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## Cutting Edge: STAT6 Signaling in Eosinophils Is Necessary for Development of Allergic Airway Inflammation

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Eosinophils are critical cellular mediators in allergic asthma and inflammation; however, the signals that regulate their functions are unclear. The transcription factor STAT6 regulates Th2 cytokine responses, acting downstream of IL-4 and IL-13. We showed previously that eosinophil-derived IL-13 plays an important role in the recruitment of T cells to the lung and the subsequent development of allergic asthma. However, whether eosinophils respond to Th2 signals to control allergic airway inflammation is unclear. In this report, we show that STAT6<sup>-/-</sup> eosinophils are unable to induce the development of allergic lung inflammation, including recruitment of CD4<sup>+</sup> T cells, mucus production, and development of airways hyperresponsiveness. This is likely due to the reduced migration of STAT6<sup>-/-</sup> eosinophils to the lung and in response to eotaxin. These data indicate that, like Th cells, eosinophils need to respond to Th2 cytokines via STAT6 during the development of allergic airway inflammation. *The Journal of Immunology*, 2015, 194: 000–000.

**A**llergic asthma is the most common form of asthma, affecting >50% of the 20 million asthma sufferers (1). Although the prevalence of this disease is increasing worldwide, it is still unclear why some predisposed individuals develop disease. The presence of lung and airway eosinophilia has been observed in both human and mouse models of allergic asthma (2). During the development of allergic asthma, T cells, in particular CD4<sup>+</sup> T cells, orchestrate the inflammatory microenvironment through production of Th2 cytokines (IL-4, IL-13, and IL-5), which contribute to the influx and maintenance of eosinophils into the airway spaces in response to cytokines. Thus, these cytokines are major regulators of the inflammatory response. Using a murine model of allergic airway disease, we (3,4) and other investigators (5,6)

showed that, on the C57BL/6 background, there is a requirement for eosinophils in the recruitment of T cells to the lungs and development of allergic airway disease. We also showed that eosinophil-derived IL-13 is critical for the development of allergic airway disease (7). However, whether eosinophils respond to Th2 signals to control allergic airway inflammation is unclear.

The STAT family of transcription factors is activated in response to different cytokine–receptor interactions (8). In particular, STAT6 is the primary transcription factor activated by Th2 cytokines IL-4 and IL-13, which play a critical role in the induction of allergic inflammation (8). Analyses of STAT6<sup>-/-</sup> mice revealed that STAT6 is required for several IL-13/IL-4–mediated processes, such as airway hyperresponsiveness (AHR) and development of lung pathology during allergic airway disease (9–13). Thus, STAT6 signaling is critical in the development of Th2-dependent allergic responses. In this study, we report that STAT6<sup>-/-</sup> eosinophils are unable to induce the development of allergic airway disease, including recruitment of CD4<sup>+</sup> T cells and eosinophils to the lung and the development of goblet cell mucus production. These data indicate that STAT6 is required for the generation and maintenance of Th2 responses, as well as for the responses of eosinophils during the development of allergic airway disease.

### Materials and Methods

#### Mice

Wild-type (WT),  $\Delta$ dblGATA (14), and IL-5–transgenic mice on a C57BL/6 background (a gift from J. Lee and N. Lee, The Mayo Clinic, Scottsdale, Arizona) (15) were used. IL-5–transgenic/STAT6<sup>-/-</sup> mice were generated by crossing IL-5–transgenic mice with STAT6<sup>-/-</sup> mice (gift of M. Bynoe, Cornell University). All experiments were approved by the Institutional Animal Care and Use Committees at Pennsylvania State University and Cornell University.

#### OVA-induced allergic asthma model and determination of AHR

WT or  $\Delta$ dblGATA mice were induced to develop allergic asthma, as previously described (7, 16). In some experiments,  $\Delta$ dblGATA mice received adoptive transfer of WT or STAT6<sup>-/-</sup> eosinophils ( $1 \times 10^6$ ), purified and

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Abbreviations used in this article: AHR, airway hyperresponsiveness;  $\Delta$ dblGATA, deletion of palindromic high-affinity GATA1 binding site upstream of the GATA1 gene; PAS, periodic acid–Schiff; WT, wild-type.

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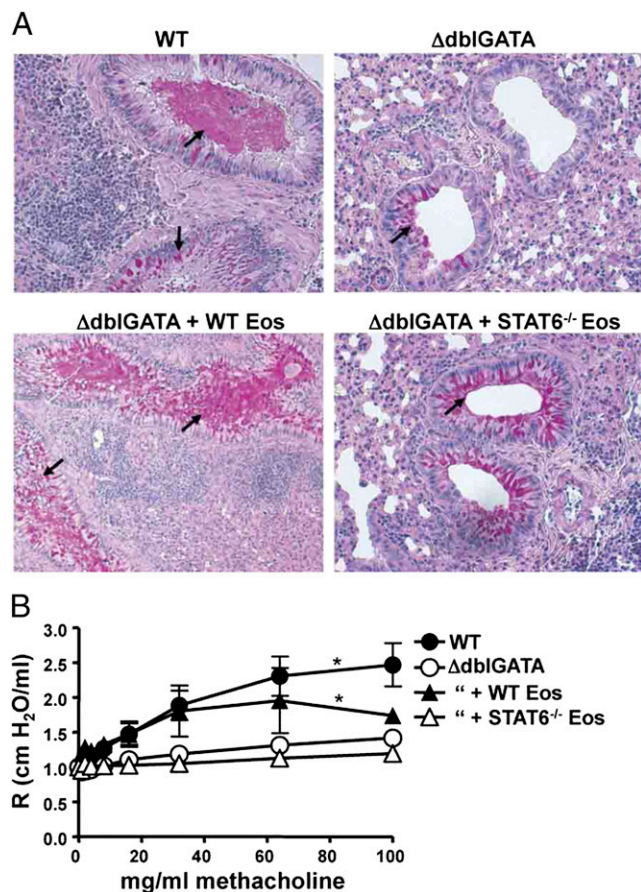


**FIGURE 1.** STAT6 expression by eosinophils is required for allergic airway inflammation. WT and  $\Delta$ dblGATA mice were immunized and challenged with OVA as described. Some mice received WT or STAT6<sup>-/-</sup> eosinophils 6 h prior to the first intranasal OVA exposure. Twenty-four hours after the last intranasal exposure, lungs were collected and analyzed by PAS to detect mucus production (arrows indicate mucus) (PAS stain at original magnification  $\times 10$ ) (A), or mice were analyzed for AHR by mechanical ventilation (B) ( $n \geq 3$  mice/group). Data are mean  $\pm$  SEM. \* $p < 0.05$  versus  $\Delta$ dblGATA or STAT6<sup>-/-</sup> eosinophil recipients.

delivered via retro-orbital injection, as previously described (7). AHR was determined using a flexiVent mechanical ventilator (SCIREQ), as previously described (7). Cells were harvested from lung tissue and analyzed as previously described (7).

#### Analysis of airway inflammation

Fixed and sectioned lungs were stained with H&E or periodic acid-Schiff (PAS) to detect inflammation and mucus (performed by the Animal Health Diagnostic Laboratory, Cornell University).

#### Eosinophil chemotaxis and analysis of cytokine production

Eosinophil chemotaxis was performed in response to recombinant eotaxin, following a 30-min pretreatment (or not) with IL-4, using a Transwell assay (Costar; 6.5 mm diameter, 5  $\mu$ m pore size), as previously described (17, 18). Eosinophils ( $1 \times 10^6$ ) were left to migrate for 2 h at 37°C. Fold change in migration was calculated as the number of cells that migrated in response to eotaxin, with or without IL-4, divided by the number of cells that migrated in response to medium alone. Purified eosinophils were cultured in media in the presence or absence of PMA/ionomycin for 2 h, and supernatants were collected and analyzed for IL-4, IL-5, and IL-13 by ELISA.

#### Quantitative RT-PCR analysis of gene expression

RNA was isolated from lung tissue, and total RNA (1  $\mu$ g) was reverse transcribed to cDNA. PCR was performed in triplicate with commercially available primers and probes, per the manufacturer's protocol (Applied Biosystems), as described previously (7).

#### Statistical analysis

Three to five mice/group were used for each experiment. Student *t* test and ANOVA were performed using Prism software to evaluate statistical significance between sample sets or multiple groups, with  $p < 0.05$  considered statistically significant.

## Results and Discussion

#### Eosinophil expression of STAT6 is required for the development of allergic airway inflammation

We showed previously that eosinophil-derived IL-13 is important in the generation of inflammation during disease (7). STAT6 mediates cytokine signaling by IL-4 and IL-13 (8), and analysis of STAT6<sup>-/-</sup> mice revealed a requirement for STAT6 in several IL-13/IL-4-mediated processes, such as AHR, and lung pathology (19). This requirement for STAT6 was largely suggested as a result of T cell and epithelial cell responses to Th2 cytokines. However, it is not clear whether eosinophils also respond to Th2 cytokine signals to regulate allergic airway inflammation. To determine whether eosinophils receive Th2 signals during the development of allergic airway disease, we generated eosinophils lacking STAT6 and used them as donors for  $\Delta$ dblGATA eosinophil-deficient mice. WT or  $\Delta$ dblGATA mice were immunized with OVA/alum, were given either WT or STAT6<sup>-/-</sup> eosinophils via the i.v. route 24 h prior to airway OVA challenge, and were analyzed for the development of allergic inflammation. We found that  $\Delta$ dblGATA mice that received STAT6<sup>-/-</sup> eosinophils did not develop classical signs of allergic lung inflammation beyond that seen in  $\Delta$ dblGATA mice that received no eosinophils, and there was no mucus in the airways, unlike in the WT mice or  $\Delta$ dblGATA mice that received WT

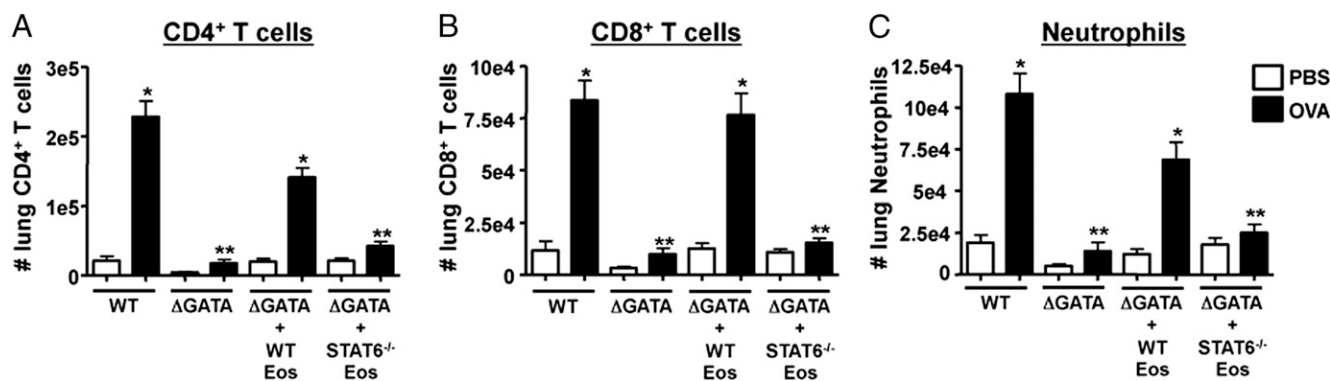


**FIGURE 2.** Eosinophil expression of STAT6 is required for recruitment of T cells to the lungs in allergic airway inflammation. WT and  $\Delta$ dblGATA mice were induced to develop allergic airway inflammation as in Fig. 1 and then cells from lung tissue were analyzed for CD4<sup>+</sup> T cells (A), CD8<sup>+</sup> T cells (B), or neutrophils (C) by flow cytometry 24 h after the last intranasal OVA exposure. Data are mean  $\pm$  SEM ( $n = 3$ –8 mice). \* $p < 0.05$  versus PBS, \*\* $p < 0.05$  versus WT OVA exposed.

eosinophils, as we reported previously (PAS scores: WT = 3.4;  $\Delta$ dblGATA = 0.2;  $\Delta$ dblGATA+WT eosinophils = 2.4;  $\Delta$ dblGATA+STAT6<sup>-/-</sup> eosinophils = 0.3, Fig. 1A (4, 6, 7, 20). Furthermore,  $\Delta$ dblGATA mice that received STAT6<sup>-/-</sup> eosinophils did not develop AHR, as determined by mechanical ventilation (Fig. 1B). These data indicate that lung inflammation and AHR that develop as a result of allergic airway disease are dependent on STAT6 expression in eosinophils and their ability to respond to Th2 cytokines.

*Eosinophil expression of STAT6 is required for the recruitment of CD4<sup>+</sup> T cells to the lung during the development of allergic airway inflammation*

We showed previously that eosinophils are required for recruitment of CD4<sup>+</sup> T cells to the lung during development of allergic airway disease, which is dependent on eosinophil expression of IL-13 (4, 6, 7, 20). Therefore, we next determined whether the lack of airway inflammation in recipients of STAT6<sup>-/-</sup> eosinophils was due to lack of recruitment of CD4<sup>+</sup> T cells to the lungs. We found that transfer of WT eosinophils into  $\Delta$ dblGATA mice rescues the recruitment of CD4<sup>+</sup> and CD8<sup>+</sup> T cells to the lung to an extent beyond that seen in  $\Delta$ dblGATA mice alone (Fig. 2A). In contrast,  $\Delta$ dblGATA recipients of STAT6<sup>-/-</sup> eosinophils exhibited reduced numbers of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the lung (Fig. 2A). This decrease in the recruitment of T cells to lung correlated with the reduced numbers of T cells in the airways, as determined by analysis of bronchoalveolar lavage fluid (Fig. 2B). Similar results were found for neutrophils (Fig. 2C). Thus, STAT6<sup>-/-</sup> eosinophils are defective in providing signals necessary for orchestrating the recruitment of CD4<sup>+</sup> and CD8<sup>+</sup> T cells required for the induction of the disease. Although it would have been useful to deliver the eosinophils directly to the lung via an intratracheal route, reconstitution via i.v. injection may more closely mimic the case in WT mice, where eosinophils in the blood are recruited to the lungs during inflammation. This would not necessarily be the case with the direct intratracheal route.

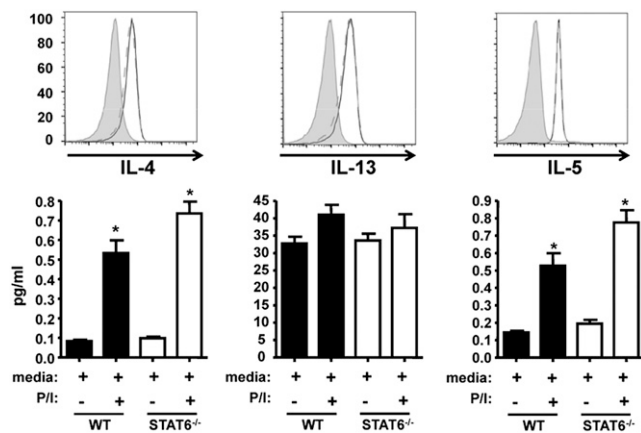


**FIGURE 3.** STAT6 expression does not affect cytokine expression in eosinophils. WT or STAT6<sup>-/-</sup> eosinophils were purified by MACS bead negative selection and analyzed for intracellular staining for the indicated cytokines by FACS (solid lines: WT eosinophils, dashed lines: STAT6<sup>-/-</sup> eosinophils, shaded graphs: control) (upper panels) or were stimulated with PMA/ionomycin for 2 h, and supernatants were analyzed for the indicated cytokines (lower panels). \**p* < 0.05 versus media control.

*The absence of STAT6 in eosinophils does not affect preformed protein for Th2 cytokines*

Eosinophils also were shown to carry preformed stores of cytokines, which allow these cells to respond rapidly upon stimulation (21). STAT6 is an important mediator of cytokine production, in part via its ability to regulate the expression of the transcription factor GATA3 (8). Because we showed previously that eosinophil-derived IL-13 is critical for their function in allergic airway disease, we analyzed WT and STAT6<sup>-/-</sup> eosinophils for the presence of Th2 cytokines; however, there was no difference between them with regard to the levels of cytokine protein secreted (Fig. 3).

*STAT6-deficient eosinophils fail to migrate to the lung during the development of allergic airway inflammation*

Global STAT6-deficient mice are unable to recruit inflammatory cells to the lungs, including eosinophils in allergic airway disease (10). However, whether eosinophils respond to Th2 signals to migrate to the lung during allergic lung inflammation is unclear. To determine whether STAT6 expression in eosinophil is necessary for the ability of these cells

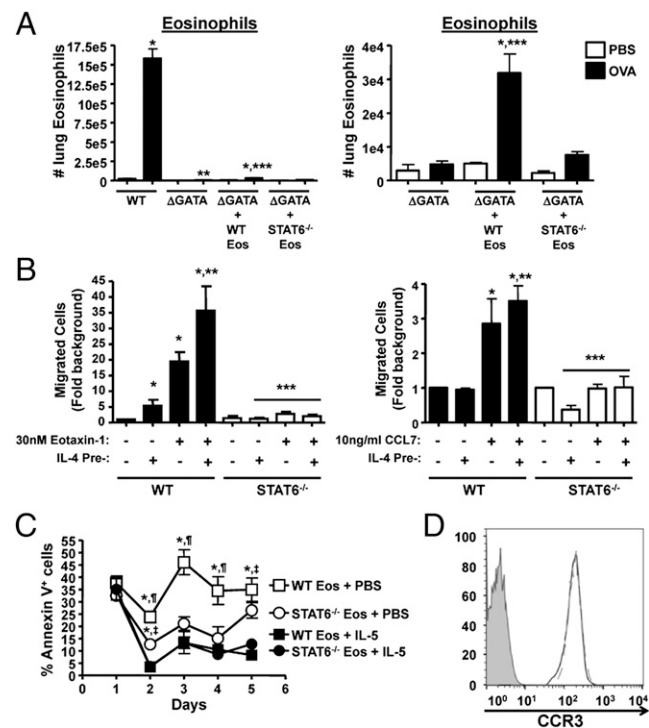


**FIGURE 4.** STAT6 regulates the recruitment of eosinophils to the lung during the development of allergic asthma. (A) Presence of eosinophils in the lungs in similar experiments to those shown in Figs. 1 and 2. Expansion of the data from  $\Delta$ dblGATA mice, with or without WT or STAT6<sup>-/-</sup> eosinophils, is shown (right panel). Data are mean  $\pm$  SEM (*n* = 4–5 mice). \**p* < 0.05 versus PBS, \*\**p* < 0.05 versus WT OVA exposed, \*\*\**p* < 0.05 versus  $\Delta$ dblGATA OVA exposed. (B) Purified WT or STAT6<sup>-/-</sup> eosinophils were analyzed for their ability to chemotax toward eotaxin-1 (left panel) or CCL7 (right panel) following pretreatment with IL-4 or PBS (as control). Data are mean  $\pm$  SEM for three experiments. \**p* < 0.05 versus PBS, \*\**p* < 0.05 versus eotaxin alone, \*\*\**p* < 0.05 versus WT equivalent conditions. (C) Purified WT or STAT6<sup>-/-</sup> eosinophils were cultured in vitro with IL-5 (2 ng/ml) or PBS for the indicated times and analyzed for survival via annexin V stain by flow cytometry. Data are mean  $\pm$  SEM from three experiments. \**p* < 0.05 PBS versus IL-5, \**p* < 0.05 versus STAT6<sup>-/-</sup> eosinophils PBS treated, \**p* < 0.05 versus STAT6<sup>-/-</sup> eosinophils IL-5 treated. (D) WT (solid line) or STAT6<sup>-/-</sup> (dashed line) eosinophils were analyzed for expression of CCR3 by flow cytometry. Shaded area indicates control staining.

to migrate to the lung during allergic inflammation, we analyzed lungs of OVA-challenged WT and  $\Delta$ dblGATA-recipient mice for the presence of eosinophils. We found that WT eosinophils transferred into  $\Delta$ dblGATA mice were able to migrate to the lung; however, STAT6<sup>-/-</sup> eosinophils transferred into  $\Delta$ dblGATA mice failed to migrate to the lung, consistent with reduced lung pathology and T cell recruitment (Fig. 4A).

The CCR3/eotaxin-1 axis selectively recruits eosinophils in vivo during the development of allergic airway disease (22–24). Recent studies showed that mouse eosinophils express type I/II IL-4Rs, and although they did not chemotax to IL-4, pretreatment with IL-4 enhanced migration to eotaxin-1 in vitro (18). Thus STAT6, via its response to the Th2 cytokine IL-4, may modulate the ability of eosinophils to migrate to the lung in vivo. Therefore, we analyzed the ability of STAT6<sup>-/-</sup> eosinophils to migrate in response to CCR3, in the presence or absence of IL-4 priming. We found that, regardless of pretreatment with IL-4, STAT6<sup>-/-</sup> eosinophils exhibited reduced migration in response to eotaxin, as well as CCL7 (Fig. 4B). Analysis of WT and STAT6-deficient eosinophils revealed no difference in survival in vivo (in IL-5-transgenic mice) or in vitro in the presence of IL-5, although STAT6<sup>-/-</sup> eosinophils exhibited better survival in the absence of IL-5 in vitro (Fig. 4C), and STAT6<sup>-/-</sup> eosinophils expressed similar levels of CCR3 compared with WT eosinophils (Fig. 4D). These data suggest that STAT6 regulates the migratory response of eosinophils to chemokines, such as eotaxin-1 and CCL7. Our results indicate that eosinophils need to respond to Th2 cytokines via STAT6 to regulate allergic lung inflammation. In particular, our data suggest that STAT6 signals regulate the ability of eosinophils to migrate.

A global deletion of STAT6 protects mice from developing allergic asthma, because these mice are defective in IL-4- and/or IL-13-dependent Th2 differentiation and IgE production (8, 9, 25). Indeed, multiple cells respond to Th2 cytokines during this disease, because STAT6 expression in both hematopoietic and structural nonhematopoietic cells was shown to be critical for the recruitment of Th2 cells to the lung and the development of allergic airway inflammation (26, 27). Although STAT6 was shown to be important for T cells to migrate to the lung via the MDC/CCR4 axis, this is due to the requirement of STAT6 for the expression of CCR4 (28). Our data extend our understanding of the function of STAT6, showing its requirement for eosinophil function in vivo and for the ability of eosinophils to migrate in response to eotaxin-1, as well as CCL7, but not via regulating the expression of CCR3, as was the case in T cells. It is possible that, similar to B cells, STAT6 plays a role in regulating cytoskeletal events that are required for optimal migration (29). Our work indicates that eosinophils specifically need to be able to respond via STAT6 to migrate and regulate T cell recruitment to the lung to cause allergic inflammation.

Although STAT6 regulates the expression of Th2 cytokines, we found that it was not required for eosinophils to express these cytokines. Rothenberg and colleagues (30) recently suggested that a feed-forward loop exists in eosinophils whereby IL-33-stimulated eosinophils release IL-4, which feeds back to stimulate the expression of a number of genes, including Retnla, CCL2, CCL7, and, in part, CCL17. Furthermore, these authors noted that the majority of IL-33-

responsive genes were IL-4/STAT6 independent, and were dependent on NF- $\kappa$ B instead, although IL-13 production was partially dependent on STAT6. Taking into account these data, we suggest that, in vivo, eosinophils need to be able to respond to Th2 cytokines, whether released by eosinophils in response to cytokines, such as IL-33, or released by other cells, such as ILC2 or Th2 cells. This STAT6-dependent response is important, in part, for the ability of eosinophils to migrate and contribute to the development of allergic airway inflammation. Thus, our work provides significant new insight into the intracellular regulation of eosinophils during disease.

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## Disclosures

The authors have no financial conflicts of interest.

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