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STAT6 Controls the Number of Regulatory T Cells In Vivo, Thereby Regulating Allergic Lung Inflammation

Nicolas J. Dorsey,*† Svetlana P. Chapaoval,*† Elizabeth P. Smith,‡ Jonathan Skupsky,§ David W. Scott,¶ and Achsah D. Keegan*†‡

STAT6 plays a central role in IL-4–mediated allergic responses. Several studies indicate that regulatory T cells (Tregs) can be modulated by IL-4 in vitro. We previously showed that STAT6−/− mice are highly resistant to allergic lung inflammation even when wild-type Th2 effectors were provided and that they have increased numbers of Tregs. However, the role of STAT6 in modulating Tregs in vivo during allergic lung inflammation has not been thoroughly investigated. To examine Treg and STAT6 interaction during allergic inflammation, STAT6−/−, STAT6xRAG2−/−, and RAG2−/− mice were subjected to OVA sensitization and challenge following adoptive transfer of OVA-specific, wild-type Th2 effectors with or without prior Treg depletion/inactivation, using anti-CD25 (PC61). As expected, STAT6−/− mice were highly resistant to airway inflammation and remodeling. In contrast, allergic lung inflammation was partially restored in STAT6−/− mice treated with PC61 to levels observed in STAT6xRAG2−/− mice. In some cases, STAT6xRAG2−/− mice were also given natural Tregs along with Th2 effectors. Adoptive transfer of natural Tregs caused a substantial reduction in bronchoalveolar lavage eosinophil composition and suppressed airway remodeling and T cell migration into the lung in STAT6xRAG2−/− mice to levels comparable to those in STAT6−/− mice. These results demonstrate the STAT6–dependent suppression of Tregs in vivo to promote allergic airway inflammation. The Journal of Immunology, 2013, 191: 1517–1528.

D4+ Th2 cells are pivotal for the induction of allergic asthma (1–4). These cells secrete IL-4, 5, and 13 cytokines and cause change in the airways, including excessive mucus production, bronchial smooth muscle thickening, and eosinophilic inflammation (1, 5–9). IL-4 and IL-13 both bind to subunits of the IL-4 receptors to cause receptor activation and initiation of a complex signaling cascade that results in phosphorylation and induction of STAT (signal transducer and activator of transcription) 6, a transcription factor (TF), which has been shown to be important for Th2 differentiation and propagation of the allergic response (10–13). This allergic asthmatic response will not normally occur because of immune tolerance to allergens established by regulatory T cells (Tregs) (14–17). Tregs are CD4+CD25+Foxp3+ T cells that regulate immune responses of effector lymphocytes (18–21). Tregs can be divided into two main groups. The first group, natural (n) Tregs, exit the thymus as CD4+CD25+Foxp3+ T cells (22–24). The second group, inducible (i) Tregs, leave the thymus as CD4+CD25+Foxp3− and convert to CD4+CD25+Foxp3+ Tregs only after Ag exposure, in the presence of TGF-β (25, 26). Both types of regulatory T cells have been shown to regulate allergic lung inflammation (16, 17, 27–29).

Various studies have indicated that immunosuppression and tolerance elicited by Foxp3+ Tregs are blocked by the Th2 cytokines IL-4/13 (15, 30, 31). In fact, different interaction may take place between the key Th2 TFs (Gata3 and STAT6) and Treg TF (Foxp3): Foxp3 can bind Gata3 to block expression of IL-5 and Th2 differentiation, whereas IL-4 and Gata3 hinder the in vitro differentiation of naive CD4+ T cells into Foxp3+ Tregs, in the presence of TGF-β, and reduce their ability to suppress T cell proliferation (15, 32). Furthermore, in vitro experiments suggest an antagonistic interaction between STAT6 and Foxp3. IL-4 suppressed in vitro TGF-β–mediated induction of Foxp3 in a STAT6–dependent manner, and a STAT6 binding site has been localized within the Foxp3 promoter (32, 33). However, the role of STAT6 in the control of Tregs during allergic airway inflammation in vivo has not been elucidated.

STAT6-deficient mice were previously shown to be highly resistant to allergic airway inflammation (34–36). This observation was not surprising, given that STAT6 is important for Ag-induced Th2 cell differentiation, Th2 migration, and other characteristics of IL-4–mediated allergic airway inflammation (10, 13, 34, 37, 38). However, we found that STAT6−/− mice were still resistant to allergic lung inflammation even when provided with wild-type (WT) bone marrow or WT Th2 effectors (34). Of additional interest, when STAT6−/− mice were crossed onto a lymphocyte-deficient genetic background (RAG2−/−), STAT6xRAG2−/− mice were able to develop moderate eosinophilic airway inflammation in the presence of WT Th2 cells (34). These results suggest that a Rag2–dependent cell type was able to efficiently suppress allergic inflammation. Furthermore, we showed that STAT6−/− mice had twice the number of CD4+CD25+Foxp3+ Tregs in their lungs and spleen compared with WT mice under both steady-state and inflammatory conditions.

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Abbreviations used in this article: ASM, airway smooth muscle; BAL, bronchoalveolar lavage; HFP, high-power field; IHC, immunohistochemistry; (T)reg, inducible (i) Treg; LN, lymph node; nTreg, natural Treg; PAS, periodic acid–Schiff; T eff, effector T cell; TF, transcription factor; Treg, regulatory T cell; Tr1, IL-10–Treg cell; WT, wild-type.
conditions, whereas STAT6+/− and STAT6−/− nTregs were equally efficient in suppressing in vitro T cell proliferation responses (34). These results suggested the hypothesis that STAT6−/− mice are highly resistant to allergic airway inflammation because they have increased numbers of Tregs. To test this hypothesis, we first depleted/inactivated Tregs in STAT6−/− mice prior to transfer of Th2 effector cells and exposed the mice to allergen, using a modified version of a classic murine OVA-induced allergic asthma model (39, 40). In this article, we demonstrate a significant restoration of autoimmune allergic inflammation owing in part to their increased Treg cell population.

Materials and Methods

Mice

BALB/c STAT6−/− mice were previously generated and described (10, 34) and were bred in the Association for Assessment and Accreditation of Laboratory Animal Care–approved animal care center at the University of Maryland, Baltimore, Maryland. STAT6+/− mice were crossed to Rag2−/− mice to generate STAT6+/− Rag2−/− mice (34, 39). WT (BALB/c) Rag2−/− and D011.10 Rag2−/− mice were purchased from Taconic (Germantown, NY). The D011.10 Foxp3GFP(KI) mice were previously generated by crossing Foxp3GFP(KI) mice on a BALB/c background with D011.10 TCR transgenics and bred thereafter in the University of Maryland, Baltimore, animal care facility (19, 41). C57BL/6 STAT6−/− mice were obtained from Dr. Jonathan Bromberg (University of Maryland, Baltimore). All procedures described were performed in agreement with the animal protocol approved by the Institutional Animal Care and Use Committee at the University of Maryland, Baltimore, School of Medicine.

FACS analysis

Single-cell suspensions made from spleens and lymph nodes (LNs) were passed through 40-μm cell strainers (BD Falcon) (42). RBCs were lysed in RBC lysing Buffer (Sigma-Aldrich, St. Louis, MO). Cells prepared from individual mice or cells pooled from mice in the same experimental group were stained using conjugated Abs to the following surface markers: CD4 (PE, PE-Cy7, or Alexa Fluor 647; BD Biosciences), CD8 (APC; BD Biosciences), CD25 (PE and PE-Cy7; BD Biosciences), mouse D011.10 TCR (D011.10xRag2−/− mice), mouse D011.10 Rag2−/−, and CD25−/−/− mice were stained using conjugated Abs to the following surface markers: CD4 (Clone PC61.5.3 clone of rat anti-mouse CD25 Ab (BioXCell)) was administered to STAT6−/− mice i.p. (0.03 mg/kg) 48 h prior to each sensitization to deplete/inactivate CD4+CD25+Foxp3+ Tregs, and Rat IgG1 isotype Ab (BioXCell) was used as a control (17, 43).

Adaptive cotransfer of CD4+ CD25+ Tregs or control CD4+ CD25− T cells and Th2 effector cells

To expand the CD4+CD25+Foxp3+ Treg cell population, D011.10 Foxp3GFP(KI) mice were injected with a complex of IL-2 Ab (JES6-1 clone; eBioscience) and recombinant murine IL-2 cytokine (PeproTech, Rocky Hill, NJ) at a 1:2 molar ratio, respectively, i.p. once daily for 3 d, as previously described (44, 45). At 3 d later, CD4+CD25+ Tregs or control CD4+CD25− T cells were isolated from harvested spleens and LNs of D011.10 Foxp3GFP(KI) mice. Then, 24 h prior to OVA sensitization, 9 × 10^3 cells were cotransferred i.p. to STAT6+/− Rag2−/− mice, along with in vivo primed Th2 effector cells (from D011.10 Rag2−/− mice) at 1:2 ratio, respectively. FACS analysis (using the above-mentioned Abs) indicates > 90% of purified CD4+CD25+ T cells are Foxp3+ (16, 46).

Allergen sensitization and challenge

Mice were immunized i.p. on days 1 and 6 with chicken egg OVA (Sigma-Aldrich) in Alum or with Alum alone. On days 12 and 14, all mice were challenged with aerosolized 1% OVA in PBS for 40 min, using an Intravac Envoy Nebulizer (39).

Assessment of allergic airway inflammation

At 48 h following the last allergen challenge, bronchoalveolar lavage (BAL) was performed on each mouse, and BAL cells were cytospun, stained, and enumerated, as described earlier (34, 39). Airway cytokine IL-5 was measured in BAL fluid by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Lung histology

As previously described (11), 10 ml PBS was used to flush lungs and to displace circulating blood. Lungs were removed and fixed in 10% formalin (Fischer Scientific, Fairland, NJ) at 25˚C for 120 min and subsequently stored in 70% ethanol. Lungs were paraffin embedded and divided into tissue sections. Deparaffinized serial lung sections were stained with H&E or periodic acid–Schiff (PAS) at the Histology Core at the University of Maryland School of Medicine. Peroxidase was inactivated by washing slides with PBS, followed by a 30-min soak in a 0.3% H2O2 solution. A 1:1000 dilution of rat anti-CDS3 (Serotec, Raleigh, NC) and a subsequent 1:200 dilution of biotinylated anti-rat mouse Abs (Vector Laboratories, Burlingame, CA) were used to stain the slides.

Evaluation of airway remodeling

Serial lung sections were stained with Masson’s trichrome after paraffin embedding to show collagen deposition (blue). Red indicates muscle fibers and keratin. Images (X40 magnification) of trichrome-stained lung sections were analyzed with National Institutes of Health Image J Software (National Institutes of Health, Bethesda, MD) to quantify the blue area stained for collagen relative to the entire area, as previously described (39, 47). On average, 40 airways per mouse group were analyzed. H&E-stained lung sections were used to determine the average thickness of the airway smooth muscle (ASM). National Institutes of Health Image J software was used to measure the cross-sectional diameter of the ASM layer at three distinct regions surrounding the airway lumen (39, 48). On average, 40 airways were measured for each mouse group.

Statistical analysis

Data averages are presented as mean ± SEM. To determine statistical significance and compare two groups, the two-tailed Student t test (Microsoft Excel) and F-test for variance (Microsoft Excel) or single factor ANOVA (Microsoft Excel) were used. A p value ≤ 0.05 or 0.001 was considered statistically significant.

Results

Treg depletion/inactivation restores allergic airway inflammation in STAT6−/− mice in an OVA-induced allergic lung inflammation model

In previous studies, we observed that BALB/c STAT6−/− mice bred and maintained in our animal facility had twice as many CD25−/−, Foxp3+ cells in the lungs and spleen as did STAT6+/− mice under basal and inflammatory conditions, using cyttoplasmic
staining to detect Foxp3 (34). To confirm this finding in additional lines, we analyzed the LN and spleens from untreated D011.10 Foxp3<sup>GFpKO</sup> and D011.10xSTAT6<sup>−/−</sup> Foxp3<sup>GFpKO</sup> mice for GFP expression (Supplemental Fig. 1A). We found that compared with D011.10 Foxp3<sup>GFpKO</sup>, mice D011.10xSTAT6<sup>−/−</sup> Foxp3<sup>GFpKO</sup> mice had five times the amount of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in their LN and twice as many Tregs in their spleen. Further, C57BL/6 STAT6<sup>−/−</sup> mice had twice the number of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs as did WT mice (Supplemental Fig. 1B). Therefore, the enhanced STAT6<sup>−/−</sup> nTregs in vivo, in addition to the TGFβ-mediated induction of iTregs shown in vitro (32). We hypothesized that if the observed increase in Treg numbers was responsible for the resistance of STAT6<sup>−/−</sup> mice to allergic airway inflammation, then Treg depletion/inactivation would re-store Th2-driven inflammation to levels observed in STAT6xRAG2<sup>−/−</sup> mice. To test this hypothesis, we provided OV A-specific Th2 cells to STAT6<sup>−/−</sup> and STAT6xRAG2<sup>−/−</sup> mice and immunized them as shown in Fig. 1A (39). In addition, 2 d prior to each sensitization, STAT6<sup>−/−</sup> mice were treated with the PC61 clone of anti-mouse CD25 Ab or a control IgG Ab. The use of PC61 to deplete/inactivate Tregs has been shown previously and is validated in Supplemental Fig. 2A (17, 43, 49–51).

Control STAT6<sup>−/−</sup> mice treated with IgG did not demonstrate a significant increase in airway eosinophilia in response to OVA priming (Fig. 1B). Eosinophils represented only 6 ± 0.9% of the cell population recovered from their BAL, and the cell composition was dominated by macrophages. However, when Tregs were depleted/inactivated from STAT6<sup>−/−</sup> mice, using PC61, the proportion of BAL eosinophils significantly increased to 16 ± 2.8% after OVA priming and challenge. STAT6<sup>−/−</sup> mice had the greatest extent of eosinophilic expansion in their BAL (40 ± 1.5%). The depletion/inactivation of Tregs from STAT6<sup>−/−</sup> mice also resulted in a 3-fold increase in total BAL eosinophils from IgG-treated STAT6<sup>−/−</sup> mice to STAT6<sup>−/−</sup> mice given PC61 (12,319 versus 39,045, respectively) (Fig. 1C). The largest numbers of eosinophils were recovered from the BAL of STAT6xRAG2<sup>−/−</sup> mice (80,715 cells). Efficiency of Treg depletion/inactivation was evaluated by FACS analyses of the lung-draining LNs after completion of the full experimental protocol (Supplemental Fig. 2B). Compared with the percentage of host CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in control STAT6<sup>−/−</sup> mice administered IgG (1.29%), the proportion of host Tregs was reduced by ∼50% in STAT6<sup>−/−</sup> mice given PC61 (0.78%). This consistent depression of Tregs was also observed when PBS was used as a control in place of IgG (data not shown).

**Histological changes in the lung following the depletion/inactivation of Tregs**

For the purpose of examining lung tissue eosinophilia, serial lung sections from STAT6<sup>−/−</sup> mice (treated with either PC61 or control Ab) and STAT6xRAG2<sup>−/−</sup> mice were stained with H&E (Fig. 2). Enlarged images can be seen in Supplemental Fig. 2C. IgG-treated STAT6<sup>−/−</sup> mice showed minor, insignificant increases in eosinophilic inflammation around airways or pulmonary vasculature after OVA priming and challenge (Fig. 2A, 2B). This result is consistent with previous observations of STAT6<sup>−/−</sup> mouse resistance to allergic lung inflammation (34). Conversely, the lungs of STAT6<sup>−/−</sup> mice with Treg depletion/inactivation revealed a significantly increased proportion of eosinophils adjacent to their airways and vasculature (19 ± 2.8% and 35 ± 1.9%, respectively) (Fig. 2A). The degree of eosinophilic inflammation in PC61-treated STAT6<sup>−/−</sup> mice was similar to that of STAT6xRAG2<sup>−/−</sup> mice, in which eosinophils composed 36% of the cells surrounding the airway lumen and almost half of the cells adjacent to the pulmonary vasculature (Fig. 2A). STAT6xRAG2<sup>−/−</sup> mice consistently developed moderate levels of eosinophilic lung inflammation. These results demonstrate that resistance to eosinophilic lung infiltration in STAT6<sup>−/−</sup> mice in the presence of WT Th2 cells can be reversed after Treg depletion/inactivation. PAS staining of lung sections revealed that in contrast to OVA-induced mucus production in WT mice, there was no increase in mucus production by epithelial cells in OVA-primed STAT6<sup>−/−</sup> or STAT6xRAG2<sup>−/−</sup> mice (Supplemental Fig. 3A). This observation was not unexpected because epithelial cell STAT6 expression is...
required for mucus production and the experimental mice were all STAT6 deficient (35–37). Although the epithelial expression of STAT6 is also required for eotaxin production (38), the production of IL-5 by Th2 cells is sufficient to support eosinophilic inflammation (36, 39).

Effect of Treg depletion/inactivation on remodeling of the airway during allergic inflammation

Airway remodeling is a hallmark feature of allergic asthma that can be analyzed by measuring the extent of collagen deposition and the thickness of the smooth muscle layer surrounding the airway lumen (52–54). To determine the extent of pulmonary collagen deposition, lung sections from STAT6−/− and STAT6xRAG2−/− mice were stained with Masson’s Trichrome (Fig. 3A). Image J software was used to quantify the average area of collagen. A small increase in collagen deposition was detected in the lungs of STAT6−/− mice given IgG (9.48 ± 0.26%) (Fig. 3B). We observed an increase in collagen in the lungs of Treg-depleted/inactivated STAT6−/− mice (14.66 ± 0.38%) to levels comparable to those found in STAT6xRAG2−/− mice (12.35 ± 0.35%).

The transverse diameter of the ASM layer surrounding the airway was quantified using Image J software analysis of H&E-stained lung sections (Fig. 3C, 3D). OVA priming and challenge increased the ASM thickness in all experimental groups. However, the average smooth muscle cell thickness in the Treg-depleted/inactivated STAT6−/− mice was increased 2-fold, compared with that in control STAT6−/− mice given IgG (Fig. 3D) (39, 47, 48). This smooth muscle layer thickness was similar to that observed in STAT6xRAG2−/− mice (Fig. 3D). These results show that STAT6−/− mice can be made more susceptible to airway remodeling by the depletion/inactivation of their Tregs.

Treg depletion/inactivation in STAT6−/− mice allows for increased T cell migration and recruitment to the lung during allergic inflammation

Previous studies have shown that Tregs are able to block egress of effector T cells (Teffs) from the site of immunization and prevent them from migrating into the tissue targeted for inflammation (55). Moreover, Tregs have demonstrated their ability to suppress adhesion between endothelial cells and Teffs and to modulate T cell recruitment (56). Therefore, we analyzed the number of CD3+ T cells that migrated into the lungs of STAT6xRAG2−/− mice by immunohistochemistry (IHC) (Fig. 4). IgG-treated STAT6−/− mice had very low numbers of CD3+ T cells in the areas adjacent to their airways (Fig. 4). However, PC61-treated STAT6−/− mice whose Tregs were

![FIGURE 2.](http://www.jimmunol.org/)

A) The percentage of eosinophils surrounding airways or blood vessels was quantified by differential counting in 9–15 HPF per group. Percentages are represented graphically ± SEM (n = 3–5 mice/group). Data are representative of three independent experiments. *p < 0.05, n.s., Nonstatistical significance (p > 0.05).

B) Representative H&E images of lung sections from OVA-primed mice adjacent to the airway lumen (left) or bordering the pulmonary vasculature (right) are shown at original magnifications ×10, ×40, and ×100. Arrowheads identify eosinophils surrounding the airway or lung vasculature.
depleted/inactivated demonstrated a significant increase in the number of CD3⁺ T cells that were able to migrate into their lungs after OVA priming and challenge; this was comparable to the high number of CD3⁺ T cells localized to the lungs of STAT6−/− mice (Fig. 4). These results suggest that the 2-fold increase in Treg numbers in STAT6−/− mice can suppress T cell migration to the lung.

FIGURE 3. Airway remodeling in Treg-depleted/inactivated STAT6−/− mice. STAT6−/− and STAT6xRAG2−/− mice were subjected to the allergic asthma protocol described in Fig. 1. (A) Masson’s trichrome stain was applied to paraffin-embedded lung sections of each mouse. Collagen stains blue; keratin, muscle fibers, and erythrocytes stain red. The cytoplasm stains reddish pink. Collagen deposition is shown in photographs at original magnifications ×10 and ×40, as indicated, and ×100 (insets). (B) National Institutes of Health Image J software was used to quantify total collagen in the lung. The average percent area of collagen ± SEM (stains blue) is represented graphically. *p < 0.001, n.s., Nonstatistical significance (p > 0.001, n = 30–50 airways per group). (C) The ASM layer thickness was evaluated in H&E-stained lung sections from each mouse group (original magnification ×40). The cross-sectional thickness of the ASM layer is delineated by arrows. (D) The transverse distance between the inner- and outermost border of the ASM layer was measured at three points adjacent to each airway, using National Institutes of Health Image J software analysis. The average diameter of ASM layer thickness (μm ± SEM) is represented graphically. *p < 0.001, n.s., Nonstatistical significance (p > 0.001).

FIGURE 4. Treg depletion during the induction of allergic lung inflammation enhances CD3⁺ T cell migration into the lung. STAT6−/− and STAT6xRAG2−/− mice were subjected to the allergic asthma protocol described in Fig. 1. Lung sections were stained with Abs to CD3. (A) CD3⁺ cells appear brown in representative images (original magnifications ×10, ×40, and ×100) of lung sections from OVA-primed mice. (B) Graphical representation of IHC data. The number of CD3⁺ cells in each lung section was quantified and graphed. Data represented as cell counts ± SEM. HPF, original magnification ×100. *p < 0.05, n.s. indicates nonstatistical significance (p > 0.05).
Adoptive transfer of transgenic, GFP-labeled CD4+CD25+ Foxp3+ nTregs suppresses allergic lung inflammation

We have previously shown that when provided with WT Th2 effectors, STAT6xRAG2−/− mice were able to develop moderate levels of eosinophilic allergic lung inflammation, whereas STAT6+/− mice were not (34). STAT6xRAG2−/− mice lack Tregs owing to their inability to develop mature T lymphocytes, whereas STAT6−/− mice had twice as many Tregs as did STAT6+/+ mice (34). Therefore, the ability of Th2 cells to drive allergic lung inflammation in STAT6xRAG2−/− mice may be due to their complete absence of Tregs. To test this possibility, we enriched CD4+CD25+ nTregs from untreated D011.10 Foxp3-GFPKI mice. We also prepared CD4+CD25− cells from these mice to use as negative controls. The enriched Treg population was 76% CD4+CD25+, whereas the control T cell population was only 4% CD4+CD25+. As expected, >90% of CD4+CD25+ Treg cells expressed Foxp3 at high levels whereas most (>95%) CD4+CD25− cells were Foxp3− (Supplemental Fig. 4A).

The CD4+Foxp3+Foxp3− nTregs or, as a control for cell transfer, CD4+CD25− T cells were adoptively cotransferred to STAT6xRAG2−/− mice with OVA-specific Th2 cells at a 1(Tregs):2 (CD4 Th2 effector cells) ratio. At 24 h later, the mice were immunized with OVA/Alum twice. All mice were challenged with aerosolized OVA twice, and 2 d later, the degree of allergic lung inflammation was assessed (Fig. 5A).

To evaluate the effectiveness of adoptive cell transfer, lung-draining LNs and spleens were collected from the mice following completion of the experiment. Supplemental Fig. 4B shows flow cytometric analysis of donor KJ126+ T cells obtained from STAT6xRAG2−/− mice. Only 1.5% of lung-draining LN cells from STAT6xRAG2−/− mice that received CD4+CD25+ T cells expressed Foxp3. In contrast, STAT6xRAG2−/− mice that received CD4+CD25+ nTregs contained a higher proportion of donor CD4+Foxp3+ T cells (5.73%) in their lung-draining LNs. A similar difference was also observed in the spleen. The percentage of CD4+Foxp3+ splenocytes increased 10-fold, from ~3.8% in STAT6xRAG2−/− mice that received CD4+CD25− T cells to ~38% in STAT6xRAG2−/− mice given Tregs.

STAT6xRAG2−/− mice that received CD4+CD25− T cells along with Th2 effectors contained a high proportion of eosinophils in their BAL in both the alum- (15%) and OVA-primed (38%) groups. Furthermore, the relatively elevated level of eosinophilia observed in the alum-primed, OVA-challenged group was suppressed by the Treg transfer. In addition, the transfer of Tregs to STAT6xRAG2−/− mice reduced the total number of BAL eosinophils 13-fold, from 92,468 to 7965 cells in the OVA-primed group; the reduction in the allergic airway response was also confirmed by histological analysis (Fig. 5B).

FIGURE 5. Adoptive transfer of nTregs into STAT6xRAG2−/− mice suppresses Th2-driven airway eosinophilia. (A) STAT6−/− and STAT6xRAG2−/− mice received in vivo primed D011.10 CD4+ Th2 effectors and were immunized twice with Alum or OVA/Alum and challenged with OVA on two occasions 6 d apart. STAT6xRAG2−/− mice also received CD4+CD25+ Tregs or CD4+CD25− T cells prepared from D011.10 Foxp3-GFPKI at a 1:Treg:2 (CD4 Th2 effector cells) ratio. At 24 h later, the mice were immunized with OVA/Alum twice. All mice were challenged with aerosolized OVA twice, and 2 d later, the degree of allergic lung inflammation was assessed (Fig. 5A).
eosinophil number was even more evident in the alum-primed group. No eosinophils were recovered from the BAL of STAT6−/− mice in this experiment (Fig. 5C).

The Th2 cytokine IL-5 modulates eosinophil development and mediates its activation (57, 58). Furthermore, IL-5 induces the release of necessary signals to allow eosinophil migration and infiltration into the airway after exposure to an allergen (58, 59). To determine if the transferred nTregs could suppress airway IL-5 production by the transferred Th2 effectors, we assessed the concentration of IL-5 in BAL fluid recovered from all experimental mice (Fig. 5D). OVA-immunized STAT6xRAG2−/− mice given CD25− T cells demonstrated a high concentration of airway IL-5 production. The amount of IL-5 recovered was substantially reduced when OVA-sensitized STAT6xRAG2−/− mice were infused with nTregs. Likewise, STAT6−/− mice treated with OVA did not elicit increased airway IL-5 secretion.

To determine the extent of eosinophilic inflammation in lung tissue sections, H&E staining was performed and the amount of inflammation was quantified (Fig. 6). Enlarged images can be seen in Supplemental Fig. 4C. Histological analysis revealed extensive eosinophilic infiltration of the airway in the lungs of control OVA-primed and -challenged STAT6xRAG2−/− mice that received CD4+CD25− T cells (Fig. 6B). The transfer of CD4+CD25+ nTregs to STAT6xRAG2−/− mice led to an approximate 3-fold reduction in the proportion of eosinophils adjacent to the airway, from 37 to 12% (Fig. 6A). As expected, very few eosinophils were observed surrounding the airways of STAT6−/− mice (9 ± 0.9%). Accumulations of eosinophils were noted around the pulmonary vasculature and in the lung interstitium in STAT6xRAG2−/− mice that were given CD4+CD25− T cells (57 ± 5.4%) (Fig. 6A). The transfer of nTregs to these mice reduced lung eosinophilia by > 50%. Similarly, a low degree of pulmonary eosinophilia was observed in STAT6−/− mice. PAS staining of lung sections did not demonstrate mucus production by epithelial cells in OVA-primed STAT6−/− or STAT6xRAG2−/− mice (Supplemental Fig. 3B), similar to findings in the PC61 studies.

nTregs suppress allergen-induced collagen deposition and ASM layer thickening in STAT6xRAG2−/− mice

To determine if airway remodeling correlated with the patterns of eosinophilic inflammation, collagen deposition and ASM thickness were measured, as previously described (39, 47, 48), in
STAT6xRAG2−/− mice that received CD4⁺CD25⁺ nTregs or CD4⁺CD25− T cells along with Th2 effectors and in STAT6−/− mice (given Th2 effectors) (Fig. 7). Collagen deposition in the larger main and lobar bronchi was present in all OVA-sensitized mouse groups, but there were differential amounts of collagen production deeper into the respiratory tree surrounding the smaller and intermediate-sized terminal bronchiolar airways (Fig. 7A). Dense deposits of collagen were present in the neighborhood of the airways of STAT6xRAG2−/− mice that received CD4⁺CD25− T cells (17.7 ± 0.48%) (Fig. 7B). In contrast, when STAT6xRAG2−/− mice were provided with nTregs, bronchiolar collagen deposition was reduced to 12 ± 0.42% (Fig. 7B). Likewise, STAT6−/− mice also demonstrated a low degree of collagen deposition (10 ± 0.4%) (Fig. 7B). These results were mirrored by ASM thickness measurements (Fig. 7C). Control STAT6xRAG2−/− mice that received CD4⁺CD25− T cells produced the highest ASM thickness, and the cross-sectional width was decreased by ∼50% when STAT6xRAG2−/− mice were given Tregs (Fig. 7D). ASM measurements were comparable between Treg-treated STAT6xRAG2−/− mice and STAT6−/− mice (17.9 μm versus 15.1 μm, respectively) (Fig. 7D).

The effect of Treg adoptive transfer on T cell migration into the lungs of STAT6xRAG2−/− mice during allergic lung inflammation

To determine if the transferred Tregs could suppress T cell migration into the lung, we quantified the number of CD3⁺ T cells in the lungs of STAT6xRAG2−/− mice and STAT6−/− mice (Fig. 8). STAT6xRAG2−/− mice infected with Th2 effectors and CD25− cells had the highest numbers of CD3⁺ T cells in their lungs. This amount was significantly reduced when STAT6xRAG2−/− mice were given Th2 effectors and CD4⁺CD25⁺ nTregs [27 ± 1.4 cells per high-power field (HPF) versus 7 ± 1.3 cells per HPF, respectively] (Fig. 8B). In fact, a similar number of CD3⁺ T cells were seen in the lungs of STAT6−/− mice and Treg-infused STAT6xRAG2−/− mice (7 ± 1.3 cells per HPF versus 5 ± 1.3 cells per HPF, respectively) in both the alum-primed, OVA-challenged and OVA-primed, OVA-challenged groups. These results demonstrate that the adoptive transfer of nTregs prevents the migration of T cells to the lungs in response to OVA inhalation.

Discussion

The studies reported in this article show that STAT6−/− mice are highly resistant to Th2-driven allergic lung inflammation in part because they have increased numbers of Tregs. Furthermore, they provide evidence that STAT6 suppresses Tregs in vivo during allergic lung inflammation. STAT6 is important for the propagation of IL-4-mediated allergic inflammation (10, 13, 35, 37). Indeed, Shimoda and colleagues (13) have shown that STAT6 plays a pivotal role in Th2 cell differentiation. Therefore, initial observations that STAT6−/− mice were highly resistant to allergic lung inflammation were not unexpected (34, 35). However, when STAT6−/− mice repeatedly failed to elicit allergic inflammation.

FIGURE 7. Airway remodeling after the transfer of Tregs into STAT6xRAG2−/− mice. STAT6−/− and STAT6xRAG2−/− mice were subjected to the allergic asthma protocol described in Fig. 4. (A) Masson’s trichrome stain was applied to paraffin-embedded lung sections of each mouse. Collagen stains blue; keratin, muscle fibers, and erythrocytes stain red. The cytoplasm stains reddish pink. Collagen deposition is shown in representative photographs at original magnifications of ×10 and ×40, as indicated, and ×100 (inset). (B) National Institutes of Health Image J software was used to quantify total collagen deposition of STAT6−/− and STAT6xRAG2−/− mice (given Th2 effectors) versus 7 ± 1.3 cells per HPF, respectively] (Fig. 8B). In fact, a similar number of CD3⁺ T cells were seen in the lungs of STAT6−/− mice and Treg-infused STAT6xRAG2−/− mice (7 ± 1.3 cells per HPF versus 5 ± 1.3 cells per HPF, respectively) in both the alum-primed, OVA-challenged and OVA-primed, OVA-challenged groups. These results demonstrate that the adoptive transfer of nTregs prevents the migration of T cells to the lungs in response to OVA inhalation.

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STAT6 activation rendered T cells resistant to regulation by TGF-β. Our study showed that the restoration of allergic lung inflammation was partially restored in STAT6−/− mice treated with PC61. Previous in vitro studies support the idea of an opposing relationship between STAT6 and Foxp3 in iTregs (32, 34). Dardalhon et al. (32) showed that IL-4 inhibited TGF-β–induced expression of Foxp3 in naive T cells in vitro by a STAT6-dependent mechanism. Furthermore, Pillmer et al. (30) found that constitutive STAT6 activation rendered T cells resistant to regulation by Foxp3 in vivo. In addition, Takaki and colleagues (33) demonstrated that STAT6 can bind directly to the Foxp3 promoter and suppress TGF-β–dependent induction of Foxp3. These findings indicate that IL-4–induced activation of STAT6 can prevent the induction of Foxp3 in naive CD4+ T cells and therefore prevents induction of iTregs in vitro.

However, the potential for in vivo modulation of Tregs by STAT6 in the context of allergic lung inflammation was unclear. In this study, we used Ab-mediated depletion/inactivation of Tregs and adoptive transfer of nTregs in a previously established model of Th2-driven allergic lung inflammation (34). In this model, STAT6−/− and STAT6xRAG2−/− mice were provided with in vivo primed, OVA-specific CD4+ Th2 cells. Th2-driven allergic lung inflammation was partially restored in STAT6−/− mice treated with PC61 to levels observed in STAT6xRAG2−/− mice. Parameters of airway remodeling were also increased by Treg depletion/inactivation. Adoptive transfer of CD4+CD25+Foxp3+ nTregs, but not CD4+CD25−Foxp3− naive T cells, at a 1:2 ratio with Th2 cells caused a substantial reduction in eosinophilic inflammation and suppressed airway remodeling in STAT6xRAG2−/− mice to levels comparable to those in STAT6−/− mice. Taken together, these data demonstrate that STAT6−/− mice are highly resistant to Th2-driven lung inflammation in part because of their enhanced Treg population. Furthermore, they suggest that in addition to promoting Th2 differentiation, STAT6 suppresses Tregs in vivo to promote allergic airway inflammation.

Our study showed that the restoration of allergic lung inflammation in STAT6−/− mice was accompanied by enhanced T cell migration into the lung, thus indicating a role for Treg modulation.
of T cell access to the lung. Furthermore, this study suggests that in addition to regulating allergic inflammation by suppressing the downstream effector action of inflammatory cells (eosinophilia, airway remodeling, IL-5 production), Tregs have another checkpoint where they also block T cell recruitment and homing into the Ag-bearing tissue. This concept aligns with findings from recent studies that demonstrated the ability of Tregs to modulate T effector trafficking in the context of experimental autoimmune encephalitis and diabetes (55, 70). Davidson’s study (55) used polyclonal Tregs in a system using an autoimmune disorder in response to a self Ag. However, we illustrated that adoptively transferred monoclonal nTregs can suppress T cell trafficking into the lungs to help prevent allergic inflammation in STAT6−/− mice. Therefore, our study reveals that regulation of T cell migration into inflamed tissue also strongly influences disease outcome for an immune-mediated disorder in response to a foreign Ag. Thus, Treg control of pathogenic Teff recruitment may be a universal mechanism used in disorders of immune dysfunction. A study by Battaglia et al. (71) showed that IL-10−/− T cells (Tr1s) block Teff migration into tissue by producing IL-10, which prompts Teffs to reduce their expression of ICAM1. In addition, Teff-mediated sequestration of Ag-bearing tissue from Teffs may provide a more efficient method of regulating allergic lung inflammation. This mechanism would be an alternative to containing inflammation within the lung after pathogenic cells have released their contents and have possibly caused more tissue damage and Ag exposure. Our analysis of the total T cell numbers in the lungs (CD3 staining) did not distinguish infiltrating Teffs from Tregs. Future studies to carefully analyze Teff and Treg localization and migration will be necessary to clarify the mechanism by which nTregs modulate Teff recruitment.

The relative roles of nTregs versus iTregs during allergic lung inflammation are still not well understood. TCR repertoire studies suggest that nTregs have TCRs that are mainly designed to recognize self Ags and to prevent autoimmune disease (22, 23). The main cognate Ag pool for iTreg TCRs is still unclear. Some studies suggest that iTregs have TCRs that can recognize both self and foreign Ags, such as allergens, to prevent immune-mediated disease (22, 23). Lewkowich et al. (17) showed that nTreg depletion/inactivation in mice during the immunization phase of the allergic asthma response suppressed dendritic cell activation and led to elevated house dust mite–induced allergic airway inflammation. This study indicates that nTregs play an important role in suppressing IL-4–driven allergic lung inflammation. Interestingly, studies by Pandiyan and colleagues (72) revealed a positive role for IL-4 in supporting nTregs in vitro; IL-4 was able to maintain Foxp3 and CD25 expression in nTregs.

However, our study establishes that in vivo, IL-4–activated STAT6 inhibits nTregs and allows for the propagation of Th2-driven lung inflammation. Our adoptive transfer of control OVA-specific CD25− cells did not show a high level of conversion of iTregs in vivo in the presence of Th2 cells. Thus, in our adoptive transfer model, the suppression of allergic inflammation and remodeling was likely mediated by the nTregs. On the basis of our in vitro studies showing an equipotent suppression of T cell proliferation by nTregs prepared from STAT6−/− and STAT6+/+ mice (34), we transferred STAT6−/− nTregs in this experiment. In future studies, it will be interesting to test whether these nTregs would have different regulatory potencies in vivo.

The definitive role of in vivo STAT6 modulation of iTregs during allergic lung inflammation has not been completely resolved. In a transgenic murine model that lacked natural Tregs, Curotto de Lafaille et al. (73) demonstrated that adaptive or inducible Tregs alone reduced chronic, but not acute, lung inflammation induced by intranasal treatment with OVA, thus indicating that iTregs are sufficient to suppress allergic lung inflammation. The protocol we used for Treg depletion/inactivation would affect both nTregs and iTregs and therefore does not distinguish between the suppressive roles of these two cell subsets during allergic lung inflammation.

In addition, a study by Joselewicz et al. (74) revealed that compared with their WT counterparts, C57BL/6 mice devoid of CNS1 (conserved noncoding sequence 1)−/− iTregs (but having intact nTregs) experienced spontaneous Th2-type airway inflammation, suggesting a nonredundant role for iTregs to block mucosal allergic inflammation in the lung. Furthermore, Xu and colleagues (29) demonstrated that the adoptive transfer of in vitro–generated iTregs to sensitized mice, before or during OVA challenge, can reduce allergic lung inflammation and improve lung function.

One specific type of Treg capable of producing large amounts of IL-10, Tr1, has been shown to significantly affect human allergic asthma (75–77). An inverse relationship exists between the levels of IL-10–secreting T cells and the severity of allergic asthma in patients. Furthermore, Aksara et al. (27) found that respiratory tolerance from allergic inflammation in mice involved induction of IL-10–secreting Tregs. Therefore, studies that elucidate the interaction between IL-4 activation of STAT6 and Tr1 induction would be of both scientific and clinical interest. Further investigation into the relative role of STAT6 in modulating nTregs, iTregs, and Tr1 during the allergic response has the potential to make significant scientific contributions to the field of allergy and immunology, but also to aid in the development of novel therapeutic clinical strategies to combat and/or prevent this immune-mediated illness in humans.

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Disclosures

The authors have no financial conflicts of interest.

References


182: 1169–1174.

188: 1739–1750.


18: 1497–1505.

12: 1527–1535.

17: 1907–1096.

1527: 5502–5510.


10: 1707–1711.

1527: 5502–5510.

33: 438–449.


17: 1907–1711.

130: 575–586.

130: 575–586.

130: 575–586.

130: 575–586.

130: 575–586.


1: 626–55.

187: 939–948.


206: 751–760.


52: 4545–4555.

51: 3838–3849.

199: 1875–1886.

188: 4417–4422.

18: 1497–1505.

188: 645–660.

16: 1249–1260.


Total Number of Lymph Node CD4+Foxp3+ Tregs

<table>
<thead>
<tr>
<th>Strain</th>
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<tr>
<td>C57BL/6 WT</td>
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</tr>
<tr>
<td>C57BL/6 STAT6^-/-</td>
<td>7.3x10^6 cells</td>
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**Additional Figure Supplemental 1**

**Elevated nTreg Frequency in STAT6-deficient Mice**

(A) Single cell suspensions of spleens and lymph nodes were prepared from naïve BALB/c D011.10xFoxp3$^{GFP^{KI}}$ mice or BALB/c D011.10xSTAT6$^{-/-}$ Foxp3$^{GFP^{KI}}$ reporter mice, who were generated by crossing STAT6$^{-/-}$ mice and D011.10x Foxp3$^{GFP^{KI}}$ mice. n=2-3 mice/group. Lymphocytes were stained with conjugated antibody to CD4 and CD25 and were assessed for CD4 and Foxp3 expression (indicated by GFP marker) using flow cytometry. Foxp3 (GFP) expression is shown in the CD4$^+$ cell population. (B) Lymph nodes were harvested from naive C57BL/6 STAT6$^{+/+}$ or STAT6$^{-/-}$ mice and stained for CD4, CD25 and cytoplasmic Foxp3 expression. The percentage of CD4$^+$ Foxp3$^+$ cells is shown. The total number of CD4$^+$Foxp3$^+$ lymphocytes for each group was calculated based on the total lymph node cells recovered from 3 mice. n=3 mice/group
Additional Figure Supplement 2

Reduced Expression of Foxp3 by host CD4⁺ T cells after Treg depletion/ inactivation

(A) Balb/c Foxp3-GFP mice were administered 0.5 mg of PC61 α-CD25 or PBS on days 1 and 5. Twenty-four hours later, lymphocytes were isolated from the LN of individual mice. (Left) Expression of CD4 (PE) and Foxp3 (GFP) was determined by flow cytometry analysis. The average percentage of CD4⁺Foxp3⁺ is shown (right). n=2 mice/group (B) To test the efficacy of PC61 to eliminate Foxp3⁺ Tregs in the context of OVA-induced allergic asthma, we harvested lymphocytes from the pulmonary lymph nodes of STAT6⁻/⁻ mice treated with IgG or PC61 at the completion of the protocol. (Left) Lung-draining lymph node cells from each experimental group were pooled and stained with antibodies to CD4, KJ126, and CD25 and stained intracellularly for Foxp3 expression prior to flow cytometry. (Right) Graphical representation of the percentage of CD4⁺ Foxp3⁺ T cells is shown. (n=4-5 pooled mice/group) Data is representative of three independent experiments. (C) Allergic airway inflammation was induced as described in Figure 1. Lung sections were stained with H&E. Enlargements of representative H&E images of lung sections from OVA-primed mice adjacent to the airway lumen (top) or bordering the pulmonary vasculature (bottom) are shown.
Additional Figure Supplement 3

Analysis of mucus production.

(A, B) Allergic airway inflammation was induced as described in Figures 1 and 5. Lung sections from the indicated experimental groups, and from (C) WT BALB/c mice as a positive control, were stained with PAS. Enlarged 40x representative PAS images of lung sections from Alum-primed mice (top) or OVA-primed mice (bottom) are shown. (n=3-5 mice/ group).
Adoptive transfer of CD4$^+$CD25$^+$ Tregs

(A) CD4$^+$CD25$^+$ Tregs or control CD4$^+$CD25$^-$ T cells were isolated from spleens and lymph nodes of D011.10 Foxp3$^{GFP(KI)}$ reporter mice using a combination of immunomagnetic separation and a series of negative selection (Easy Sep Negative Selection CD4$^+$ T cell enrichment) and 2 (for CD4$^+$CD25$^-$ T cells) or 4 (for CD4$^+$CD25$^+$ Tregs) rounds of positive selection. Cell subsets were analyzed by FACS for surface expression of CD4 and CD25 (top) and expression of Foxp3 (GFP) (bottom) in the CD4$^+$CD25$^+$ populations of control (CD4$^+$CD25$^-$) or Treg (CD4$^+$CD25$^+$) cell samples (n=9 mice). Data is representative of two independent experiments. (B) Allergic lung inflammation was induced using OVA in STAT6$^{-/-}$ and STAT6xRAG2$^{-/-}$ mice as described in Materials and Methods. Lung-draining LN cells and splenocytes from STAT6xRAG2$^{-/-}$ mice given CD4$^+$CD25$^+$ Tregs or CD4$^+$CD25$^-$ T cells were pooled and surface-stained with antibodies to CD4 and KJ126, prior to flow cytometry and assessed for Foxp3 expression (GFP) by FACS analysis. Foxp3 expression is shown in the CD4$^+$KJ126$^+$ donor cell population. (n=5 mice/group). Data is representative of two independent experiments. (C) Allergic airway inflammation was induced as described in Figure 5. Lung sections were stained with H&E. Enlargements of the representative H&E images of lung sections from OVA-primed mice adjacent to the airway lumen (top) or bordering the pulmonary vasculature (bottom) are shown. (n=3-5 mice/group)