Blockade of STAT3 in T Cells Inhibits Germinal Center Reactions against Intranasal Allergens

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Abstract

Understanding the developmental mechanisms of humoral immunity against intranasal antigens is essential for the development of therapeutic approaches against air-borne pathogens as well as allergen-induced pulmonary inflammation. Follicular helper T (Tfh) cells expressing CXCR5 are required for humoral immunity by providing IL-21 and ICOS costimulation to activated B cells. However, the regulation of Tfh cell responses against intranasal antigens remains unclear. Here, we found that the generation of Tfh cells and germinal center B cells in the bronchial lymph node against intranasal proteinase antigens was independent of TGF-β. In contrast, administration of STAT3 inhibitor STA-21 suppressed the generation of Tfh cells and germinal center B cells. 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 specific for the intranasal antigens. 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 that T cell-intrinsic STAT3 is required for the generation of Tfh cells to intranasal antigens and that targeting STAT3 might be an effective approach to ameliorate antibody-mediated pathology in the lung.

Key Words: STAT3, Intranasal allergens, Tfh cell, Germinal center reactions, Immunoglobulin

INTRODUCTION

Induction of antigen-specific antibodies is one of the key features of adaptive immunity upon encountering environmental antigens including infectious agents as well as allergens. While the generation of high-affinity antibodies to infectious agents is beneficial to hosts, the production of IgE can trigger anaphylactic shock and allergic tissue inflammation (Gould et al., 2003; Gould and Sutton, 2008). The generation of high-affinity antibodies and memory B cell requires help from T cells by facilitating germinal center reactions (Liu et al., 1992; MacLennan, 1994; Crotty, 2011). In particular, our respiratory system is exposed to an external environment and continuously encounters various types of foreign antigens such as airborne pathogens, pollution, and allergens (Pauli et al., 1988; Albright and Goldstein, 1996). However, the cellular and molecular mechanisms governing the development of germinal center reactions to airborne antigens remain unclear. Elucidating such mechanisms will pave the development of efficacious vaccines to airborne pathogens and therapeutic approaches for the treatment of allergic lung inflammation.

Upon antigenic stimulation, naïve CD4+ T cells can be differentiated into at least 5 distinct effector T cells including Th1, Th2, Th17, follicular helper T (Tfh), and Foxp3+ regulatory T cells depending on microenvironmental cytokine milieu (Dong, 2006; Cua and Tato, 2010; Nurieva and Chung, 2010). Among them, 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 (Breitfeld et al., 2000; Vinuesa et al., 2005; Crotty, 2011). 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 (Batten et al., 2010; Eto et al., 2011). In addition to these cytokine signals, interaction of the activated T cells with B cells activated with the same antigen at T:B border is required for the differentiation of Tfh
cells (Chang and Chung, 2014). 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 (Doganci et al., 2005; Neveu et al., 2010; Rincon and Irvin, 2012). Hence, Tfh cells are likely induced and play a crucial role in modulating antibody responses in allergic inflammation as well as in pulmonary infections.

Lung environment is unique in that it favors the differentiation of Th2 cells and Th17 cells rather than that of Th1 cells. For instance, Listeria monocytogenes infection via parental routes triggers Th1 cell dominant responses with little Th2 and Th17 cell responses (Pepper et al., 2010; Chung et al., 2013). The same pathogen, however, induces Th17 dominant responses with little Th1 cells in the lung when administered via intranasal route. Similarly, protein antigens including allergens predominantly trigger Th2 cell responses in the lung while the same antigens elicit Th1 and Th17 cell responses (Lloyd and Hessel, 2010; Zhao et al., 2013). The mechanisms by which lung environment preferentially induces Th2 and Th17 cells while suppressing Th1 cell responses are unclear; however, the differentiation of allergen-induced Th17 cells depends on TGF-β. In addition, the regulation of Tfh cell immunity in the lung remains to be determined.

In the present study, we aimed to dissect the kinetics of Tfh cells and germinal center reactions as well as molecular requirements of such immunity against intranasal antigens by using an allergen-induced animal model of lung inflammation. Our results indicate that Tfh cell responses preceded the development of germinal center B cell reactions as well as the production of allergen-specific immunoglobulins. In addition, the pulmonary Tfh cell responses were independent of TGF-β, but depended on STAT3 in a T cell intrinsic manner.

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). STAT3flox/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 a 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 of 7 μg of proteinase extracted from Aspergillus melleus (Sigma, St Louis, MO, USA) and 20 μg of Ovalbumin (Ova; Grade V, Sigma, St Louis, MO, USA) (Asp/Ova) in 50 μl of PBS (Katy, TX, USA) every two days for a total of five times (day 0, 2, 4, 6, 8). Sixteen hours after the last challenge, all mice were euthanized and the bronchial lymph nodes, superficial cervical lymph nodes and sera were obtained for further analysis. For TGF-β neutralization experiments, mice were injected intraperitoneally with 200 μg of an anti-TGF-β neutralizing antibody (1D11, BioXCell, West Lebanon, NH, USA) or their corresponding IgG1 control (MOPC-21, 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 0.5 mg/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 0.25 mg/kg STA-21 or vehicle every other day for 9 days (day 1, 3, 5, 7).

Flow cytometry

For T cell analysis, the 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-CXCR5 (eBioscience, San Diego, CA, USA). For phenotype analysis, FITC-conjugated anti-CD86 (eBioscience, San Diego, CA, USA), PerCP-Cy5.5-conjugated anti-CD133 (Biolegend, San Diego, CA, USA) and FITC-conjugated anti-CD86 (BD bioscience, San Jose, CA, USA) were used. 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).

ELISA

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 3-fold or 5-fold serial dilution onto plates precoated 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).

Adoptive transfer studies

To examine the role of STAT3 on CD4+ T cells, naïve CD4+ T cells were isolated from either STAT3flox/flox–CD4-Cre(+)OT-II or STAT3flox/flox–CD4-Cre(-)OT-II mice by using a CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated naive CD4+ T cells (10x10^5 cells/transfer) were transferred into Tcrb−/− mice. In some experiment, naïve CD4+ T cells from STAT3flox/flox–CD4-Cre(+)OT-II mice (CD45.2+) and B6.SJL×OT-II mice (CD45.2+/CD45.1−) were mixed at a 5:1 ratio before transferring them into Tcrb−/− mice. Next day, the recipients were intranasally injected with Asp/Ova and the induction of Tfh cells, germinal center B cells, plasma B cells and Ova-specific immunoglobulin was 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.

ELISA

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Fig. 1. Kinetic analysis of Tfh and germinal center response in the intranasally challenged mice. Mice were intranasally injected with Asp/Ova every 2 days for 9 days and analyzed on day 3, 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 bronchial lymph nodes or superficial cervical lymph nodes from allergen-injected mice are plotted. Numbers around the outlined area are the percentage of cells expressing each marker. (C) Ova-specific immunoglobulins were detected in sera obtained from mice on day 3, 9 and 13. Data are means ± SEM. n=3.
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

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 allergic challenges, we employed an animal model of allergic asthma induced by Aspergillus protease and ovalbumin (Asp/Ova), as previously described (Chung et al., 2013; Lim et al., 2013). Groups of C57BL/6 mice were intranasally challenged with Asp/Ova every other day before lymphoid cells from bronchial lymph node (bLN) or superficial cervical lymph node (scLN) were analyzed for the frequencies of CXCR5+PD-1+ Tfh cells, GL7+CD95+ germinal center B cells, and B220-CD138+ plasma cells. As shown in Fig. 1A, B, the magnitudes of Tfh cells, germinal center B cells and plasma cells were all remarkably higher in the bLN than those in the scLN. Kinetic analysis of Tfh cell responses in the bLN 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 bLN, 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 bLN, the serum levels of Ova-specific IgE, IgG1, IgG2b were highest at day 13 compared with those of day 3 and day 9 (Fig. 1C). The levels of Ova-specific IgG2c appeared to be higher at day 9 than those of day 13. These results indicate that bLN is the lymphoid organ 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 bLN. TGF-β signal is required for the differentiation of Th17 cells and regulatory T cells; however, its role in the Tfh cell and germinal center responses has been controversial (McCarron and Marie, 2014; Schmitt et al., 2014; Marshall et al., 2015). 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, GL7+CD95+ germinal center B cells and B220-CD138+ plasma cells in the bLN compared with those of a control Ab-
treated group (Fig. 2). 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 recent study showed that STAT3 differentially regulates pulmonary Th17 and Th2 cells (Lim et al., 2015). STAT3 signal in T cells is known to mediate the generation of Tfh cells (Nurieva and Chung, 2010). Therefore, we next asked...
**Fig. 5.** STAT3-deficient CD4⁺ T cell failed the development of germinal center response in the bronchial lymph nodes. (A) The proportion of GL7⁺CD95⁺ cells among B220⁺ cells and B220⁻CD138⁺ cells among lymphocytes in the bronchial lymph nodes 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.

**Fig. 6.** 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 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 bronchial lymph nodes was 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.
if inhibition of STAT3 impacts 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. 3A, 3B shows the frequency of CCR5<sup>−</sup>PD-1<sup>−</sup> Tfh cells was lower in the STA-21-treated group than in the vehicle-treated group. Similarly, the frequencies of GL7<sup>−</sup>CD95<sup>+</sup> germinal center B cells and B220<sup>−</sup>CD138<sup>−</sup> plasma cells were significantly decreased in the STA-21 treated group (Fig. 3C, 3D). These data indicate that a STAT3 inhibitor STA-21 suppressed the generation of Tfh cell and germinal center B cell responses in the bLN 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<sup>+</sup> T cell during antigen-specific responses in vivo, we crossed OT-II mice harboring T cell receptor specific to Ova<sub>323-339</sub> in the context of MHC II with mice lacking STAT3 in CD4<sup>+</sup> cells (CD4<sup>STAT3<sup>−/−</sup></sup>) to obtain CD4<sup>STAT3<sup>−/−</sup></sup> OT-II mice. CD4<sup>+</sup> T cells from CD4<sup>STAT3<sup>−/−</sup></sup> OT-II mice or CD4<sup>STAT3<sup>−/−</sup></sup> OT-II mice were isolated and adoptively transferred into Torb<sup>−</sup> mice before the recipients were challenged with intranasal Asp/Ova. Compared with that of the CD4<sup>STAT3<sup>−/−</sup></sup> OT-II T cell recipients, the frequency of CCR5<sup>−</sup>PD-1<sup>−</sup> Tfh cells in the recipients of CD4<sup>STAT3<sup>−/−</sup></sup> OT-II T cell was significantly diminished (Fig. 4). On the other hand, the frequency of CCR5<sup>−</sup>PD-1<sup>−</sup>Foxp3<sup>−</sup> follicular regulatory T (Tfr) cells was not reduced in the latter group. These data indicate that STAT3 signal pathway in CD4<sup>+</sup> T cell was essential for the generation of Tfh cells.

We next sought to determine whether STAT3-deficiency in CD4<sup>+</sup> T cell also impacted the magnitude of germinal center responses. As shown in Fig. 5A, 5B, there was a marked decrease in the percentage of GL7<sup>−</sup>CD95<sup>+</sup> germinal center B cells in the recipients of CD4<sup>STAT3<sup>−/−</sup></sup> OT-II T cell compared with that in the recipients of CD4<sup>STAT3<sup>−/−</sup></sup> OT-II T cell. Similarly, the percentage of B220<sup>−</sup>CD138<sup>−</sup> 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. 5C). Collectively, these data indicate that the STAT3 signal in antigen-specific CD4<sup>+</sup> T cells not only mediated Tfh cell differentiation but also facilitated 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 were due to T cell-intrinsic function of STAT3 or the lack of appropriate signals from STAT3-sufficient T cells, we mixed STAT3-sufficient OT-II T cells (CD45.1<sup>+</sup>/CD45.2<sup>−</sup>) with CD4<sup>STAT3<sup>−/−</sup></sup> OT-II T cells before transferring them into Torb<sup>−</sup> mice. Compared with STAT3-sufficient OT-II T cells, CD4<sup>STAT3<sup>−/−</sup></sup> OT-II T cells showed a significantly less percentage of CCR5<sup>−</sup>PD-1<sup>−</sup> Tfh cell in the same recipients (Fig. 6). These data indicate that T cell intrinsic STAT3 signal was crucial for the differentiation of naive T cells into Tfh cells in vivo in the bLN upon intranasal allergenic challenges.

**DISCUSSION**

Regulatory mechanism of germinal center reactions in the BALT remains unclear. In the present study, we found that the generation of Tfh cells and germinal center B cell responses was 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 bLN, (ii) STAT3-deficient T cells failed to be differentiated into Tfh cells in the bLN, (iii) Tcrb<sup>−</sup> 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 suggest that targeting STAT3 signaling in T cells might be effective to suppress the production of unnecessary humoral immunity to inhaled allergens.

TGF-β signal is required for the differentiation of Th17 cells and induced regulatory T cells. TGF-β has been originally suggested to be indispensable for the generation of Tfh cells in vivo (Nurieva et al., 2008), or even inhibit Tfh cell immunity (Mc Carron and Marie, 2014). However, a recent study showed that TGF-β signal enhances the differentiation of human Tfh cells by promoting the generation of Bcl6<sup>+</sup>RORγT<sup>+</sup> T cells upon STAT3 and STAT4 activation (Schmitt et al., 2014). Furthermore, TGF-β signal has been shown to facilitate Tfh cells during acute viral infection by attenuating IL-2 signals (Marshall et al., 2015). In the present study, however, administration of neutralizing antibody to TGF-β exhibited little effects on the frequencies of Tfh cells, germinal center B cells, and plasma cells. Hence, it is likely that blockade of TGF-β can minimally affect the generation of allergen-specific immunoglobulins in the BALT.

STAT3 activation is a common requirement for the differentiation of Th17 cells and Tfh cells (Nurieva et al., 2008). STAT3 has been shown to antagonize STAT5 signal by competing their common binding sites (Yang et al., 2011) during Th17 cell differentiation. STAT5 signal is also a negative regulator of Tfh cell differentiation (Johnston et al., 2012; Nurieva et al., 2012), 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 bLN. 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. Our recent study showed that STAT3 deficiency significantly increased the frequency of pulmonary Th1 cells (Lim et al., 2015). 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 (Kemp and Lockey, 2002; Akin, 2015). Suppression of allergen-specific humoral responses might provide beneficial therapeutic ef-
ffects in patients with allergy. Our findings propose that 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 including allergic asthma.

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