73 ± 1.307 , $p < 0.0001$). To our surprise, while T cells producing IFN γ and IL-4/5 simultaneously were relatively rare in the STAT3-sufficient mice, more than half of Th2 cells in the $CD4^{STAT3-/-}$ mice co-produced IFN γ ($CD4^{STAT3+/+}$ vs $CD4^{STAT3-/-}$; 2.001 ± 0.237 vs 22.78 ± 1.089 , $p < 0.0001$) (Fig. 5B, lower panels). The frequency of IFN γ and IL-17 co-producing $CD4^+$ T cells was also slightly increased in the $CD4^{STAT3-/-}$ mice ($CD4^{STAT3+/+}$ vs $CD4^{STAT3-/-}$; 1.010 ± 0.086 vs 2.052 ± 0.160 , $p < 0.0001$). Taken together, these results demonstrate that STAT3-

deficiency significantly enhanced the expression of Th2 cytokines by $CD4^+$ T cells in the airway, even by IFN γ -producing T cells.

3.5. Cell-intrinsic role of STAT3 on pulmonary Th2 cells

Our results thus far showed that STAT3-deficiency led to defective Th2 cell responses in the bLN, but it remarkably enhanced Th2 cell responses in the airway. In addition to conventional $CD4^+$ T cells, $CD8^+$ T cells, NKT cells and some of the dendritic cells in $CD4^{STAT3-/-}$ mice are also deficient in STAT3 as they express CD4 during their ontogeny.

To rule out the involvement of STAT3 in non- $CD4^+$ T cells and to directly compare STAT3-sufficient and STAT3-deficient T cells in the same in vivo environment, we adoptively transferred 1:1 mixture of ovalbumin-specific naïve $CD4^+$ T cells from $CD4^{STAT3-/-}$ OT-II mice ($CD45.2^+/CD45.2^+$) or $CD45.1^+/CD45.2^+$ OT-II (STAT3-sufficient) mice into B6.SJL ($CD45.1^+/CD45.1^+$) congenic mice before the recipients were intranasally challenged with Asp/Ova. Although both STAT3-sufficient and STAT3-deficient OT-II T cells equally migrated into the secondary lymphoid organs (Fig. S1), the size of the latter population after intranasal Asp/Ova was significantly less than that of the former population (Fig. 6A). Compared with $CD4^{STAT3+/+}$ OT-II donor population, the frequencies of Th1 cells were consistently enhanced in the $CD4^{STAT3-/-}$ OT-II T cell population isolated from the bLNs, the lung as well as the BAL, while the opposite was true for Th17 cells (Fig. 6B–D). However, although the frequency of Th2 cells among $CD4^{STAT3-/-}$ OT-II T cell population was decreased in the bLN, it substantially increased in the BAL samples (Fig. 6B–D). We observed very similar results when each OT-II donor T cell population was separately transferred into B6.SJL recipient mice (Fig. S2). These results indicate that

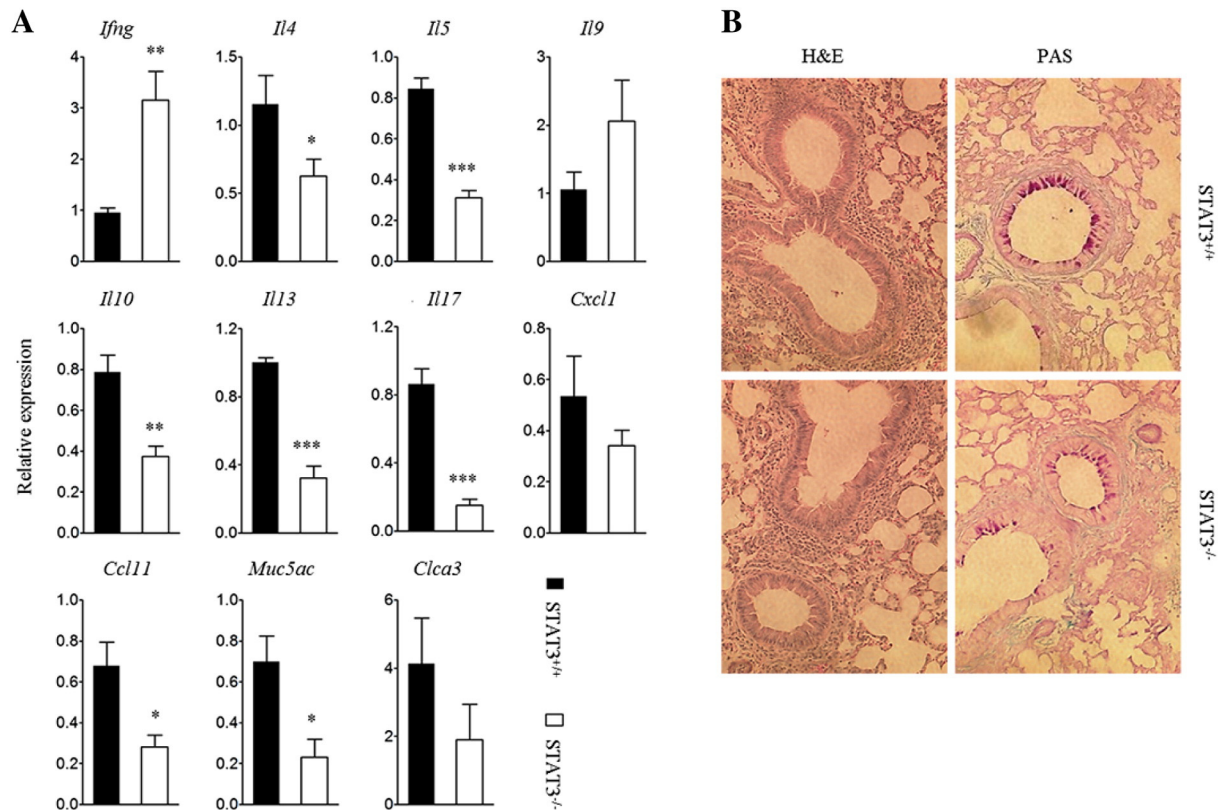


Fig. 4. Comparative analysis of asthma-related genes and lung histology between $CD4^{STAT3+/+}$ and $CD4^{STAT3-/-}$ mice. Groups of $CD4^{STAT3+/+}$ and $CD4^{STAT3-/-}$ mice were treated as described in Fig. 2, and the levels of indicated gene transcripts in the lung were determined by quantitative RT-PCR analysis (A). H&E and PAS staining of the lungs were visualized by light microscope ($\times 20$ magnification) (B). Data represent three independent experiments. *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$ in comparison with $CD4^{STAT3+/+}$ group.

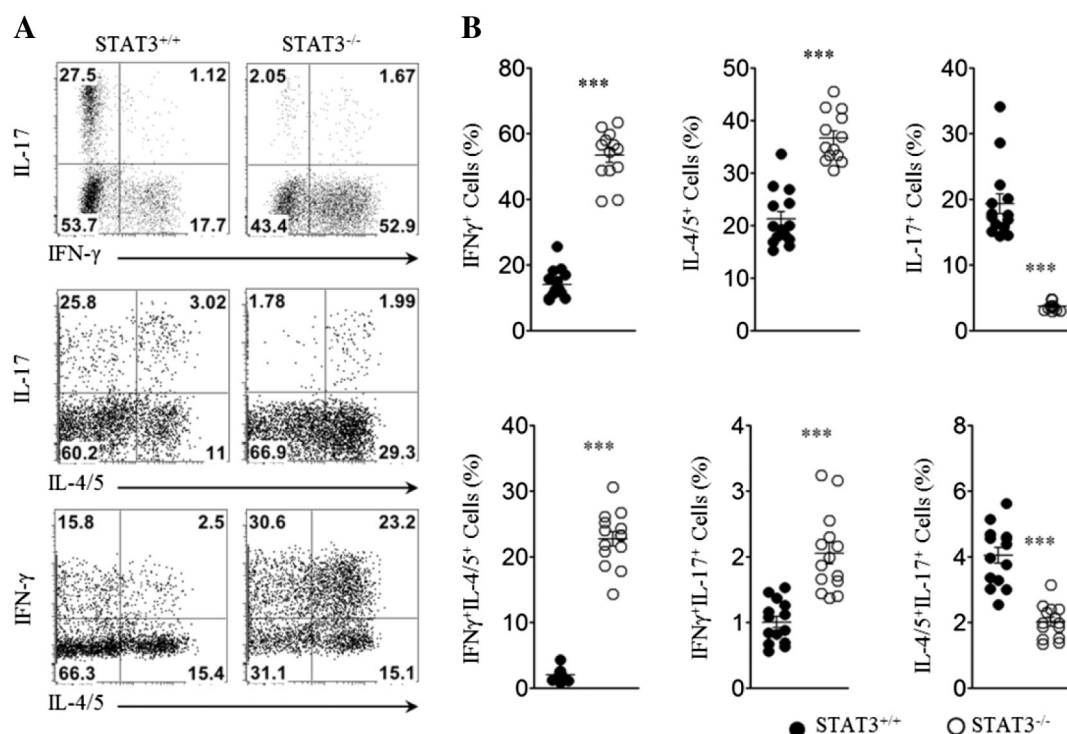


Fig. 5. Comparative analysis of CD4⁺ T cells in the bronchoalveolar lavage of allergen challenged CD4^{STAT3+/+} and CD4^{STAT3-/-} mice. Groups of CD4^{STAT3+/+} and CD4^{STAT3-/-} mice were treated as described in Fig. 2, and the expression of indicated cytokines by CD4⁺ T cells from the bronchoalveolar lavage was analyzed by intracellular staining. Representative FACS plots were shown in A. Data are mean $\pm$ SD (B). Data are pooled from three independent experiments. ***, $p < 0.001$ in comparison with CD4^{STAT3+/+} group.

the opposing effects of STAT3-deficiency on Th2 cell responses between the bLNs and the airway were cell-intrinsic.

4. Discussion

Understanding the functions of STATs on helper T cell immunity during the development of asthma is important not only because it will provide mechanistic insights into the pathogenesis of the disease, but also it may pave the development of therapeutics for the treatment of this pulmonary disease. In the present study, we aimed to delineate the role of STAT3 on pulmonary helper T cell responses in an animal model of allergic asthma, particularly focusing on Th2 and Th17 cells as they are the major pathogenic T cells in airway inflammation. Our results showed that T cell-specific STAT3-deficiency resulted in consistent increased Th1 cell responses and decreased Th17 cell responses in the bLN, and in the lung as well as in the BAL upon intranasal allergenic challenges. In addition, our results demonstrated that although allergen-specific Th2 cell responses were dramatically decreased in the bLNs of the T cell-specific STAT3-deficient mice, they were substantially enhanced in the BAL of the same mice. Adoptive T cell transfer studies convincingly showed that these opposing functions of STAT3 on Th2 cell responses were T cell-intrinsic rather than due to the changes of microenvironment as a result of STAT3-deficiency in neighboring cells. Our findings propose a previously unappreciated site-dependent control of Th2 cell differentiation and/or maturation by STAT3 during allergic lung inflammation.

Crucial contribution of STAT6 to the differentiation of Th2 cells has been clearly established [50–52]. In addition, a recent study reported STAT3 as another critical STATs required for optimal Th2 cell differentiation through direct binding to Th2 cell-associated gene loci and through facilitating STAT6 to bind its target genes [37]. Consistent with this finding, our results showed defective Th2 cell differentiation in STAT3-deficient T cells in vitro as well as almost completely diminished Th2 cell responses in the bLN of CD4^{STAT3-/-} mice after intranasal challenges with allergens in vivo. However, the defective Th2 cell

responses observed in the bLN of CD4^{STAT3-/-} mice were disappeared when we analyzed helper T cell population in the lung. Furthermore, the BAL cells from the CD4^{STAT3-/-} mice harbored significantly increased frequency of Th2 cells compared with STAT3-sufficient control mice. Hence STAT3 differentially regulates the expression of Th2 cell cytokines in T cells depending on the anatomic location during allergic lung inflammation. The reason for the discrepancy between the previous study and the present study is not clear, but it is possibly due to the type of allergens used and/or the differences in asthma induction protocols because the former study utilized intraperitoneal injection of alum-adsorbed ovalbumin and subsequent intranasal ovalbumin whereas we challenged mice with fungal proteinase plus OVA to trigger allergic lung inflammation. It is also possible that the reduced cellularity of allergen-specific T cells in the lung rather than defective Th2 cell responses resulted in diminished allergic inflammation. Compared with those in the bLNs where allergen-specific T cell activation begins, T cells found in the BAL are likely further differentiated T cells after multiple rounds of antigenic- and cytokine stimulation. Thus we propose that STAT3 is required for initial activation of Th2 cell program, but inhibits the production of Th2 cytokines at later stages of T cell differentiation. How STAT3 plays these opposing roles on Th2 cells while consistently inhibits or promotes Th1 and Th17 cell program, respectively, is unclear at the moment.

The clinical severity of asthma is correlated with the level of IL-17 in sputum and circulation of patients [53–55], and growing evidence suggests that IL-17 mediates neutrophilic lung inflammation [13,14]. Interestingly, a recent study has reported that IL-17-producing Th2 cells promote exacerbation of allergic asthma, and their findings highlight the plasticity of Th2 cells [35]. In STAT3-deficient T cells, we observed reduced frequency of IL-17-producing Th2 cells, most likely due to the defective expression of IL-17 in the absence of STAT3 signal. To our surprise, however, we repeatedly observed a significantly increased frequency of CD4⁺ T cells co-expressing IFN-γ and IL-4/5 in the CD4^{STAT3-/-} mice, especially in the BAL samples. More than 50% of IL-4/5-producing helper T cells were found to co-express IFN-γ

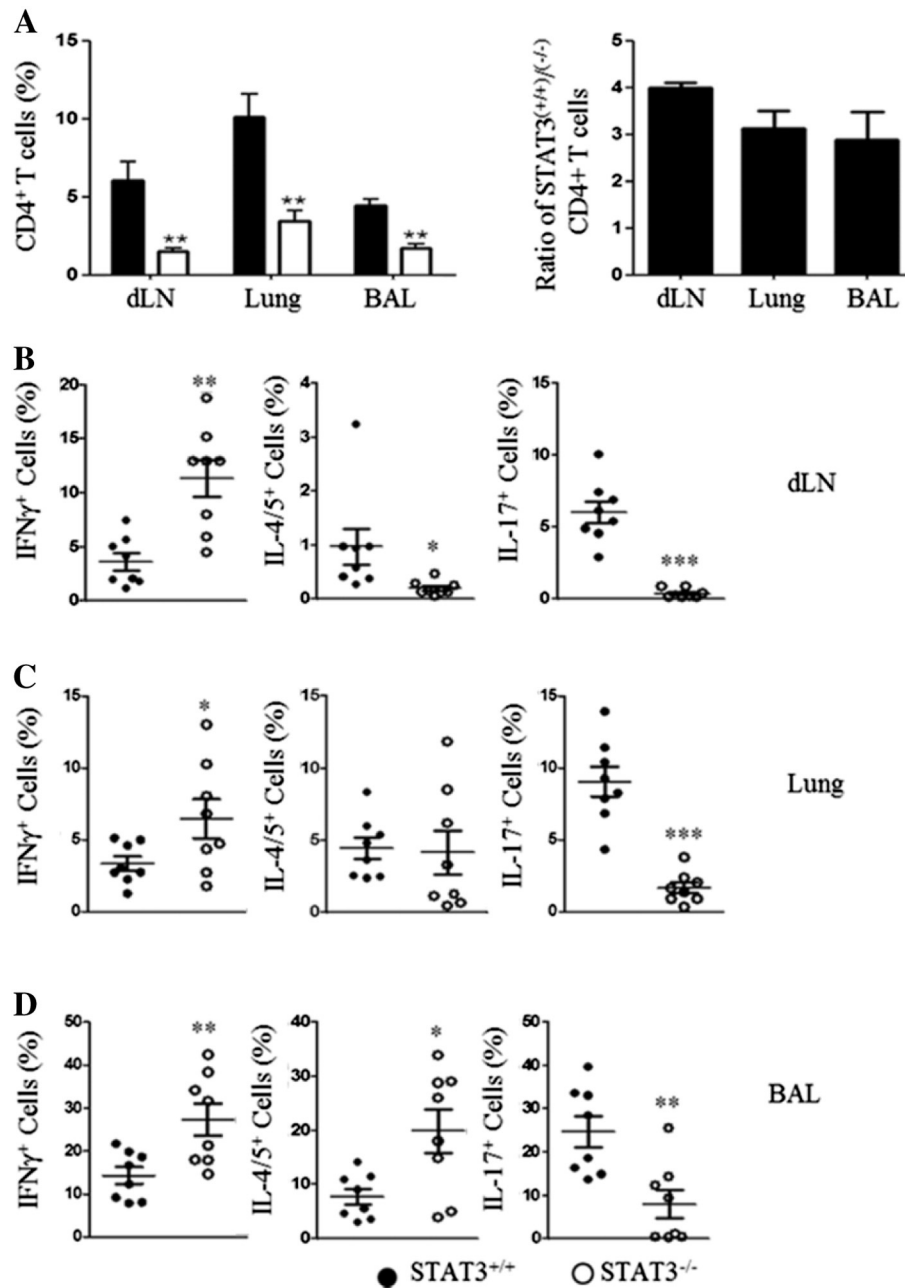


Fig. 6. T cell-intrinsic STAT3-deficiency differentially impacts on Th2 cell but consistently suppresses or increases Th17 cell and Th1 cell, respectively. CD4⁺ T cells isolated from STAT3-sufficient B6.SJL $\times$ OT-II (CD45.1⁺/CD45.2⁺) or CD4⁺STAT3^{-/-} OT-II mice (CD45.2⁺/CD45.1⁺) were mixed (1:1 ratio) and transferred into B6.SJL (CD45.1⁺/CD45.1⁺) congenic mice. The recipient mice were intranasally challenged with Asp/Ova every other day for four times. One day after the last challenge, lymphoid cells were isolated from the BAL fluid, lungs, and bronchial lymph nodes. The prevalence of each donor population was analyzed by CD45.1 and CD45.2 surface staining, and was shown as the percentages among CD4⁺ T cells (left panel) or as the ratio of STAT3-sufficient to STAT3-deficient donor T cells (right) (A). The frequencies of IFNγ⁺, IL-4/5⁺ and IL-17⁺ producers among each donor T cell population were determined by intracellular cytokine staining (B–D). Data are mean $\pm$ SEM and represent two independent experiments. *, $p < 0.05$ or **, $p < 0.01$ or ***, $p < 0.001$ in comparison with CD4⁺STAT3^{-/-} OT-II T cells.

simultaneously. These CD4⁺ T cells with ‘Th1–Th2 hybrid’ phenotype are conventional allergen-specific helper T cells since the same population was also observed when STAT3-deficient OT-II T cells were analyzed in our adoptive transfer studies. This is quite an unexpected finding since the strong antagonism of T-bet and GATA-3 has been well-established, and thus IFNγ-producers seldom produce Th2 cytokines and vice versa [56–58]. The development of these IFNγ-producing Th2 cells (or IL-4-producing Th1 cells) is negatively regulated by T cell-intrinsic STAT3 signals.

In summary, we found that cell-intrinsic STAT3 signals have diverse, and some time opposing, roles on the development of helper T cell subsets during allergen-induced lung inflammation. Our findings provide a

novel insight into the complexity in the mutual regulation of T helper subsets, and propose that careful considerations are required for the development of STAT3-targeting therapeutic approaches.

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Appendix A. Supplementary data

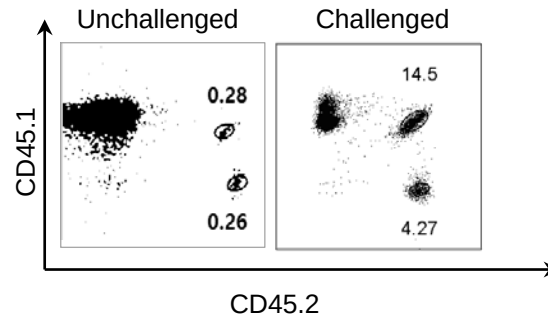
Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.intimp.2015.03.051>.

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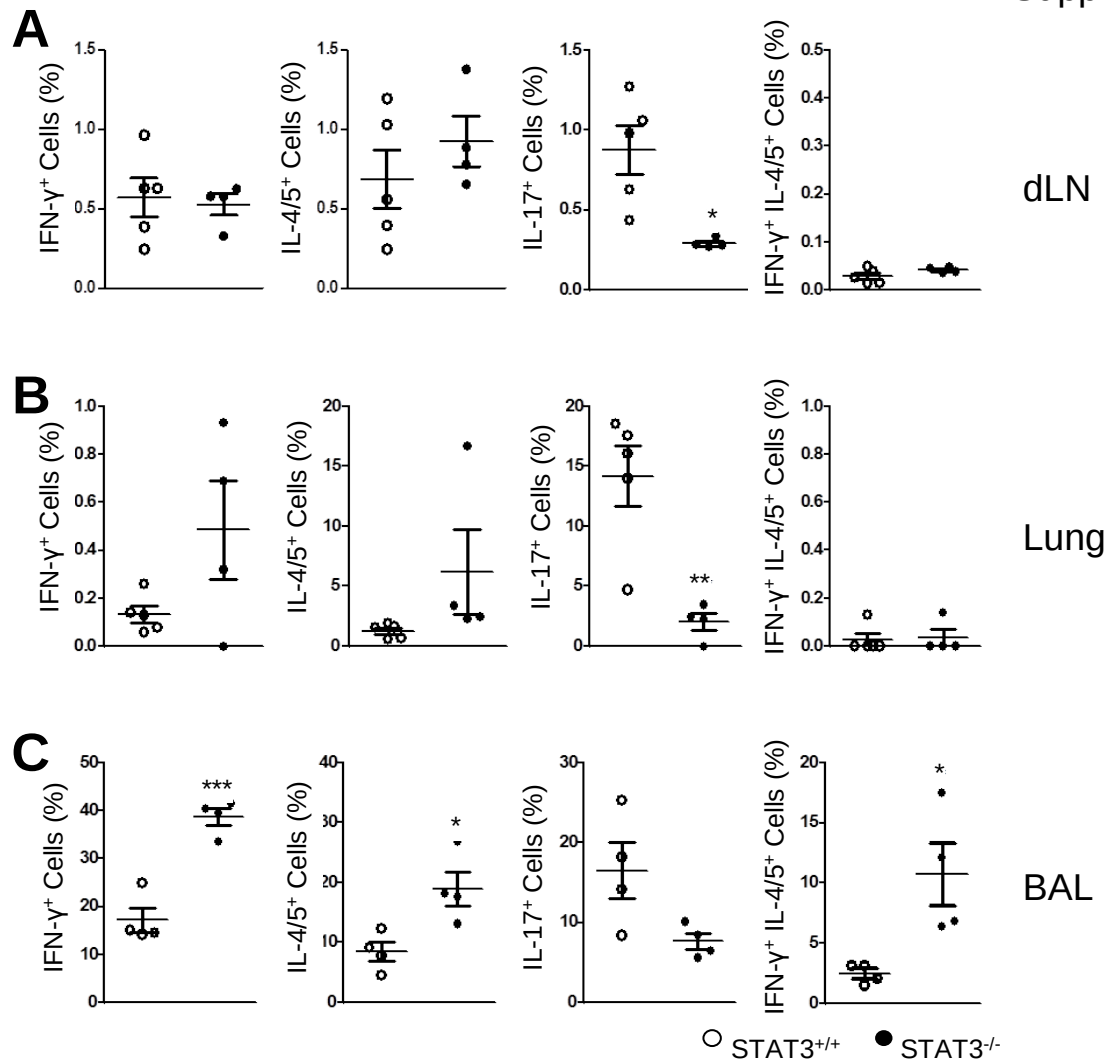
Supplementary Table 1. Primers for quantitative real-time PCR analysis

	Forward	Reverse
<i>Ifng</i>	5'-GATGCATTTCATGAGTATTGCCAAG-3'	5'-GTGGACCACTCGGATGAGCTC-3'
<i>Il4</i>	5'-AGATCACGGCATTTCGAACG-3'	5'-TTTGGCACATCCATCTCCG-3'
<i>Il5</i>	5'-CGCTCACCGAGCTCTGTTG-3'	5'-CCAATGCATAGCTGGTGATTTTT-3'
<i>Il9</i>	5'-GTGACATACATCCTTGCCCTC-3'	5'-GTGGTACAATCATCAGTT GGG-3'
<i>Il10</i>	5'-ATAACTGCACCCACTTCCCAGTC-3'	5'-CCCAAGTAACCCTTAAAGTCCTGC-3'
<i>Il13</i>	5'-GCTTATTGAGGAGCTGAGCAACA-3'	5'-GGCCAGGTCCACACTCCATA-3'
<i>Il17</i>	5'-CTCCAGAAGGCCCTCAGACTAC-3'	5'-GGGTCTTCATTGCGGTGG-3'
<i>Cxcl1</i>	5'-TGGCTGGGATTCACCTCAAGAACA-3'	5'-TGTGGCTATGACTTCGGTTTGGGT-3'
<i>Il22</i>	5'-CATGCAGGAGGTGGTACCTT-3'	5'-CAGACGCAAGCATTTCTCAG-3'
<i>Ccl11</i>	5'-ATTGT GTTGTGTTGTTTGCTTGC-3'	5'-GTCAGCCTGGTCTACACAGTGA-3'
<i>Muc5ac</i>	5'-CCATGCAGAGTCCTCAGA -3'	5'-TTACTGGAAAGGCCCAAG -3'
<i>Clca3</i>	5'-AATGATGAGCCCTACACCGAACA-3'	5'-AGTGAGCCCACTCA TGGACAAAG-3'
<i>Actb</i>	5'-TGGAATCCTGTGGCATCCATGAAAC-3'	5'-TAAAACGCAGCTCAGTAACAGTCCG-3'
<i>Tbx21</i>	5'-CAACAACCCCTTTGCCAAAG-3'	5'-TCCCCCAAGCAGTTGACAGT-3'
<i>Gata3</i>	5'-AGAACCGGCCCTTATGAA-3'	5'-AGTTCGCGCAGGATGTCC-3'
<i>Rorc</i>	5'-CCGCTGAGAGGGCTTCAC-3'	5'-TGCAGGAGTAGGCCACATTACA-3'
<i>Foxp3</i>	5'-GGCCCTTCTCCAGGACAGA-3'	5'-GCTGATCATGGCTGGGTTGT-3'



Supplementary Figure 1. Identification of donor T cell populations in an adoptive T cell transfer experiment.

CD4⁺ T cells isolated from STAT3-sufficient B6.SJL×OT-II (CD45.1⁺/CD45.2⁺) or CD4^{STAT3}^{-/-} OT-II mice (CD45.2^{+/+}) were mixed (1:1 ratio) and transferred into B6.SJL (CD45.1^{+/+}) congenic mice. The recipient mice were intranasally challenged with Asp/Ova every other day for four times. Lymphoid cells isolated from unchallenged or challenged mice were stained with anti-CD45.1 and anti-CD45.2 to identify the source of the donor T cells. Numbers indicate the percentages of each population among total CD4⁺ T cells.



Supplementary Figure 2. Expression of Th1, Th2 and Th17 cytokines by STAT3-deficient OT-II T cells in intranasal Asp/Ova challenged mice.

CD4⁺ T cells isolated from CD4^{STAT3+/+} OT-II or CD4^{STAT3-/-} OT-II mice (CD45.2^{+/+}) were transferred into B6.SJL (CD45.1^{+/+}) mice. The recipient mice were intranasally challenged with Asp/Ova every other day for four times. Lymphoid cells from the indicated organs or tissues were restimulated with PMA and ionomycin in the presence of brefeldin A and monensin, and the expression of IFN γ -, IL-4/5- and IL-17 by donor T cells were measured by intracellular cytokine staining. Data are mean $\pm$ SD. *, $p < 0.05$ or **, $p < 0.01$ or ***, $p < 0.001$ in comparison with CD4^{STAT3+/+} OT-II T cells.