약학석사학위논문

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알레르기성 폐 염증에서 STAT3를 통한 제2형 보조 T세포와 여포 보조 T세포 반응의 조절

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이 논문을 약학석사학위논문으로 제출함

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Abstract

Regulation of Th2 and Tfh cell responses during allergic lung inflammation via STAT3

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Understanding the developmental mechanisms of adoptive immunity against intranasal antigens is essential for the development of therapeutic approaches against air-borne pathogens as well as allergen-induced pulmonary inflammation. Allergic lung inflammation entails both humoral immunity and cell-mediated type

2 responses. T helper 2 (Th2) cells trigger the activation of IgE producing B cells, mast cells and eosinophils. Follicular helper T (Tfh) cells expressing CXCR5 are required for humoral immunity by producing IL-21 and ICOS costimulation to activated B cells. It has been established that signal transducer and activator of transcription 3 (STAT3) plays essential roles during the differentiation of the T helper 17 (Th17) cells from naïve helper T cells. However, its role on Th2 responses to allergens remains incompletely understood. By employing T cell-specific STAT3 deficient mice, we demonstrated that STAT3 in T cells plays diverse role on Th2 cells depending on their locations in animal models of allergic asthma. In mediastinal lymph nodes (mLNs), 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-suficient 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. STAT3 signal in T cells is known to mediate the generation of Tfh cells as well as Th17 cells. We found that administration of STAT3 inhibitor STA-21 suppressed the generation of Tfh cells and germinal center B cells in the mLNs against intranasal proteinase antigens. Compared with wild-type OT-II T cells, STAT3-deficient OT-II T cells transferred into recipients lacking T cells not only showed significantly reduced frequency Tfh cells, but also induced diminished IgG as well as IgE antibody production specific for the intranasal allergens. Co-transfer study of wild-type OT-II and STAT3-deficient OT-II T cells revealed that the latter failed to differentiate into Tfh cells. These findings demonstrate the dynamic and opposing roles of STAT3 during the development of Th2 cells from mLNs to the airway and requirement of STAT3

for the generation of Tfh cells to intranasal antigen in the mLNs; thus, these results propose the need of careful consideration on STAT3-targeting approaches for the treatment of lung diseases.

keywords : STAT3, intranasal allergen, Th2 cell, Tfh cell, germinal center reactions, immunoglobulin

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Introduction

Allergic asthma is the most frequent type of asthma and triggered by inhaled allergen. The symptoms of allergic asthma are difficulty in breathing, coughing, wheezing, chronic pulmonary eosinophilia, excessive airway mucus production and high IgE level in serum for sensitive response of bronchus against the foreign allergen. These pathological features are commonly attributed to activities of allergen specific helper T cell responses via the recruitment of inflammatory cells into the airway and the assistance of the production of allergen specific immunoglobulin by B cells.

Upon antigenic stimulation, naïve $CD4^+$ T cells can be differentiated into at least 5 distinct effector T cells including T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), follicular helper T (Tfh), and regulatory T (Treg) cells depending on microenvironmental cytokine milieu [1, 2, 3]. T cell precursors that interact with antigen-presenting cells (APCs) differentiate into allergen-specific Th2 cells and contribute to IL-5 mediated promotion of eosinophil recruitment, IgE production by B cells and allergic response induced by activation of mast cells. It has been documented that allergen-specific Th17 cells mediate neutrophilic asthma by producing IL-17A and IL-17F [4]. Among $CD4^+$ T cells, Tfh cells are known to play a crucial role in facilitating germinal center reactions by providing IL-4 and IL-21 as well as ICOS costimulation to B cells. These 'helps' from Tfh cells trigger the survival and proliferation of B cells, isotype-switching of immunoglobulin, affinity maturation of immunoglobulin, and the differentiation of plasma cells and

memory B cells from activated B cells [5, 6, 7]. Cytokines from innate immune cells including IL-6, IL-12 and IL-27 have been identified to induce the initial differentiation of Tfh cells by inducing IL-21 via STAT3, along with other cytokines, in activated T cells [8, 9]. In addition to these cytokine signals, interaction between activated T cells and B cells activated with the same antigen at T:B border is required for the differentiation of Tfh cells [10]. Aerosol allergens efficiently induce Th2 cell responses and the production of IgE and IgG1 in animal models of allergic inflammation. The involvement of Tfh cells as well as the requirement of pulmonary Tfh cells in this process is relatively unclear. Allergens and aerosol pathogens are known to induce the production of IL-6, a potent inducer of IL-21 [11, 12, 13]. Hence, Tfh cells are likely induced and play a crucial role in modulating antibody responses in allergic inflammation as well as in pulmonary infections.

The medications for allergic asthma patients are steroids that can regulate inflammation and have immunosuppressive effects. Inhaled corticosteroids which are steroid hormones are effective for suppressing Th2 cell-driven eosinophilic asthma [14, 15]. However, long-term use of corticosteroids may affect the growth of young patients and increase a fungal infection [16, 17].

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 [18, 19, 20]. In addition, Tfh cell responses which are involved in IgE class switching play important roles in causing allergic asthma,

but regulation of Tfh cell responses against intranasal allergens is not understood [21]. STAT proteins were identified as being essential role in helper T cell differentiation from naïve precursors [22, 23]. It is established that STAT6 is necessary for the development of Th2 cell generation but 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 of T cells in allergic inflammation according to recent papers [24]. Also, it is well documented that STAT3 is required for the development of Tfh cells.

On the basis of these data, we can surmise that STAT3 delivers intracellular signals crucial for both Th2 and Tfh cells. In addition, targeting STAT3 might be an effective therapeutic approach for inhibiting both helper T cell response and therefore the treatment of allergic asthma.

Materials and methods

Mice

C57BL/6 mice were purchased from Orient (Gyeonggido, Republic of Korea). OT-II, B6.SJL(CD45.1) and *Tcrb*$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). *STAT3*$^{flox/flox}$*CD4-Cre* mice were kindly provided by Drs. C. Dong (MD Anderson Cancer Center, Houston, TX, USA) and S. Akira (Osaka University, Osaka, 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 protocol approved by Institutional Animal Care and Use Committee Seoul National University (SNU-140602-2-2).

Allergen-induced lung inflammation

Mice were anesthetized with isoflurane (Piramal, Bethlehem, PA, USA) and were intranasally administered with a mixture 7 μg of proteinase extracted from Aspergillus melleus (Sigma, St Louis, MO, USA) and 20 μg of Ovalbumin (Grade V, Sigma, St Louis, MO, USA) (Asp/Ova) in 50 μL of PBS (GenDEPOT, Katy, TX, USA) every two days for a total of four or five times (day 0, 2, 4, 6 or 0, 2, 4, 6, 8). Sixteen hours after the last challenge, all mice were euthanized and the mediastinal lymph nodes (mLNs), superficial cervical lymph nodes (scLNs), lungs, bronchoalveolar lavage(BAL) fluid and sera were obtained for further analysis. For TGF-β neutralization experiments, mice were injected intraperitoneally with TGF-β neutralizing antibody (1D11, BioXCell, West Lebanon, NH, USA) or their corresponding IgG1 control (MOPC21, BioXCell, West Lebanon, NH, USA) three

times every two days (day 0, 2, 4). For STAT3 inhibition experiments, mice were treated with intraperitoneal injections of 0.5mg/kg STA-21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or vehicle every two days for 9 days (day 0, 2, 4, 6, 8) and were treated with intranasal injections of 0.25mg/kg STA-21 or vehicle every other day for 9 days (day 1, 3, 5, 7).

Isolation of lymphoid cells from the lungs

We surgically removed the lungs and teased into small pieces by using gentleMACS^{TM} dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and then digested them with RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) containing 10 % of FBS (GenDEPOT, Katy, TX, USA), 0.5 mg/mL of collagenase (Roche, Switzerland), 2 mg/mL of Dispase (Invitrogen, Carlsbad, CA, USA), and 30 μg/mL of DNase I (Sigma, St Louis, MO, USA) 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 lymphocytes separation medium (MP Biomedicals, Solon, OH, USA) and were harvested from interphase.

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, USA) following trachea was cannulated. Obtained BAL fluid was centrifuged, supernatant was collected and IFN-γ, IL-5, IL-17 concentrations were determined by ELISA (all from Biolgend, San Diego, CA, USA). Cells in the pellet were resuspended with PBS and attached 3,000 of cells onto slide glass by

cytospin, and were stained with Diff-Quik (Siemens, Newark, DE, USA) for differential cell counts. We calculated absolute numbers of each cell population based on total cell counts.

Flow cytometry

For T cell analysis, the cells obtained from mice were incubated for 4 h with PMA (100 ng/mL, Sigma, St Louis, MO, USA) and ionomycin (1 μM, Sigma, St Louis, MO, USA) in the presence of Brefeldin A and Monensin (all from eBioscience, San Diego, CA, USA). Cells were stained with Pacific Blue-conjugated anti-CD45.1 (Biolgend, San Diego, CA, USA), Pacific Blue or PerCP-Cy5.5-conjugated anti-CD45.2 (Biolgend, San Diego, CA, USA) and PerCP-Cy5.5 or APC-Cy7-conjugated CD4 (Biolgend, San Diego, CA, USA) for gating of $CD4^+$ T cells. Cells were incubated in fixation buffer (eBioscience, San Diego, CA, USA) for 20 min at 4°C and washed with intracellular staining buffer (eBioscience, San Diego, CA, USA). Intracellular cytokine staining was performed by using Alexa488 or Brilliant Violet 711-conjugated anti-IFN-γ (eBioscience or Biolegend, San Diego, CA, USA), PE or FITC-conjugated anti-IL-17A (Biolgend, San Diego, CA, USA), Alexa647-conjugated anti-IL-4 (Biolgend, San Diego, CA, USA) and APC-conjugated anti-IL-5 (Biolgend, San Diego, CA, USA), PE-conjugated anti-IL-13 (eBioscience, San Diego, CA, USA). For Tfh cell analysis, cells were stained with PerCP-Cy5.5-conjugated anti-CD4, and biotinylated anti-CXCR5 followed by PE- or APC-conjugated streptavidin. PerCP-Cy5.5-conjugated anti-CD45.1 and Pacific Blue-conjugated anti-CD45.2 were additionally used for surface staining. All antibodies were purchased from Biolegend (San Diego, CA, USA). These cells were permeabilized with a Foxp3 staining kit (eBioscience, San Diego, CA, USA),

and further stained with APC-conjugated anti-Foxp3 (Biolgend, San Diego, CA, USA). For phenotypic analysis, FITC-conjugated anti-PD-1 (eBioscience, San Diego, CA, USA) was used. For B cell analysis, the cells were stained with APC-conjugated anti-B220 (Biolegend, San Diego, CA, USA), PE-conjugated anti-CD95 (eBioscience, San Diego, CA, USA), PerCP-Cy5.5-conjugated anti-CD138 (Biolegend, San Diego, CA, USA) and FITC-conjugated anti-GL7 (BD bioscience, San Jose, CA, USA). These cells were analyzed by FACSAria III or FACSVerse (BD bioscience, San Jose, CA, USA) and data were analyzed using software called Flowjo (TreeStar, Ashland, OR, USA).

T cell differentiation in vitro

$CD4^+$ T cells were isolated from either *CD4*$^{STAT3-/-}$OT-II or *CD4*$^{STAT3+/+}$OT-II mice by $CD4^+$ isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and then $CD25^-CD62L^{high}CD44^{low}$ cells were sorted by Aria III cell sorter (BD BioScience, San Jose, CA, USA). These naïve $CD4^+$ T cells (1 x 10^5/well) were co-cultured with bone marrow-derived dendritic cells (1 x 10^4/well) in RPMI 1640 medium containing 10 % of FBS and soluble anti-CD3 (0.5 μg/mL) (BD BioScience, San Jose, CA, USA) for 4 days. For Th2 or Th17 $CD4^+$ T cell induction, cells were stimulated with either IL-4 (10 ng/mL) (PeproTech Inc., Rocky Hill, NJ, USA) plus IL-2 (10 ng/mL) (PeproTech Inc., Rocky Hill, NJ, USA) or LPS (100 ng/mL) (Sigma, USA) plus TGF-β (2 ng/mL) (PeproTech Inc., Rocky Hill, NJ, USA), respectively.

ELISA

To determined antigen-specific cytokines production from mLN cells, 1 × 10^6 of

cells were cultured in the presence of 0, 5, 25 μg/mL Ova for 3 days. The amounts of IFN-γ, IL-4, IL-5, IL-17 in cultured supernatant were measured by ELISA kit (all from BioLegend, San Diego, CA, USA). All assays were done according to the manufacturer's instructions. Sera from intranasally challenged mice with Asp/Ova were collected, and Ova-specific IgM, IgE, IgG1, IgG2b and IgG2c antibodies were measured by ELISA. Briefly, serum samples were added in a 3-fold or 5-fold serial dilution onto plates pre-coated with 5 μg/mL Ova. Ova-specific antibodies were detected with HRP conjugated goat anti-mouse IgM, IgE, IgG1, IgG2b, and IgG2c antibodies (Southern Biotechnology Associates, Birmingham, AL, USA).

Quantitative real-time RT-PCR

Total RNA was obtained from the lung cells or $CD4^+$ T cells with TRIzol (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized with amfiRivert reverse transcriptase (GenDEPOT, Houston, TX, USA). Level of mRNA transcript were quantified with iTaq-SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and the ABI-PRISM 7900 detection system (Applied Biosystems, Foster City, CA, USA).` Data were normalized to expression of the *Actb*. The following primer pairs were used: *Ifng*; forward, 5'-GATGCATTCATGAGTATTGCCAAG-3', reverse, 5'-GTGGACCACTCGGA-TGAGCTC-3', *Il4*; forward, 5'-AGATCACGGCATTTTGAACG-3', reverse, 5'-TTTGGCACATCCATCTCCG-3', *Il5*; forward, 5'-CGCTCACCGAGCTCTGTTG-3', reverse, 5'-CCAATGCATAGCTGGTGATTTTT-3', *Il9*; forward, 5'-GTGAC-ATACATCCTTGCCTC-3', reverse, 5'-GTGGTACAATCATCAGTTGGG-3', *Il10*; forward, 5'-ATAACTGCACCCACTTCCCAGTC-3', reverse, 5'-CCCAAGTAACC

CTTAAAGTCCTGC-3', *Il13*; forward, 5'-GCTTATTGAGGAGCTGAGCAACA-3', reverse, 5'-GGCCAGGTCCACACTCCATA-3', *Il17*; forward, 5'-CTCCAGAAG-GCCCTCAGACTAC-3', reverse, 5'-GGGTCTTCATTGCGGTGG-3', *Cxcl1*; forward, 5'-TGGCTGGGATTCACCTCAAGAACA-3', reverse, 5'-TGTGGCTA-TGACTTCGGTTTGGGT-3', *Il22*; forward, 5'-CATGCAGGAGGTGGTACCTT-3', reverse, 5'-CAGACGCAAGCATTTCTCAG-3', *Ccl11*; forward, 5'-ATTGTGTTG TTTGTTTGCTTGC-3', reverse, 5'-GTCAGCCTGGTCTACACAGTGA-3', *Muc5ac*; forward, 5'-CCATGCAGAGTCCTCAGA -3', reverse, 5'-TTACTGGA-AAGGCCCAAG -3', *Clca3*; forward, '5-AATGATGAGCCCTACACCGAACA-3', reverse, 5'-AGTGAGCCCACTCA TGGACAAAG-3', *Actb*; forward, 5'-TGGAATCCTGTGGCATCCATGAAAC-3', reverse, 5'-TAAAACGCAGCTCAG-TAACAGTCCG-3', *Tbx21*; forward, 5'-CAACAACCCCTTTGCCAAAG-3', reverse, 5'-TCCCCCAAGCAGTTGACAGT-3', *Gata3*; forward, 5'-AGAACCGG-CCCCTTATGAA-3', reverse, 5'-AGTTCGCGCAGGATGTCC-3', *Rorc*; forward, 5'-CCGCTGAGAGGGCTTCAC-3', reverse, 5'-TGCAGGAGTAGGCCACATTA-CA-3', *Foxp3*; forward, 5'-GGCCCTTCTCCAGGACAGA-3', reverse, 5'-GCTGATCATGGCTGGGTTGT-3'.

Adoptive transfer studies

To examine the role of STAT3 on $CD4^+$ T cells, naïve $CD4^+$ T cells isolated from either $CD4^{STAT3-/-}$OT-II or $CD4^{STAT3+/+}$OT-II mice by using a $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated naïve $CD4^+$ T cells (10×10^6 cells/transfer) were transferred into B6.SJL or $Tcrb^{-/-}$ mice. In some experiment, naïve $CD4^+$ T cells from $STAT3^{flox/flox}CD4\text{-}Cre(+)$OT-II mice ($CD45.2^{+/+}$) and B6.SJL×OT-II mice ($CD45.2^+/CD45.1^+$) were mixed at a 1:1 or

5:1 ratio before transferring them into B6.SJL or *Tcrb*$^{-/-}$ mice. Next day, the recipients were intranasally injected with Asp/Ova were analyzed as described. Staining with anti-CD45.1 and anti-CD45.2 was used to distinguish each donor T cell population during flow cytometric analysis.

Statistics

Data were analyzed with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Statistics were calculated with the two-tailed Student's t-test. p values are presented within each figure or figure legend.

Results

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

To examine the role of STAT3 on helper T cell responses in allergic lung inflammation, 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 $CD4^{STAT3-/-}$ mice, in which *Stat3* was selectively deleted in T cells, or $CD4^{STAT3+/+}$ 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 generate IL-17 producers (Figure 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 (Figure 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 (Figure 1B), although they showed increased levels of *Ifng* and *Tbx21* transcript. Quantitative real-time RT-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 (Figure 1C), indicating that Th2 cell lineage program was hampered by STAT3-deficiency in T cells during *in vitro* differentiation.

Decreased Th2 cell responses in STAT3-deficient T cells in the mediastinal 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 Asp/Ova. Our previous works showed that the intranasal challenges induce not only Th2 cell responses but also Th17 and Th1 cell responses [25, 26]. We intranasally challenged $CD4^{STAT3-/-}$ mice and $CD4^{STAT3+/+}$ mice with Asp/Ova every other day for total four times. One day after the last challenge, we obtained lymphoid cells from the mLNs, stimulated them with Ova *ex vivo*. Consistent with the well-known role of STAT3 on Th17 cell differentiation [27, 28], we observed a significantly decreased amount of IL-17 in the supernatant of $CD4^{STAT3-/-}$ lymphoid cells compared with control (Figure 2). Conversely, the amount of IFN-γ was significantly higher in the former than the latter group, presumably due to the inhibitory role of STAT3 on Th1 cell [29, 30]. Consist with the data in Figure 1, the production of type 2 cytokines IL-4 and IL-5 was almost completely abolished in the $CD4^{STAT3-/-}$ lymphoid cells (Figure 2, lower panels). The observed decreased Th2 cell responses in the mLNs were, at least in part, consistent with a recent study. Collectively, the mLNs of $CD4^{STAT3-/-}$ mice showed almost complete decrease of both Th17 and Th2 cell responses whereas they exhibited increased Th1 cell responses to intranasal allergens.

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 Figure 3, we observed a significantly increased IFN-γ-producing $CD4^+$ T cells among total $CD4^+$ T cells and decreased IL-17-producing $CD4^+$ T cells, which correlated well with Th1- and Th17-cytokine profiles of the mLNs in Figure 2. Interestingly, although the Th2

responses of the mLNs 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 (Figure 3A&B). In addition, a unique population of IFN-ɣ and IL-4/5 co-producing CD4^{+} T cell was significantly increased while that of IL-4/5 and IL-17 co-producing CD4^{+} T cells was decreased in the lung of the former group (Figure 3A& B). Thus, although the decrease in Th17 cell responses in the lung of *CD4*$^{STAT3-/-}$ mice were consistent with those seen in the mLNs, the Th2 cell responses in the same mice were not defective in the lungs.

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

The observed differences in Th2 cell responses between the mLNs and the lungs prompted us to analyze helper T cell subsets in the BAL. Consistent with those in the mLNs and the lungs, the frequency of IFN-ɣ-producers among CD4^{+} T cells in the BAL fluid was significantly increased in the *CD4*$^{STAT3-/-}$ mice while that of IL-17-producers was significantly decreased in the same mice (Figure 4A&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. To our surprise, while T cells producing IFN-ɣ 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-ɣ (Figure 4B, lower panels). The frequency of IFN-ɣ and IL-17 co-producing CD4^{+} T cells were also slightly increased in the *CD4*$^{STAT3-/-}$ mice. 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-ɣ-producing T cells.

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 mLNs, 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 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-suficient and STAT3-deficient T cells in the same *in vivo* environment, we adoptively transferred 1:1 mixture of Ova-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, the size of the latter population after intranasal Asp/Ova was significantly less than that of the former population (Figure 5A). 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 mLNs, lungs as well as BAL, while the opposite was true for Th17 cells (Figure 5B-D). However, although the frequency of Th2 cells among $CD4^{STAT3-/-}$ OT-II T cell population was decreased in the mLNs, it substantially increased in the BAL samples (Figure 5B-D). These results indicate that the opposing effects of STAT3-deficiency on Th2 cell responses between mLNs and the airway was cell-intrinsic.

Kinetic analysis of Tfh cell and germinal center B cell responses against intranasal allergens

As a first step to dissect germinal center reactions in the bronchus-associated lymphoid tissues (BALT) upon allergenic challenges, we employed an animal model of allergic asthma induced by Asp/Ova. Groups of C57BL/6 mice were intranasally challenged with Asp/Ova every other day before lymphoid cells from mLNs or scLNs were analyzed for the frequencies of $CXCR5^{+}PD-1^{+}$ Tfh cells, $CD95^{+}GL7^{+}$ germinal center B cells, and $CD138^{+}B220^{-}$ plasma cells. As shown in Fig. 6A, B, the magnitudes of Tfh cells, germinal center B cells and plasma cells were all remarkably higher in the mLNs than those in the scLNs. Kinetic analysis of Tfh cell responses in the mLNs showed that the frequency of Tfh cell peaked at day 7, and it gradually decreased afterward. On the other hand, the frequencies of both germinal center B cells and plasma cells peaked on day 13 in the mLNs, indicating that the Tfh cell responses preceded germinal center B cell responses by about 6 days in this experimental setting. Similar to the kinetics of germinal center B cells and plasma cells in the mLNs, the serum levels of Ova-specific IgE, IgG1, IgG2b were highest at day 13 compared with those of day 3 and day 9. The levels of Ova-specific IgG2c appeared to be higher at day 9 than those of day 13. These results indicate that mLNs are the lymphoid organs where active germinal center reactions occurred upon intranasal challenges of allergens and that the optimal time point to dissect germinal center reactions is between day 7 and day 13 in this experimental setting.

Effects of TGF-β blockade on pulmonary Tfh cell responses against intranasal allergens

We next sought to determine the factors that regulate the observed germinal center reactions in the mLNs. TGF-β signal is required for the differentiation of Th17 cells and Treg cells; however, its role in the Tfh cells and germinal center responses has been controversial. Administration of anti-TGF-β in mice challenged with intranasal Asp/Ova resulted in little differences in the frequencies of $CXCR5^{+}PD-1^{+}$Tfh cells, $CD95^{+}GL7^{+}$ germinal center B cells and $CD138^{+}B220^{-}$ plasma cells in the mLNs compared with those of a control antibody treated group (Figure 7A, B). These results demonstrate that blockade of TGF-β had little impact in the generation of Tfh cell responses as well as germinal center B cell responses.

A STAT3 inhibitor STA-21 dampens pulmonary Tfh cell responses

Our previous results showed that STAT3 differentially regulates pulmonary Th17 and Th2 cells. STAT3 signal in T cells is known to mediate the generation of Tfh cells. Therefore, we next asked if inhibition of STAT3 impact the germinal center reactions generated in response to intranasal allergens. Mice were intranasally challenged with Asp/Ova and were additionally administered with a STAT3 inhibitor STA-21 or vehicle. Although it did not reach statistical significance, Fig. 8A, 8B shows the frequency of $CXCR5^{+}PD-1^{+}$ Tfh cells was lower in the STA-21-treated group than that in the vehicle-treated group. Similarly, the frequencies of $CD95^{+}GL7^{+}$ germinal center B cells and $CD138^{+}B220^{-}$ plasma cells were significantly decreased in the STA-21 treated group. These data indicate that a STAT3 inhibitor STA-21 suppressed the generation of Tfh cell and germinal center B cell responses in the mLNs upon intranasal allergenic challenges.

Ablation of STAT3 in T cells blocks pulmonary Tfh cell responses and germinal center reactions

The observation that a STAT3 inhibitor suppressed pulmonary Tfh cell and germinal center B cell responses prompted us to determine the role of T cell-intrinsic STAT3 in the germinal center reactions to intranasal allergens. To determine the function of STAT3 in $CD4^+$ T cell during antigen-specific responses *in vivo*, we adoptively transferred $CD4^+$ T cells from *CD4*$^{STAT3+/+}$OT-II mice or *CD4*$^{STAT3-/-}$ mice into *Tcrb*$^{-/-}$ mice before the recipients were challenged with intranasal Asp/Ova. Compared with that of the *CD4*$^{STAT3+/+}$OT-II T cell recipients, the frequency of $CXCR5^+PD-1^+$ Tfh cells in the recipients of *CD4*$^{STAT3-/-}$OT-II T cell was significantly diminished (Figure 9A, 9B). On the other hand, the frequency of $CXCR5^+PD-1^+Foxp3^+$ follicular regulatory T (Tfr) cells was not reduced in the latter group (Fig. 9A, 9B). These data indicate that STAT3 signal pathway in $CD4^+$ T cell was essential for the generation of Tfh cells.

We next sought to determine whether STAT3-deficiency in $CD4^+$ T cell also impacted the magnitude of germinal center responses. As shown in Fig. 10A, 10B, there was a marked decrease in the percentage of $CD95^+GL7^+$ germinal center B cells in the recipients of *CD4*$^{STAT3-/-}$OT-II T cell compared with that in the recipients of *CD4*$^{STAT3+/+}$OT-II T cell. Similarly, the percentage of $CD138^+B220^-$ plasma cells was lower in the former group although it was not statistically significant. More importantly, we also found a significant reduction in the amounts of Ova-specific IgM, IgG2b and IgG2c as well as, to less extent, IgG1 antibodies in the former mice (Fig. 10C). Collectively, these data indicate that the STAT3 signal in antigen-specific $CD4^+$ T cells not only mediated Tfh cell differentiation

but also facilitates germinal center B cell responses and subsequent antibody production in response to intranasal allergens *in vivo*.

T cell intrinsic STAT3 is required for the differentiation of pulmonary Tfh cells *in vivo*

To further examine if the observed defective Tfh cell responses was due to T cell-intrinsic function of STAT3 or due to the lack of appropriate signals from STAT3-sufficient T cells, we mixed STAT3-sufficient OT-II T cells ($CD45.1^{+}/CD45.2^{+}$) with $CD4^{STAT3-/-}$ OT-II T cells before transferring them into *Tcrb*$^{-/-}$ mice. Compared with STAT3-sufficient OT-II T cells, $CD4^{STAT3-/-}$ OT-II T cells showed a significantly less percentage of $CXCR5^{+}PD-1^{+}$ Tfh cell in the same recipients (Fig. 11A, 11B). These data indicate that T cell intrinsic STAT3 signal was crucial for the differentiation of naïve T cells into Tfh cells *in vivo* in the mLNs upon intranasal allergenic challenges.

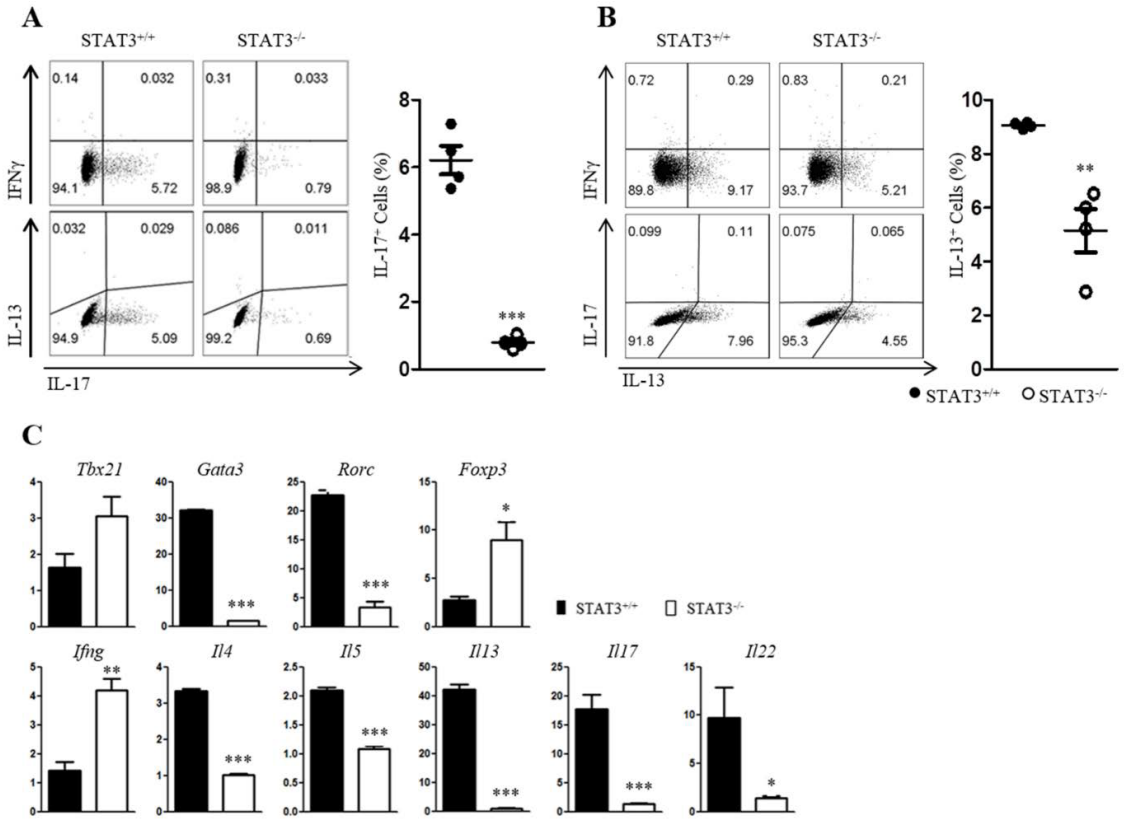

Figure 1. Effects of STAT3-deficiency on the *in vitro* differentiation of Th17 cell and Th2 cell

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-ɣ 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.

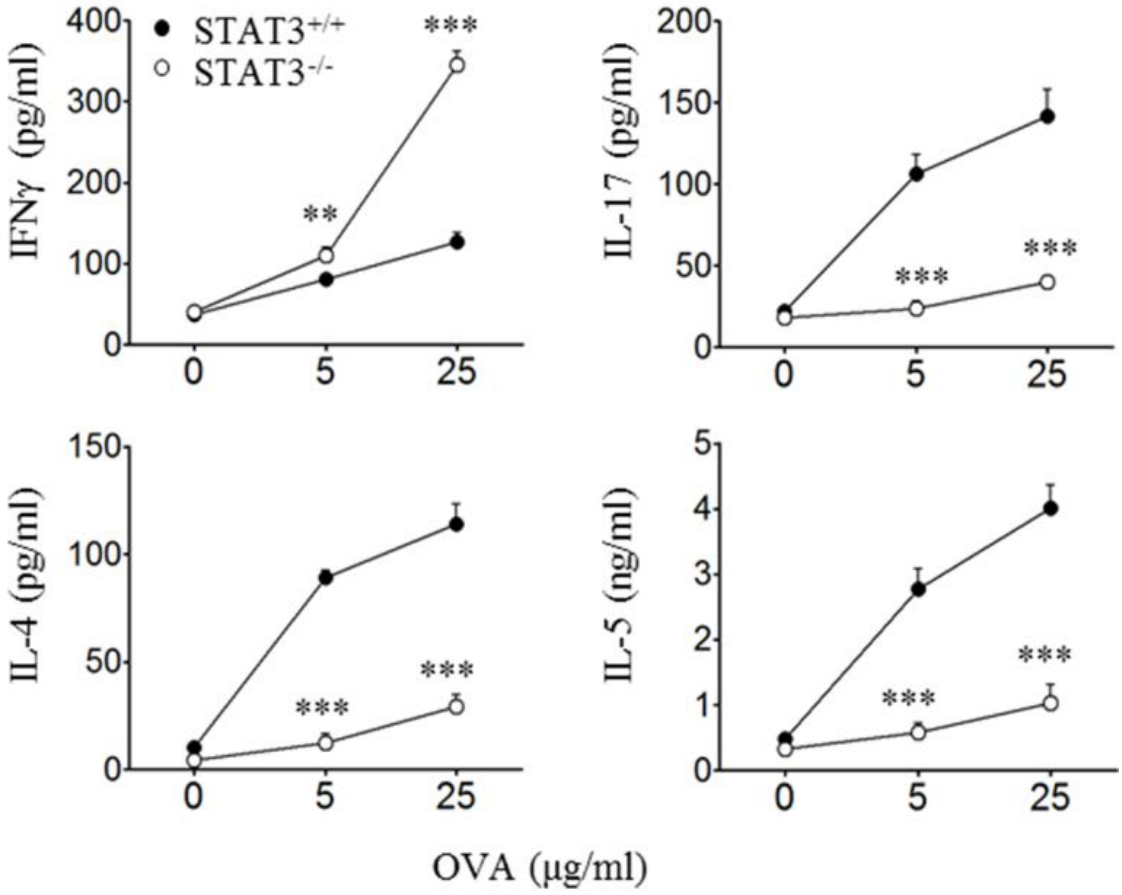

Figure 2. Effects of STAT3-deficiency on cytokine production by T cells from the mediastinal lymph nodes of mice challenged with intranasal allergens

Groups of $CD4^{STAT3+/+}$ and $CD4^{STAT3-/-}$ mice (n=4-7) were intranasally injected with Asp/Ova every other day for four times. One day after the last challenge, lymphoid cells from the mLNs 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.

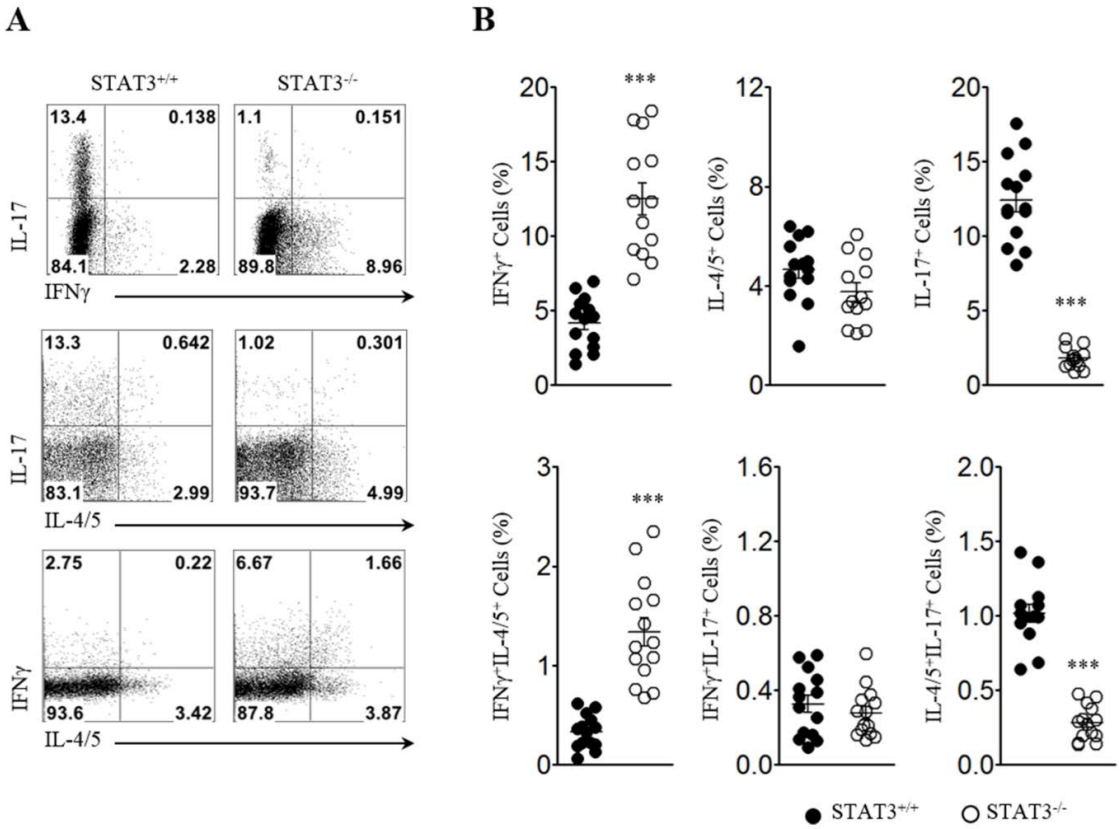

Figure 3. Effects of STAT3-deficiency on cytokine production by T cells from the lungs of allergen challenged mice

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 ± SD (B). Data are pooled from three independent experiments. ***, $p<0.001$ in comparison with *CD4*$^{STAT3+/+}$ group.

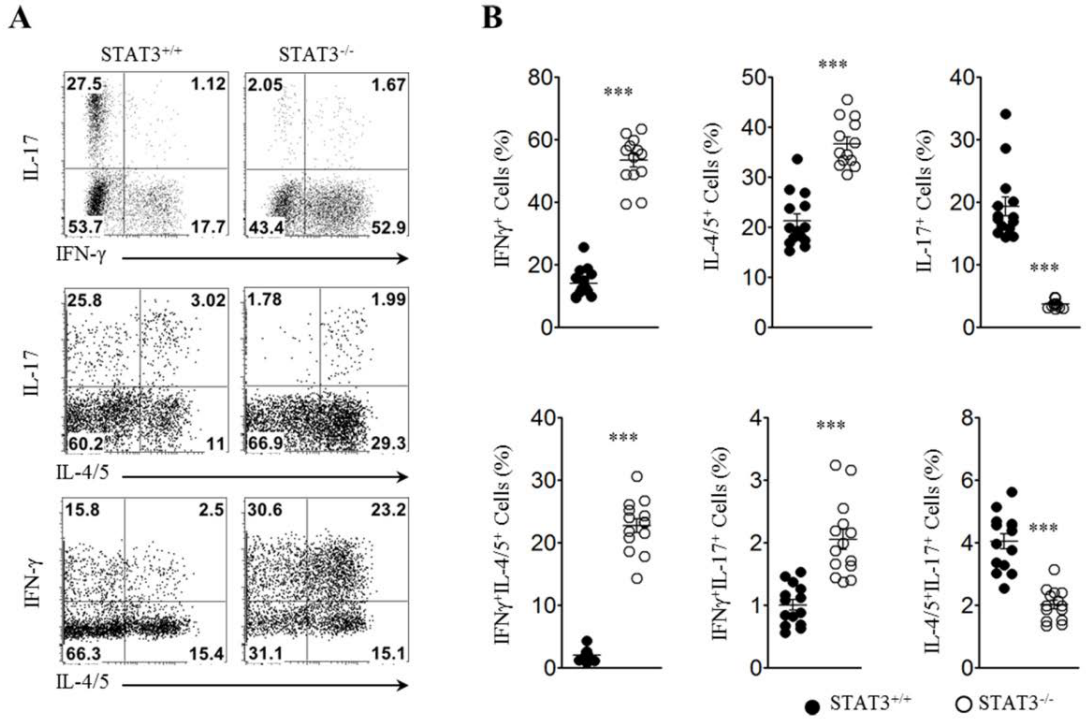

Figure 4. Comparative analysis of CD4^{+} T cells in the bronchoalveolar lavage of allergen challenged *CD4*$^{STAT3+/+}$ and *CD4*$^{STAT3-/-}$ mice

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

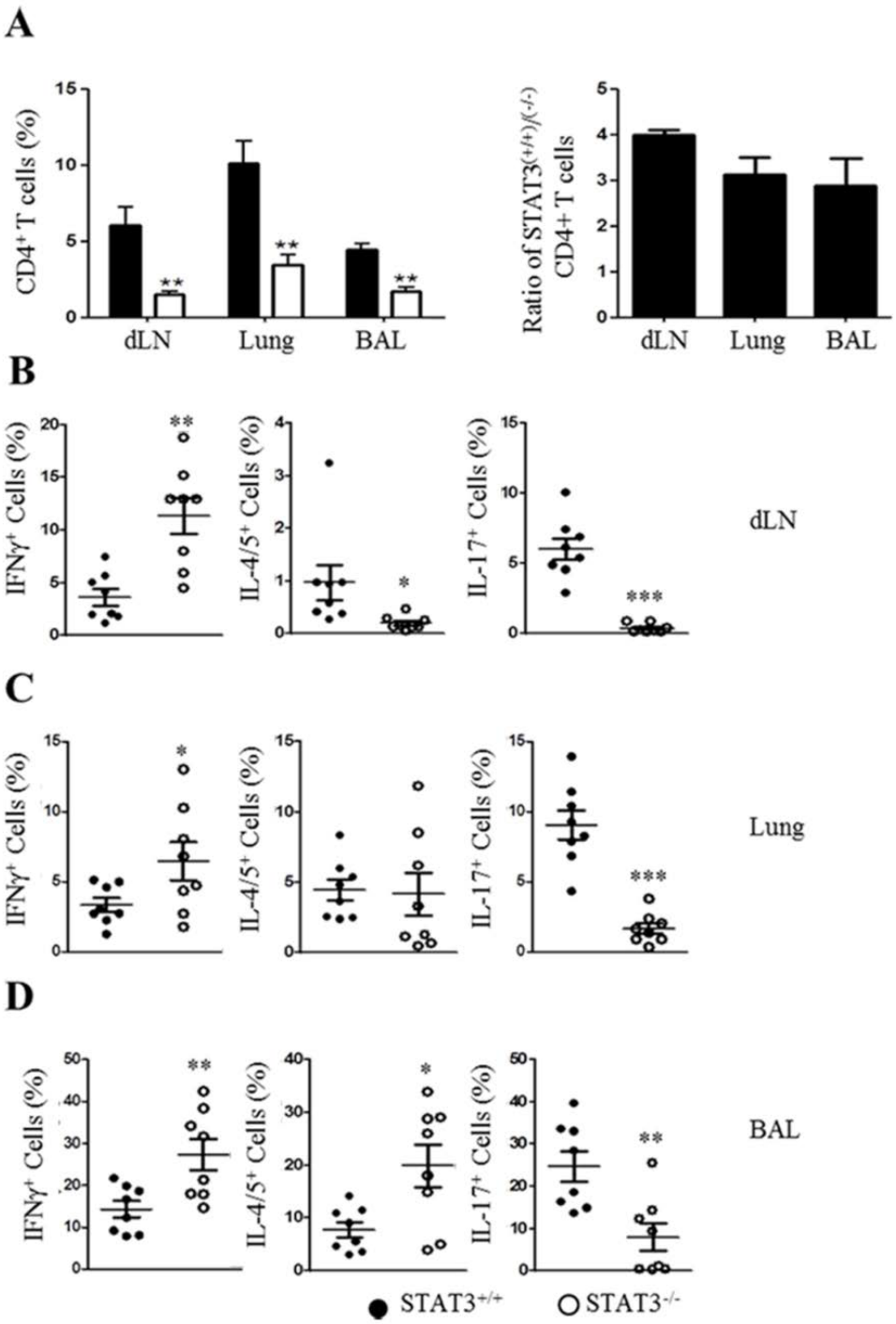

Figure 5. T cell-intrinsic STAT3-deficiency differentially impacts on Th2 cell but consistently suppresses or increases Th17 cell and Th1 cell, respectively

$CD4^+$ T cells isolated from STAT3-sufficient B6.SJL×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. One day after the last

challenge, lymphoid cells were isolated from the BAL fluid, lungs, and mLNs were obtained. 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 IFN-γ-, IL-4/5- and IL-17-producers among each donor T cell population were determined by intracellular cytokine staining (B-D). Data are mean ± 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.

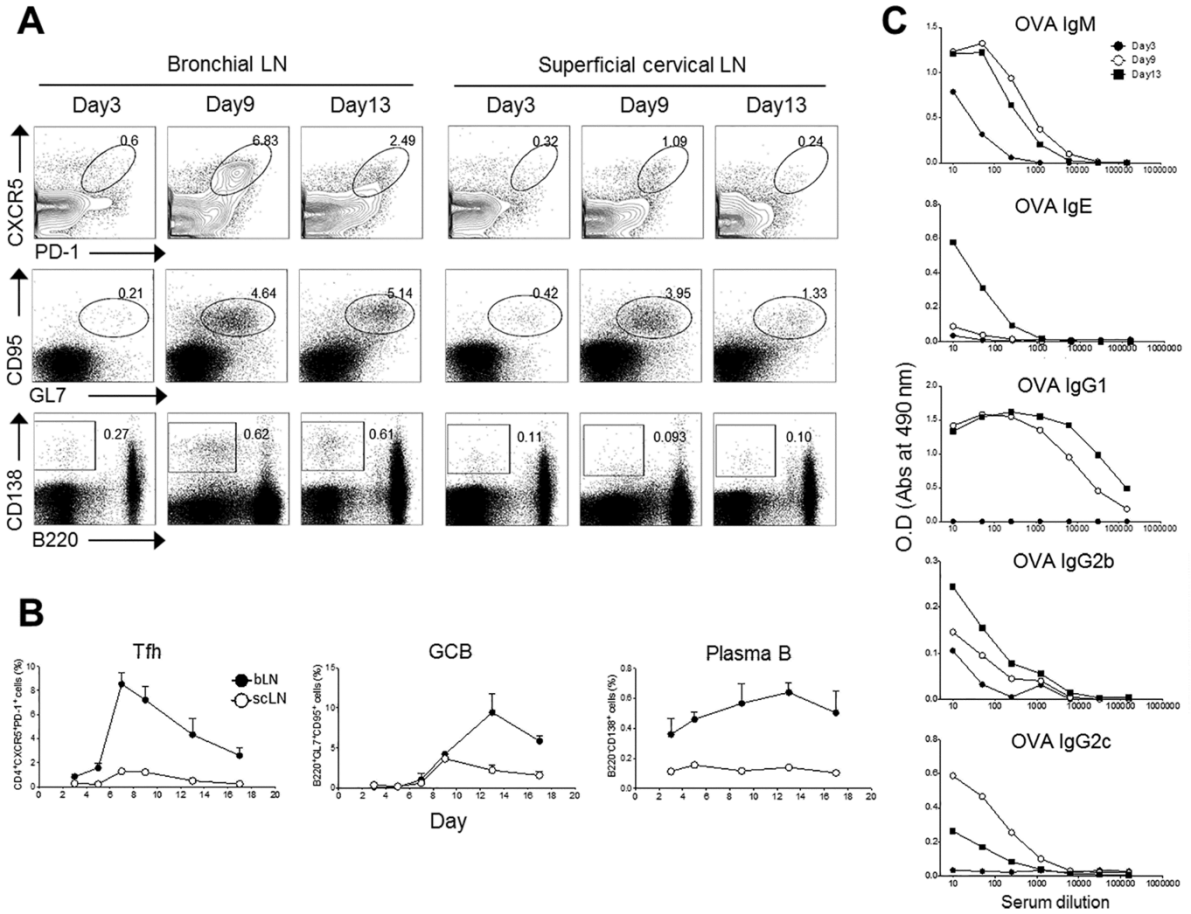

Figure 6. Kinetic analysis of Tfh and germinal center response in the intranasal challenged mice

Mice were intranasally injected with Asp/Ova every 2 days for 9 days and analyzed on day3, 5, 7, 9, 13 and 17. (A & B) The proportions of $CXCR5^{+}PD-1^{+}$ cell among $CD4^{+}$ cells, $CD95^{+}GL7^{+}$ cells among $B220^{+}$ cells and $CD138^{+}B220^{-}$ cells among lymphocytes in mLNs or scLNs from allergen-injected mice are plotted. Numbers around the outlined area is the percentage of cells expressing each marker. (C) Ova-specific immunoglobulins were detected in sera obtained from mice on day3, 9 and 13. Data are means ±SEM. n=3.

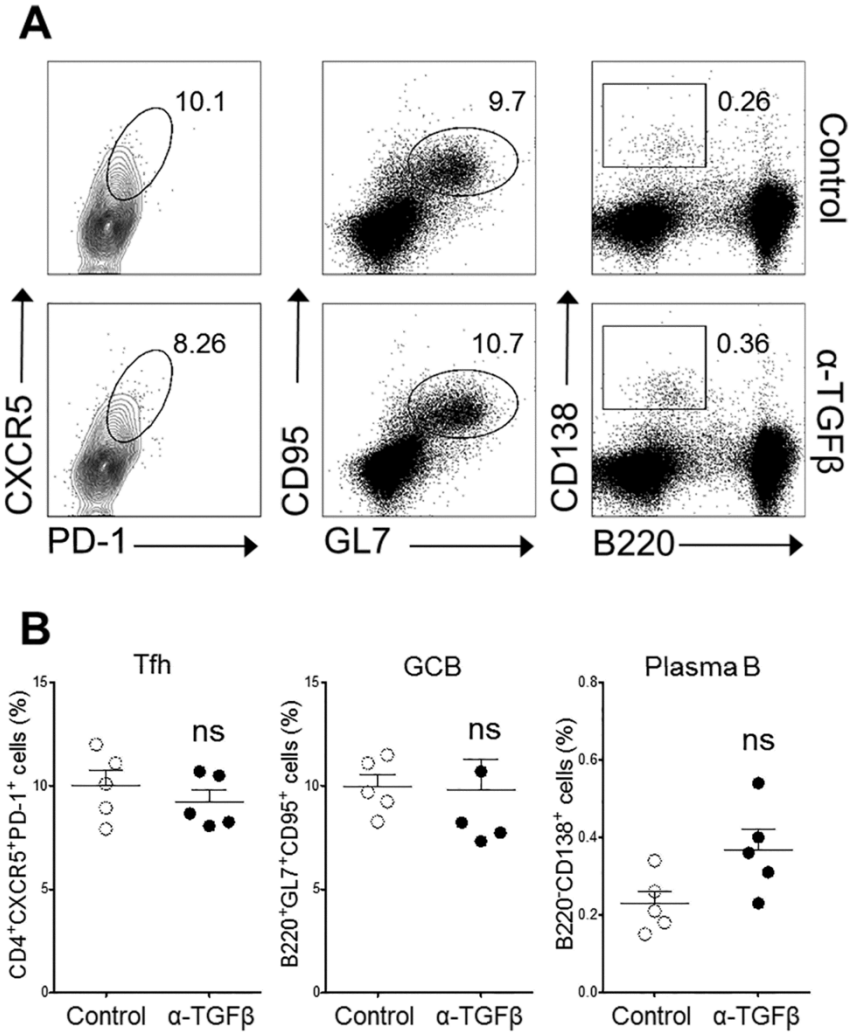

Figure 7. TGF-β neutralizing antibodies are inefficient in controlling Tfh and germinal center reactions

Mice were intraperitoneally injected with α-TGF-β antibody(1d11) or control antibody and challenged with allergen every 2 days for 9 days. (A) The left portion shows the analysis of the first day after the last injection and challenge of $CXCR5^{+}PD-1^{+}$ cells among $CD4^{+}$T cells in mLNs. In the middle, the expression of GL7 and CD95 on B cells is shown. On the right, representative flow cytometric analysis of cell surface CD138 and B220 expression in mLNs is shown. Numbers around the outlined area is the percentage of cells expressing each marker. n=4-5. ns=not significant in comparison with the control antibody injected group (B).

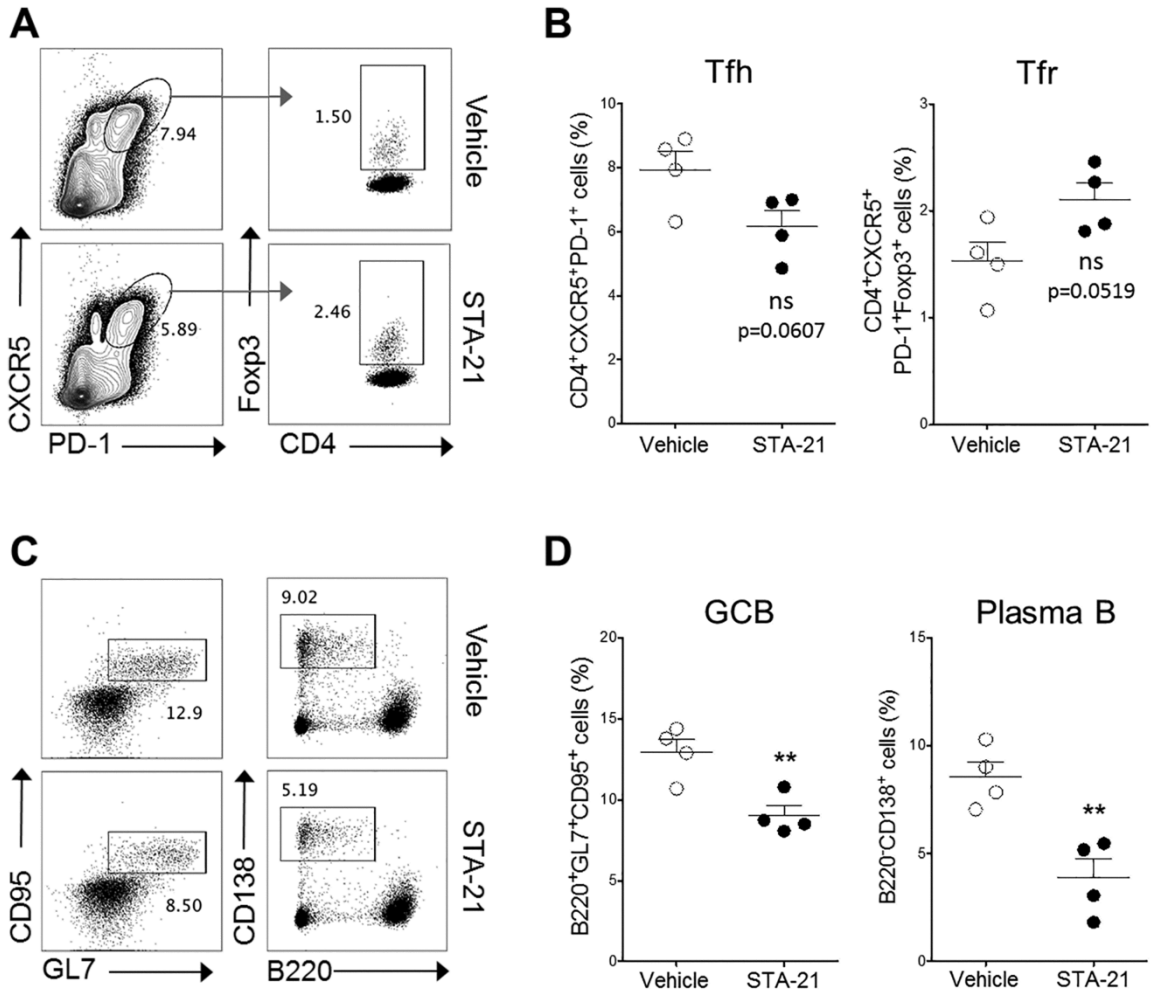

Figure 8. STAT3 inhibitor STA-21 regulates Tfh cell generation and germinal center reactions

(A) Expression of CXCR5 and PD-1 among $CD4^+$ T cells in the mLNs of Asp/Ova challenged mice, followed by intraperitoneal treatment with STA-21 or vehicle. Analysis of the proportion of $Foxp3^+$ cells among $CXCR5^+PD\text{-}1^+$ cells. (C) The percentage of $CD95^+$ $GL7^+$ cells among $B220^+$ B cells and $CD138^+$ $B220^-$ cells among lymphocytes in the mLNs. Numbers around the outlined area indicate the percentage of cells expressing each marker. Data are means ±SEM (B & D). n=4. ns=not significant, ** P <0.01 in comparison with vehicle treated mice.

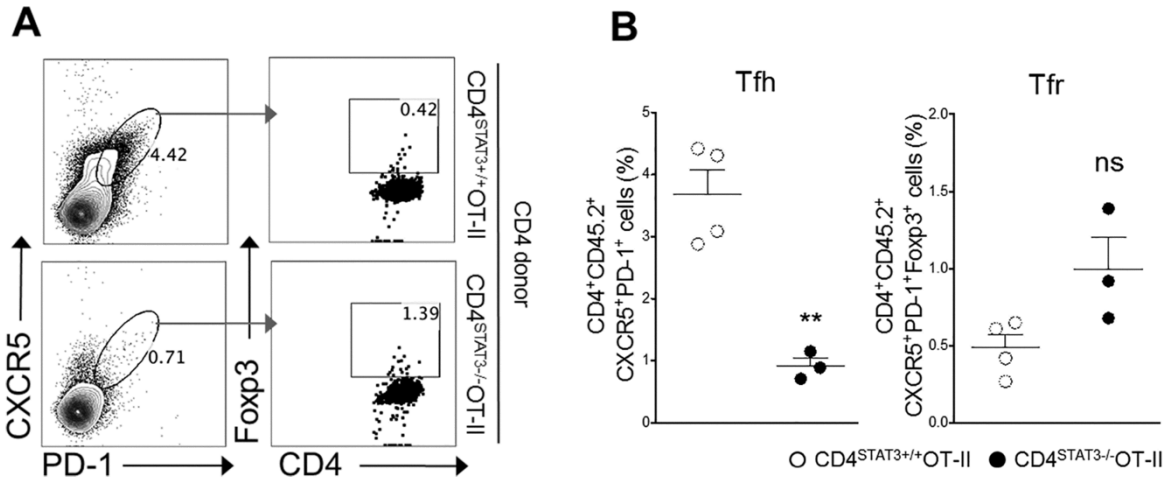

Figure 9. Generation of Tfh cells requires STAT3 in $CD4^+$ T cell

(A) Expression of CXCR5 and PD-1 among $CD4^+CD45.2^+$ T cells in the mLNs of *Tcrb*$^{-/-}$ recipients given that the $CD4^+$ T cells from *CD4*$^{STAT3+/+}$OT-II mice or *CD4*$^{STAT3-/-}$ OT-II mice, followed by intranasal challenge with Asp/Ova every 2 days for 9 days. Analysis of the proportion of $Foxp3^+$ cells among $CXCR5^+PD-1^+$ cells. Numbers around the outlined area indicate the percentage of cells expressing each marker. Data are means ±SEM (B). n=3,4. ns=not significant, ** P <0.01 in comparison with *CD4*$^{STAT3+/+}$OT-II $CD4^+$T cells.

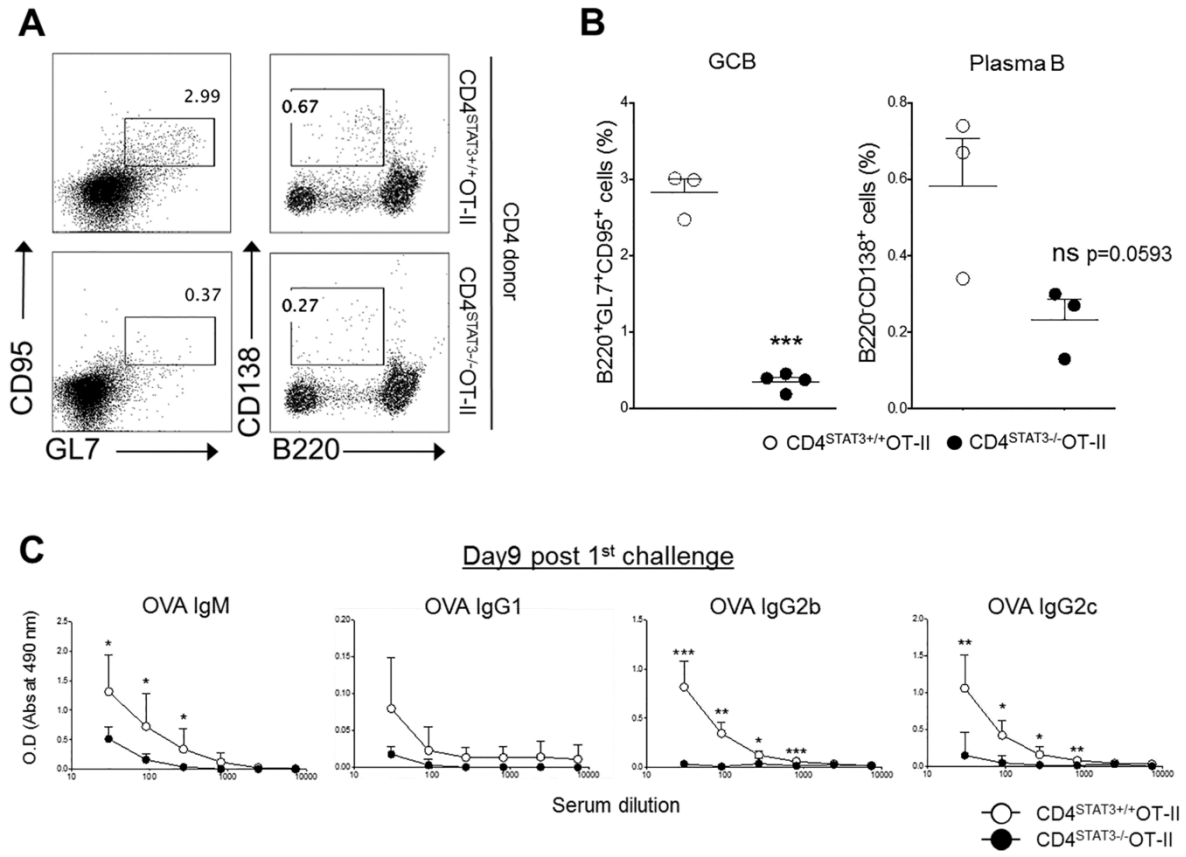

Figure 10. STAT3-deficient CD4^{+} T cell failed the development of germinal center response in the mediastinal lymph nodes

(A) The proportion of GL7^{+}CD95^{+} cells among B220^{+} cells and B220^{-}CD138^{+} cells among lymphocytes in the mLNs of *Tcrb*$^{-/-}$ recipient mice given that the CD4^{+} T cells from *CD4*$^{STAT3+/+}$OT-II mice or *CD4*$^{STAT3-/-}$OT-II mice, followed by intranasal challenge with Asp/Ova every 2 days for 9 days. Numbers around outlined areas indicate the percentages of cells expressing GL7^{+}CD95^{+} on B cells or B220^{-}CD138^{+} on lymphocytes. (C) Level of immunoglobulin specific for Ova in the sera obtained from *Tcrb*$^{-/-}$ recipient mice. Data are means SEM (B & C). n=3. ns=not significant, * P<0.05, ** P <0.01, *** P<0.001 in comparison with lymphocyte of recipient mice that were given the *CD4*$^{STAT3+/+}$OT-II CD4^{+}T cells.

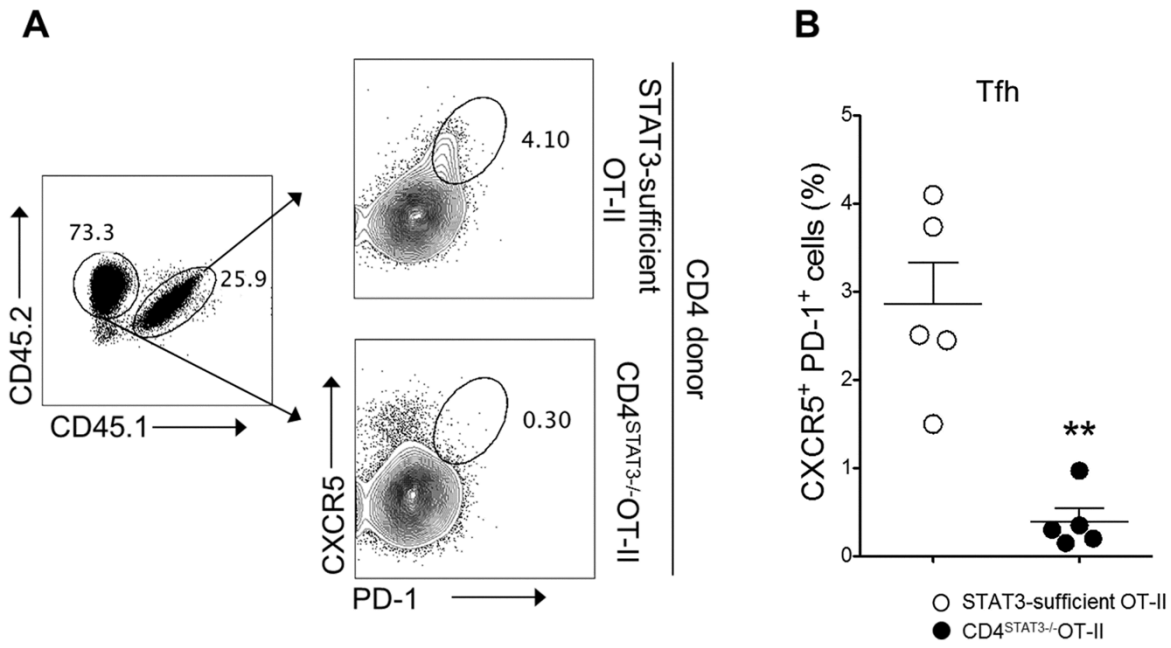

Figure 11. T cell-intrinsic STAT3 is required for Tfh cell generation in BALT

$CD4^+$ T cells isolated from STAT3-sufficient B6.SJLxOT-II ($CD45.1^+/CD45.2^+$) or *CD4*$^{STAT3-/-}$OT-II mice ($CD45.2^{+/+}$) were mixed (1:5 ratio) and transferred in to *Tcrb*$^{-/-}$ mice. The recipient mice were intranasally challenged with Asp/Ova every other day, five times. (A) On the first day after the last challenge, the expression of CXCR5 and PD-1 in $CD4^+$ T cells among each donor T cell population from the mLNs were analyzed by flow cytometry. Numbers around the outlined area indicate the percentages of cells expressing CXCR5 and PD-1. Data are means ±SEM (B). n=5. ** $P < 0.01$ in comparison with CD4 cells of STAT3-sufficient OT-II mice.

Discussion

Activation of STAT proteins is an essential requirement for the differentiation of helper T cells from naïve T cells. Therefore, understanding the role of STAT proteins 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 found that T cell-specific STAT3-deficiency resulted in consistent increased Th1 cell responses and decreased Th17 cell responses in the mLNs, lungs and BAL upon intranasal allergenic challenges. In addition, our results demonstrated although allergen-specific Th2 cell responses were dramatically decreased in the mLNs 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. In respect to humoral immunity, development of Tfh cells and germinal center responses were independent of TGF-β. By contrast, T cell intrinsic STAT3 signal appeared to be necessary for the pulmonary Tfh cells and germinal center B cell responses since (i) a STAT3 inhibitor STA-21 decreased the frequencies of Tfh cells and germinal center B cells in the mLNs, (ii) STAT3-deficient T cells failed to be differentiated into Tfh cells in the mLNs, (iii) *Tcrb*$^{-/-}$ recipients of STAT3-deficient T cells exhibited significantly reduced germinal center B cells and plasma cells, and diminished levels of allergen-specific IgG and IgE in an animal model of allergic lung inflammation induced by intranasal

fungal protease allergens. Our findings propose a previously unappreciated site-dependent control of Th2 cell differentiation and requirement for the generation of Tfh cells and germinal center B cell responses by STAT3 during allergic lung inflammation.

STAT3 activation is a common requirement for the differentiation of Th17 cells and Tfh cells [31]. 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 [24, 32]. 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 mLNs of $CD4^{STAT3-/-}$ mice after intranasal challenges with allergens *in vivo*. However, the defective Th2 cell responses observed in the mLNs of $CD4^{STAT3-/-}$ mice were disappeared when we analyzed helper T cell population in the lungs. 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. STAT3 has been shown to antagonize STAT5 signal by competing their common binding sites [33] during Th17 cell differentiation. STAT5 signal is also a negative regulator of Tfh cell differentiation [34, 35], suggesting that the balance between STAT3 and STAT5 is crucial for the Tfh cell generation. Thus enhancement of STAT5 signaling pathway or blockade of STAT3 possibly attenuates Tfh cell immunity. Indeed, we observed that

administration of a STAT3 inhibitor significantly decreased the size of germinal center reactions in the mLNs. Mechanistic studies convincingly showed that blockade of STAT3 signal in T cells was sufficient to significantly attenuate the generation of Tfh cells, germinal center B cells, plasma cells as well as the production of allergen-specific IgG and IgE. In addition, STAT3 deficiency significantly increased the frequency of pulmonary Th1 cells. Thus, STAT3 suppresses Th1 cells but promotes Tfh cells and Th17 cells in the BALT in response to intranasal allergens.

Production of allergen-specific immunoglobulin including IgE is known to mediate local and systemic anaphylactic responses by stimulating degranulation of mast cells. Suppression of allergen-specific humoral responses might provide beneficial therapeutic effects in patients with allergy. When it comes to Tfh cell-mediated humoral immunity to inhaled allergen, inhibition of STAT3 signaling in T cells can be a promising target in the development of therapeutic approaches for the treatment of allergic lung inflammation. However, cell-intrinsic STAT3 signal have diverse, and some time opposing, roles on the development of helper T cell subsets during allergen-induced lung inflammation; thus, our findings propose that thoroughtful considerations are required for the development of STAT3-targeting therapeutic approaches.

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국문초록

공중항원에 의한 적응면역의 발달 기전에 대한 이해는 공중병원균 또는 알레르겐으로 유도된 폐 염증반응의 치료법에 접근하기 위해 필요하다. 알레르기성 폐 염증반응은 체액성 면역과 세포 매개성 2 형 면역반응을 모두 수반한다. 제 2 형 보조 T 세포는 IgE 생성 B 세포, 비만세포, 호산구의 활성화를 유도하며, CXCR5 를 발현하는 여포 보조 T 세포는 IL-21 생성과 활성화된 B 세포에 ICOS 공동자극을 함으로써 체액성 면역을 매개한다. STAT3 는 미경험 T 세포가 제 17 형 도움 T 세포로 분화하는 데 필수적인 역할을 하지만 알레르겐에 대한 제 2 형 도움 T 세포 반응에서 STAT3 의 역할은 불확실하다. 본 연구에서는 T 세포 특이적으로 STAT3 가 결핍된 생쥐에 알레르기성 천식 동물 모델을 적용함으로써 제 2 형 도움 T 세포의 위치에 따라 T 세포 내에서 STAT3 가 다양한 역할을 하고 있다는 것을 증명하였다. 기관지 림프절에서 STAT3-결핍 T 세포의 제 2 형 도움 T 세포 사이토카인 생성이 크게 감소하였다. 폐의 STAT3-결핍 T 세포의 $CD4^+$ 세포 중에서 제 2 형 도움 T 세포의 빈도는 정상 T 세포와 비교하였을 때 비슷하였다. 그에 반해, 기도에서 STAT3-결핍 T 세포는 제 2 형 도움 T 세포 사이토카인의 생성이 정상 T 세포에 비해 상당이 증가하였다. 또한, T 세포 내에서의 STAT3 신호는 제 17 형 도움 T 세포 뿐 아니라 여포 도움 T 세포의 분화를 매개한다고 알려져있다. 본 연구에서는 종격 림프절에서 STAT3 저해제인 STA-21 가 공중 단백질분해효소 항원에 대한 여포 도움 T 세포 분화와 배발생반응을 억제한다는 것을 확인하였다. T 세포가 결핍된 수혜자 생쥐에 양자전이된 STAT3-결핍 OT-II T 세포는 야생형 OT-II T 세포에 비해 여포 도움 T 세포의 빈도가 감소하는 결과를 보였으며, 공중알레르겐 특이적인 면역글로불린 G 와 면역글로불린 E 의 약화를 유도하였다. 야생형 OT-II T 세포와

STAT3-결핍 T 세포의 공동-이식 실험에서 STAT3-결핍 T 세포는 여포 도움 T 세포로 분화되지 못했다. 이러한 발견은 STAT3 가 종격 림프절에서 기도까지 일어나는 제 2 형 도움 T 세포의 발달에 역동적이고 대립되는 역할을 하고 있으며, 기관지 림프절에서는 공중항원에 대한 여포 도움 T 세포의 분화에 필요하다는 것을 입증한다. 그러므로 본 연구는 폐질환 치료를 위한 STAT3-표적화 접근법은 주의깊은 고려가 필요하다는 것을 제안한다.

주요어 : STAT3, 공중알레르겐, 제 2 형 보조 T 세포, 여포 보조 T 세포, 배중심반응, 면역글로불린

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