STAT6 Activation Confers upon T Helper Cells Resistance to Suppression by Regulatory T Cells

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Recent studies have highlighted characteristics of T regulatory cells (Tregs) that underlie their suppressive function. However, mechanisms that override their suppressive function in the context of an adaptive immune response are not well understood. In the lungs of mice undergoing allergic inflammation, appreciable numbers of Tregs were identified that possessed suppressive function as assayed ex vivo. We investigated whether the Th2-promoting cytokine IL-4 played a permissive role that superseded Treg function, thereby allowing the development of allergic inflammation. IL-4 signaling via the IL-4Rα-STAT6 axis was required to maintain Foxp3 expression in Tregs and promote their proliferation. However, the results of both in vivo experiments involving adoptive transfer of Tregs into Ag-sensitized vs naive animals and in vitro suppression assays performed with or without exogenous IL-4 showed the ability of IL-4 to compromise Treg-mediated suppression. Use of retrovirally expressed, constitutively active STAT6 revealed that the underlying mechanism was not IL-4-mediated dysfunction of Tregs but involved the resistance of T helper cells to Treg-mediated suppression that would permit the development of an adaptive immune response. Our data suggest that infectious tolerance, mediated by membrane-bound TGF-β expressed by Tregs, is compromised by the competing effects of IL4-induced signaling in naive CD4+ T cells. The Journal of Immunology, 2009, 183: 155–163.

Asthma is a common disease that affects > 15 million people in the United States and is characterized by mucus hypersecretion, elevated serum IgE, eosinophilic infiltration in the airways, and airway hyperresponsiveness (1). The success of immunomodulatory treatments such as steroids and in some instances Ag-mediated therapy strongly suggests an immunological contribution to the allergic form of disease (2). The immune responses seen in experimental allergic airway inflammation, as well as in atopic human asthmatics, are characterized by a predominance of Th2 cytokines such as IL-4, IL-5, and IL-13. With regard to effects on T cells, IL-4 plays a key role as has been demonstrated in a number of studies. The IL-4R is composed of the IL-4Rα-variant in the lungs of mice undergoing allergic inflammation, appreciable numbers of Tregs were identified that possessed suppressive function as assayed ex vivo. We investigated whether the Th2-promoting cytokine IL-4 played a permissive role that superseded Treg function, thereby allowing the development of allergic inflammation. IL-4 signaling via the IL-4Rα-STAT6 axis was required to maintain Foxp3 expression in Tregs and promote their proliferation. However, the results of both in vivo experiments involving adoptive transfer of Tregs into Ag-sensitized vs naive animals and in vitro suppression assays performed with or without exogenous IL-4 showed the ability of IL-4 to compromise Treg-mediated suppression. Use of retrovirally expressed, constitutively active STAT6 revealed that the underlying mechanism was not IL-4-mediated dysfunction of Tregs but involved the resistance of Th cells to Treg-mediated suppression that would permit the development of an adaptive immune response. Our data suggest that infectious tolerance, mediated by membrane-bound TGF-β expressed by Tregs, is compromised by the competing effects of IL4-induced signaling in naive CD4+ T cells. The Journal of Immunology, 2009, 183: 155–163.

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Materials and Methods

**Mice**

All mice were obtained from The Jackson Laboratory and were maintained in the Department of Laboratory Resources at the University of Pittsburgh (Pittsburgh, PA). Male animals were used at 6–8 wk of age. Maintenance and use of laboratory animals were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

**Flow cytometric analysis**

Single-cell suspensions obtained from lung tissue were used for flow cytometric analysis. Frequencies of effector T cells (CD4+CD25+Foxp3+) and Tregs (CD4+CD25+Foxp3+) were examined with three-color flow cytometry using anti-CD4 (BD Pharmingen), anti-CD25 (BD Pharmingen), and anti-Foxp3 (eBioscience) labeled with PercPC, PE, and FITC, respectively. Before staining, cells were blocked for 15 min on ice with Fc block (10% normal mouse serum). Cell populations (2 × 10^5 events) were assessed using a FACSCalibur flow cytometer with CellQuest software (BD Pharmingen) and analysis was performed using FlowJo (Tree Star). The frequency of positive cells was calculated by subtracting the value obtained with the respective isotype controls.

**Cell isolation and stimulation**

Mice were sacrificed and lungs were removed and minced. Minced lungs were incubated in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (Gemini Bio-Products), DNase I (30 μg/ml type IV bovine pancreatic DNase I; Roche), and collagenase I (0.7 mg/ml; Sigma-Aldrich) for 45 min at 37°C in a shaking water bath. Single-cell suspensions of lungs were obtained by passing the digested lung tissue through a 70-μm nylon strainer (BD Falcon). Mononuclear cells were enriched by performing discontinuous Percoll (Sigma-Aldrich) gradient centrifugation (from 20 to 55%). Cells collected on the 55 and 45% layers were passed through a 35-μm nylon strainer and counted by trypan blue exclusion. In the case of Tregs isolated from Foxp3EGFP knock-in mice (where EGFP is enhanced GFP), after passing cells through a 70-μm nylon strainer the CD4+ T cells were isolated from the suspension using anti-CD4 microbeads (Miltenyi Biotec). GFP+CD4+ Tregs were isolated from the positively selected CD4+ cell population by cell sorting. In some experiments, splenic CD4+CD25+ Tregs were isolated by the enrichment of mononuclear cells through a density gradient followed by cell sorting. Tregs were suspended in PBS and adoptively transferred (2 × 10^5 cells per recipient) to animals. Tregs (CD4+CD25+), Th cells (CD4+CD25−), and APCs were isolated from the spleens of 6- to 8-wk-old male BALB/cByJ mice. Spleens were minced, filtered through 70-μm nylon screens, and mononuclear cells were enriched by density gradient centrifugation (lymphocyte separation medium; MP Biomedicals). Tregs and Th cells were isolated using a Treg magnetic bead isolation kit (Miltenyi Biotec). CD4+ T cells were obtained as a byproduct of Treg isolation, and CD4+ cell-depleted cells were used as APCs. Unless indicated, all cells were cultured in complete medium (Click’s medium supplemented with 10% FBS (Gemini Bio-Products), gentamicin (50 mg/L; Gemini Bio-Products), l-glutamine (2 mM; Invitrogen), and 2-ME (55 μM; Invitrogen). T cells were activated with agonistic soluble anti-CD3e mAb (2 μg/ml; BD Biosciences) and gamma-irradiated (2000 rad) APCs as a source of costimulation in most experiments. Some experiments included the addition of exogenous IL-2 (50 U/ml) or IL-4 (20 ng/ml) as indicated.

**Adaptive transfer of Tregs during experimentally induced allergic airway inflammation**

A model for the inhibition of experimentally induced airway inflammation was described in detail previously (11). Briefly, mice received i.p. injections of Tregs from OVA-tolerized mice followed by an i.p. injection of OVA/alum to prime the animals 15 min later (11, 20). After 6 days, animals received a booster i.p. injection of OVA/alum. Mice were rested for 1 wk, subjected to challenge with 1% aerosolized OVA (20 min/day) for 7 consecutive days, and analyzed 20–24 h later. In a variation of this approach, recipient animals received Tregs from tolerized mice after two i.p. immunizations with OVA/alum before the first day of the 7-day challenge with 1% aerosolized OVA. Control mice received sham injections of PBS and were subjected to the full inflammation model involving two i.p. injections of OVA/alum followed by challenge with aerosolized OVA. A minimum of four mice were included in each group in each experiment.

**Bronchoalveolar lavage (BAL)**

At 20–24 h after the last aerosol challenge in the models described above, BAL was conducted on the animals and cell differentials were completed as previously described (11).

**[3H]Thymidine incorporation assay**

Cells were cultured in round-bottom, 96-well plates with soluble anti-CD3e (2 μg/ml) and 3 × 10^6 gamma-irradiated APCs per well (if used in an assay, Th cells were added at one Th cell to one APC). Freshly isolated Tregs were added at various ratios to Th cells as indicated in Figs. 4 and 5, with Th cells maintained at constant numbers. Cells were cultured for 48 h and then pulsed with [3H]thymidine (1 μCi/well, PerkinElmer) and incubated for an additional 18 h. Incorporation of [3H]thymidine was measured by harvesting cell debris and DNA onto glass fiber filters followed by liquid scintillation counting (Wallac). All conditions were performed in triplicate, and data are presented as mean cpm ± SD.

**CFSE dilution assay**

CFSE (1 μM; Invitrogen) was used as described previously (21). The percentage of divided cells was determined by dividing the number of Th cells undergoing at least one cell division by the total number of live CFSE-labeled cells and multiplying the result by 100.

**Western blotting**

Nondenaturing cell lysis buffer with 1% Triton (Cell Signaling) was used to make total cell extracts, and Western blotting techniques were used to analyze equal amounts of protein as described previously (22). Membranes were probed with anti-STAT6 mAb (Cell Signaling) at a 1/1000 dilution. Expression of β-actin (Novus) was used to confirm equal protein loading.

**Retroviral infection of Tregs and Th cells**

A retroviral construct encoding constitutively active STAT6 (caSTAT6) was used (23). The retroviral construct containing caSTAT6 cDNA and the empty vector were purified and a packaging cell line (Phoenix cells) was infected to generate retrovirus. Infection of Tregs and Th cells was performed as previously described (21).

**Statistics**

Results shown are mean values ± SD. Wilcoxon matched pairs test, Mann-Whitney U test, or Student’s t test were used to assess statistical significance, and differences were considered significant where p ≤ 0.05.

**Results**

**Similar numbers of Tregs in the lungs of tolerized mice and those with airway inflammation**

Although Tregs can act as potent inhibitors of the immune system, the failure to maintain immunological tolerance to essentially ubiquitous Ags in patients with autoimmune and allergic diseases suggests that Tregs may be ineffective in these situations. We used a murine model of allergic airway inflammation using Ag sensitization followed by challenge. The model of tolerance involves repeated exposure to aerosolized OVA followed by testing mice for tolerance by subjecting the mice to the inflammation-inducing regimen as previously described by us (11). In each case, we examined the relative numbers of CD4+CD25+Foxp3− (the majority of these cells most likely being Th cells) vs CD4+CD25−Foxp3+ (the majority of these cells most likely being Tregs) cells in the lung. We have noted, however, that Foxp3 expression does not always endow suppressive functions (24), just as Foxp3+ cells may include other types of Tregs such as Th3 and Tr1 cells (25, 26). As expected, in naive as well as in tolerized animals the number of Foxp3− cells was lower than that of Foxp3+ cells (Fig. 1). In mice immunized for inflammation, the relative number of Foxp3+ cells was higher than that of Foxp3+ cells (Fig. 1). In mice immunized for inflammation, the relative number of Foxp3+CD4− cells termed Tregs hereafter was similar to that observed in tolerized mice (Fig. 1). It should be noted that in each case a Wilcoxon matched pair test was conducted to assess statistical significance between Tregs and Th cells in the same mice,
FIGURE 2. Tregs isolated from lungs of mice with allergic airway inflammation retain their immunosuppressive functions

Given that inflammation was induced in mouse lungs despite the presence of adequate numbers of Tregs, we wondered whether these Tregs possessed suppressive function. Toward this end, we used Foxp3EGFP knock-in mice to be able to identify Foxp3+CD4+ T cells in the lungs under conditions of inflammation. EGFP+Foxp3+ Tregs were isolated by cell sorting from the lungs of mice with airway inflammation, and splenic CD4+ Th cells from naive mice were used as target Th cells to assess the suppressive function of the Tregs. When assayed in the absence of any exogenous IL-4, the EGFP+ T cells isolated from the lungs were suppressive in a dose-dependent fashion (Fig. 2). The suppressive function was, however, compromised when IL-4 was added to the coculture. The result of this experiment showed that lung-derived Tregs were not dysfunctional per se because they were able to exercise suppressive function in the absence of IL-4. However, when present in an IL-4-rich environment, as would happen under conditions of allergic inflammation, their suppressive function could not be realized. This phenomenon of coexistence of large numbers of Tregs with Th cells in areas of inflammation has been noted in other studies as well (27–33). Thus, the logical next question was why Tregs were rendered ineffective under conditions of inflammation.

Tregs are able to inhibit the sensitization phase of airway inflammation regardless of their ability to respond to IL-4, but they require IL-4 receptor signaling to suppress responses to Ag challenge

Given that the data shown in Fig. 2 show that IL-4 prevented Tregs from exerting suppressive effects on Th cells, we asked how IL-4 provided a permissive role during the adaptive immune response to an allergen. Our focus on IL-4 and its influence on Th cells vs Tregs in the context of allergic inflammation was stimulated by a recent finding concerning IL-2, which showed that the crucial role provided by IL-2 in the peripheral maintenance of Tregs under homeostatic conditions stems from the selective expression of IL-2.
inflammation in the airways of the mice. As shown to the model of allergic inflammation and were analyzed for in-

tion of experimental airway inflammation in the recipients even 

mice were adoptively transferred. Unlike the sit-

uation where Tregs drastically reduced inflammation when transferred before sensitization (Fig. 3A), the transfer of WT CD25\(^+\) cells post sensitization and before Ag challenge reduced the number of eosinophils by \(-50\%\), whereas Tregs isolated from tolerized IL-4R\(^-\) mice had no discernible inhibitory effect on eosinophil numbers as assessed in the BAL fluid (Fig. 3B). Therefore, the ability of Tregs to respond to IL-4 appears to be important for the inhibition of responses to Ag challenge in previously sensitized animals (Fig. 3B), although it is irrelevant for their ability to block Ag sensitization (Fig. 3A). It is important to note that in previously sensitized animals a robust Th2 response, including the production of IL-4, would have been already underway that was not the case before Ag sensitization. These results led us to investigate whether IL-4 inhibited Treg function directly or made Th cells more resistant to the inhibitory effects of Tregs.

In vitro, exogenous IL-4 maintains Foxp3 expression and promotes Treg proliferation but inhibits Treg-mediated suppression of Th cells

Because in the data shown in Fig. 3 the introduction of Tregs into the lungs after allergen sensitization decreased the ability of the Tregs to suppress inflammation and the further lack of IL-4R signaling totally compromised the ability of the Tregs to suppress inflammation, we first determined the effect of IL-4 on Foxp3 expression in Tregs that is required for the suppressive function of Foxp3-expressing Tregs. Approximately 90% of freshly isolated spleen CD25\(^+\) cells from naive mice express Foxp3 (data not shown), but after 3 days in culture the percentage decreases substantially to a frequency of 37% (Fig. 4A), which is in agreement with results reported previously (36). In the absence of IL-4, the overall viability of cells was 30.5% after 3 days in culture. The Foxp3\(^+\) cells shown in the illustration were all viable based on their forward scatter/side scatter properties. Propidium iodide staining could not be performed to identify live cells, because Foxp3 staining necessitated cell permeabilization. The addition of IL-4 allowed for the maintenance of Foxp3 expression and also increased the viability of WT cells from 30.5 to 61.2% and that of STAT6-deficient Tregs from 36.1 to 49.8%; but, as expected, it did not improve the viability of IL-4R-deficient Tregs (data not shown). IL-4-mediated increase in Foxp3\(^+\) cells was not observed with IL-4R- or STAT6-deficient cells, showing that STAT6-dependent IL-4 signaling is required in the maintenance of Foxp3 expression in naturally occurring Tregs (Fig. 4A). However, this observation is different from the negative effect of STAT6 on de novo Foxp3 expression in CD4\(^+\) T cells (37). Similar to the maintenance of Foxp3 expression in the presence of IL-4, the frequency of CD25\(^{\text{high}}\) cells was also higher when IL-4 was included in the culture and was partially maintained in STAT6\(^-\) cells (Fig. 4A). It should be noted that in the presence of IL-4 the frequency of CD25\(^{\text{high}}\) population was 86.3% but that of Foxp3\(^+\) cells was slightly less (74%), which suggests that the small population of CD25\(^{\text{high}}\) cells that was not Foxp3\(^+\) represented Th cells that were copurified with Tregs. The maintenance of Foxp3 expression in Tregs by STAT6 signaling seems to be in contrast to the negative effect of STAT6 on the de novo induction of Foxp3 in naive CD4\(^+\) T cells (37).

We noted a population of low CD4 expressers within the Foxp3\(^+\) or CD25\(^+\) population. The lower CD4 expressers were not dying cells based on their scatter properties. Without an added cytokine, the frequency of the low CD4 expressers in WT Tregs was 45.5%. However, it was reduced to 14% in the presence of IL-4 (or IL-2-not shown). It is possible that IL-4 and IL-2 up-regulate the expression of the coreceptor CD4 on Tregs to promote TCR signaling. To distinguish between the antiapoptotic effects of
IL-2 on the cells vs the effects on proliferation or other parameters, we investigated the effect of a combination of IL-4 and IL-2, which gave essentially similar results obtained with either cytokine alone (data not shown).

To assess the effect of IL-4 on Treg proliferation, CD25+ cells were isolated from naive WT, STAT6−/−, and IL-4Rα−/− mice and used as shown. CD4-depleted WT splenocytes were irradiated (2000 rad) and used as APCs (3 × 10^6 per well) in conjunction with soluble anti-CD3 (2 μg/ml) to stimulate Tregs alone (A and B) or Th cell/Treg cells in coculture (C). A, Tregs were cultured for 3 days and Foxp3 or CD25 expression was analyzed by flow cytometry. Without IL-4, the frequency of viable cells was 30.5, 36.1, and 37.3% of total cells for Tregs isolated from WT, STAT6−/−, and IL-4Rα−/− mice respectively after 3 days in culture. In the presence of IL-4, the corresponding live cell frequencies were 61.2, 49.8, and 38.8%. Gating for live cells was based on forward and side scatter, which in routine assays match data obtained with propidium iodide/annexin staining. B and C, cells were stimulated in the presence or absence of IL-4 (20 U/ml) as indicated. B, Tregs were cultured for 2 days, pulsed with [3H]thymidine for 18 h, and analyzed by liquid scintillation counting (Treg to APC ratio was 1:1). Data shown are mean ± SD. C, Th cells were labeled with CFSE and cultured with Tregs in the indicated ratios for 3 days. Cells were stained and analyzed by flow cytometry. The y-axis shows the percentage of Th cells that divided one or more times. Numbers in dot plots are the percentages of cells in the quadrants. D, Th cells and Tregs were isolated from WT, STAT6−/−, or IL-4Rα−/− mice. Th cells labeled with CFSE were cocultured in all possible combinations as indicated for a CFSE dilution assay. Assays were conducted in the presence or absence of exogenous IL-4 (20 ng/ml). Data shown are means ± SD and represent average of data from two independent experiments.

On the in vivo data, these results suggested that IL-4 might actually enhance Treg-mediated suppression of airway inflammation, because both Treg proliferation and Foxp3 expression are associated with the enhancement of suppressive function (21).

To address the impact of IL-4 on Treg function, we labeled Th cells with CFSE, which allowed us to exclusively follow Th proliferation in the Treg/Th mix. Th cells were labeled with CFSE and all possible combinations of Tregs and Th cells from WT, STAT6−/−, and IL-4Rα−/− mice were included to directly assess the influence of IL-4-signaling in Tregs vs Th cells in...
Treg-mediated suppression. In data presented in Fig. 4C, each set of three bars represents the suppressive function of all three types of Tregs on a single type of Th cell, which is indicated on the x-axis. Similar heights of bars within each triplicate indicated that the type of Treg had little influence on suppression irrespective of the presence or absence of IL-4. In contrast, the height of the first triplicate set was greater than the other two and was increased by the presence of IL-4, demonstrating that IL-4 decreased suppression by Tregs (38, 39) that was dependent on functional IL-4Rα and STAT6 in Th cells. It was interesting to note that in the presence of IL-4, IL-4Rα-deficient Tregs were less efficient than STAT6-deficient Tregs in suppressing Th cell proliferation (Fig. 4D). This was probably because of the lack of the second pathway, IRS-2/Akt, downstream of IL-4Rα in IL-4Rα-deficient Tregs that has been associated with T cell proliferation and survival (40–42). Tregs unable to trigger this pathway in response to IL-4 would have both survival and proliferation compromised in addition to Foxp3 expression (Fig. 4A) and can be expected to be less efficient than STAT6-deficient Tregs with this pathway intact in exercising suppressive effects, as we observed. Collectively, our data indicated that whereas IL-4-induced STAT6 activation promotes proliferation of both Tregs and Th cells and increases T cell resistance to suppression, it did not appreciably influence the suppressive function of Tregs when cells were able to respond to IL-4. Rendering Tregs completely unresponsive to IL-4 made Th cells less sensitive to Treg-mediated suppression, probably due to the presence of fewer Tregs in culture. Thus, the previous in vitro data of Pace et al. on the ability of IL-4 to reduce Treg suppressive function was also evident in these experiments (38, 39). However, we performed additional experiments to determine whether this impairment of the suppressive function of Tregs in the presence of IL-4 was due to effects on both Tregs and Th cells. This question was particularly relevant because the in vitro data presented in Fig. 4C showed that IL-4 signaling in Tregs was not the only thing that mattered in the ability of IL-4 to dampen Treg function.

**Constitutive STAT6 signaling in Tregs does not impair Treg function**

Although STAT6 in Th cells appeared to be necessary for IL-4-mediated resistance to Tregs, it was unclear whether STAT6 activation alone in Th cells would promote their resistance to Tregs. To address this question, cells were infected with a retrovirus encoding a constitutively active form of STAT6 (STAT6VT, referred to here as caSTAT6) (43). The bicistronic retroviral construct also contained the gene for GFP, which allowed both the monitoring of infection efficiency by flow cytometry as well as the isolation of caSTAT6-expressing cells by cell sorting. The functionality of the caSTAT6 protein was evaluated by the infection of Tregs from STAT6−/− mice and the observation of proliferation. Cells expressing caSTAT6 proliferated significantly better than cells infected with the same retrovirus lacking the caSTAT6 gene (Fig. 5A). This was in agreement with data presented in Fig. 4B showing the proliferation-inducing effects of IL-4 on Tregs and additionally demonstrating that activated STAT6 downstream of IL-4-signaling is an inducer of CD4+ T cell proliferation. Because retroviral infection involves prestimulation of cells with IL-2, which also promotes Treg proliferation, as expected the proliferation-promoting effect of caSTAT6 was less pronounced compared with that of IL-4 added to freshly isolated Tregs from naive mice (Fig. 4B).

Despite the promotion of Treg proliferation by IL-4, caSTAT6 caused only a minimal change in suppressive function, showing that IL-4 signaling via STAT6 does not directly alter Treg function on a per cell basis (Fig. 5B). The similar suppressive function of vector- and caSTAT6-transduced Tregs, despite the increased proliferation of the latter, suggested more than a numbers game as far as Tregs were concerned and prompted us to focus on Th cells to study the effect of IL-4 signaling. The percentage of Tregs expressing Foxp3 transduced with caSTAT6 was similar to that of vector-transduced Tregs at the end of the culture period. The mean fluorescence intensity of Foxp3 in caSTAT6-transduced Tregs was slightly lower than that in the control Tregs (Fig. 5C). Thus, in agreement with data shown in Fig. 4, STAT6 expression in naturally occurring Tregs did not compromise Foxp3 expression.

**STAT6 signaling is sufficient for enhancement of Th cell resistance to Treg-mediated suppression**

We first ensured the expression of caSTAT6 in Th cells by infecting splenic STAT6−/− CD4+ Th cells with the retrovirus and analyzing for STAT6 protein expression by Western blotting techniques (Fig. 6A). To characterize the Th cells infected with caSTAT6, cells were sorted according to GFP expression and stimulated with anti-CD3 and APC. After 3 days of stimulation, transduced cells were analyzed for GATA-3 and T-bet expression by Western blotting techniques and for the cytokine levels in the culture supernatant. Both vector- and caSTAT6-transduced Th cells expressed T-bet, although caSTAT6-transduced cells expressed less T-bet. However, GATA-3 was not detected in vector- or caSTAT6-transduced cells (Fig. 6B). In retroviral transductions, cells are stimulated to vigorously proliferate for 40 h in the presence of IL-2 before retroviral infection. Because caSTAT6 was...
introduced only 40 h after cell activation, it was not surprising that T-bet, but not GATA-3, was expressed. Interestingly, IL-13 was introduced only 40 h after cell activation, it was not surprising that Th cell proliferation was different in the results because of the difference in culture location. Based on similar observations in other disease models, it is clear that while cells mediating immune responses to either foreign or self-Ags can be readily identified at sites of inflammation, Tregs also develop and coexist with Th cells during inflammation (27–33). In experimental autoimmune encephalitis, a model of multiple sclerosis, although Tregs were found to infiltrate the CNS, similar to our findings and those of others in allergic airway inflammation (Fig. 1 and Ref. 33), these Tregs were unable to prevent disease (32). Similarly, in sarcoidosis, a granulomatous disease of the lung, Tregs present in abundance around the granulomas were unable to inhibit TNF-α and IFN-γ production by effector T cells (29). So, why is inflammation induced if inflammatory conditions can promote Treg numbers concurrently with Th cell numbers? We focused our attention on IL-4 to explain this phenomenon. This was due to the fact that IL-4 promotes proliferation of not only Th cells but also of Tregs, as shown previously in vitro studies (38) and supported by our own data (Fig. 4). This effect is similar to that of IL-2, which has been shown to promote Treg proliferation and boost its suppressor function (14–16). IL-2 is also critical for both the central and peripheral maintenance of Tregs (45, 46). STAT5b, downstream of IL-2 receptor signaling, has been shown to be important for Treg accumulation and suppressor function in humans (14). Although IL-4 was described as inhibiting Treg suppression in vitro (39), the role of STAT6, a major mediator of IL-4 effector function, in causing Th resistance but keeps Tregs functionally competent so that when T effector cells contract later in inflammation, the Tregs are poised to take over and restore homeostasis. In a similar fashion, spontaneous IL-2 secretion by lung effector T cells in sarcoidosis has been implicated in Treg expansion around the granulomas (29). The balance between the competing effects of IL-4 or IL-2 on Treg vs T effector function.

Discussion

The present study shows that constitutive STAT6 signaling in a Th cell impairs the suppressive potential of Tregs. The biological significance of this finding lies in the scenario of allergen-induced, increased Th cell numbers accompanied by eosinophilia in the airways even though Tregs also increase in numbers at the same location. Based on similar observations in other disease models, it is clear that while cells mediating immune responses to either foreign or self-Ags can be readily identified at sites of inflammation, Tregs also develop and coexist with Th cells during inflammation (27–33). In experimental autoimmune encephalitis, a model of multiple sclerosis, although Tregs were found to infiltrate the CNS, similar to our findings and those of others in allergic airway inflammation (Fig. 1 and Ref. 33), these Tregs were unable to prevent disease (32). Similarly, in sarcoidosis, a granulomatous disease of the lung, Tregs present in abundance around the granulomas were unable to inhibit TNF-α and IFN-γ production by effector T cells (29). So, why is inflammation induced if inflammatory conditions can promote Treg numbers concurrently with Th cell numbers? We focused our attention on IL-4 to explain this phenomenon. This was due to the fact that IL-4 promotes proliferation of not only Th cells but also of Tregs, as shown previously in vitro studies (38) and supported by our own data (Fig. 4). This effect is similar to that of IL-2, which has been shown to promote Treg proliferation and boost its suppressor function (14–16). IL-2 is also critical for both the central and peripheral maintenance of Tregs (45, 46). STAT5b, downstream of IL-2 receptor signaling, has been shown to be important for Treg accumulation and suppressor function in humans (14). Although IL-4 was described as inhibiting Treg suppression in vitro (39), the role of STAT6, a major mediator of IL-4 effector function, in causing Th cell resistance to Treg function when both are present together has not been investigated before this study.

We show that although IL-4 promotes Treg proliferation and maintains Foxp3 expression, Tregs are functionally compromised under conditions of inflammation. This is an excellent example of how the same cytokine maintains the dynamics of inflammation by exercising its function on both Th cells and Tregs. When an adaptive immune response picks up, it induces Th resistance but keeps Tregs functionally competent so that when T effector cells contract later in inflammation, the Tregs are poised to take over and restore homeostasis. In a similar fashion, spontaneous IL-2 secretion by lung effector T cells in sarcoidosis has been implicated in Treg expansion around the granulomas (29). The balance between the competing effects of IL-4 or IL-2 on Treg vs T effector function.
likely determines the outcome of antigenic stimulation in terms of tolerance induction or the magnitude of inflammation induced. So, how might the chronic activation of STAT6 render cells unresponsive to Tregs? GATA-3 is the master regulator of Th2 differentiation, as we and others previously established (22, 47, 48). STAT6 is required during the early stages of Th2 differentiation to promote GATA-3 expression, but subsequently GATA-3 autoregulates its own expression. STAT6 has been also shown to rescue Th cells from an anergy checkpoint after priming to induce T cell proliferation and differentiation (49). STAT6 activation is promoted by multiple signals during Th cell differentiation, which includes those emanating from CD28 and ICOS. Tregs mediate suppression by multiple mechanisms, many of which involve TGF-β (26). We have previously shown that soluble TGF-β inhibits Itk activation and Ca²⁺ flux in T cells, thereby inhibiting GATA-3 activation to cause inhibition of Th2 differentiation (12). More recently, we have shown that membrane-bound TGF-β activates Notch1 in target Th cells and that cooperation between these two pathways causes inhibition of Th2 responses in the lung (11, 20). The question then arises as to how TGF-β-induced suppressive mechanisms are overridden by effector cytokines such as IL-4 and IL-13 secreted by Th cells during allergic inflammation.

In a recent report, Tregs expressing membrane-bound TGF-β were found to induce Foxp3 expression in naive Th cells that imparted suppressive properties to these cells in a process of infectious tolerance (50). Interestingly, in another work published recently, STAT6 was shown to inhibit TGF-β1-induced Foxp3 induction via direct binding to the Foxp3 promoter (37). Similarly, GATA-3 was also shown to bind to the Foxp3 promoter, thereby blocking TGF-β1-induced Foxp3 expression (51). Collectively, these observations provide a molecular basis for our finding that caSTAT6 renders Th cells unresponsive to suppression by Tregs. First, these recent reports would explain why Tregs would be more suppressive irrespective of IL-4Ra signaling when transferred to presensitized animals (Fig. 3A). Under these conditions, the Tregs would be able to proliferate in response to both IL-2 and IL-4 because recently activated Th cells would supply both of these cytokines, and Tregs would have the added advantage of being able to induce infectious tolerance by inducing Foxp3 into Th cells, rendering them suppressive as well (50). The induction of Foxp3 would be in competition with the opposing effects of STAT6 and GATA-3 induction in the freshly activated Th cells. In contrast, when the Tregs are administered after sensitization (Fig. 3B), the mice already have at their disposal effector cells with high levels of GATA-3 expression that would block Foxp3 induction in these already differentiated Th2 cells (51). Second, the availability of less IL-2 would cause the Tregs to depend more on IL-4 for Foxp3 expression and proliferation, which is why the Tregs were still functional in inhibiting the effector cells (albeit to a lesser extent compared with transfer into animals before sensitization) when they were derived from WT mice (Fig. 3B and Ref. 50), but their suppressive function was severely impaired in the absence of IL-4 signaling (Fig. 3B). In the in vitro studies shown in Figs. 5 and 6, expression of caSTAT6 would prevent Foxp3 induction in the Th cells (43) but would be of less consequence in Tregs, which already express Foxp3. This suggests that the process of infectious tolerance competes with the opposing process of Th cell differentiation into different lineages during an adaptive immune response. Given that Tregs can be detected in large numbers not only in the context of allergic inflammation but other types of inflammation as well (27–33), there are at least two explanations for why an adaptive immune response is launched despite the power of infectious tolerance. First, once TCR stimulation plus adequate costimulation drives Th cell proliferation during an adaptive immune response, factors such as STAT6 and GATA-3 and possibly additional transcription factors as well prevent de novo Foxp3 induction to allow an adaptive immune response to take hold. Second, under conditions of inflammation Tregs may not express adequate levels of latency-associated peptide, membrane-bound TGF-β on their cell surface, which would be interesting to study in the future. Reciprocally, protocols that promote tolerance such as persistent inhalation of aerosolized Ag may be particularly effective in promoting the expression of membrane-bound TGF-β as we previously reported (11, 20). These TGF-β-expressing Tregs would be effective in inducing infectious tolerance via the induction of Foxp3 in naive CD4⁺ T cells.

Our results also have relevance to cytokine-based therapeutic control of asthma. The IL-4 variant pitrakinra, which potently inhibits the binding of both IL-4 and IL-13 to IL-4Rα, was recently shown to significantly alleviate asthma symptoms (52). These results may be explained by the ability of pitrakinra to block STAT6 signaling downstream of IL-4Rα in effector T cells, thereby relieving resistance to Treg suppression. Because other cytokines such as IL-6, also secreted by Th2 cells, may supplant IL-4 in maintaining Treg proliferation and Foxp3 expression as we and others have shown (21, 53), the suppressive effect of Tregs concurrent with a blockade of effects of IL-4/IL-13 on Th cells by agents such as pitrakinra may significantly disable the effector function of the latter. Thus, our data suggest that in the context of active inflammation in asthma patients, blocking the dominant stimulatory effects of IL-4 and IL-13 via STAT6 on disease-associated Th2 cells would likely result in a net beneficial effect. The results of our experiments also suggest that inhibition of IL-4/IL-13 effects in vivo combined with the adoptive transfer of activated Tregs could result in even greater clinical efficacy.

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**Disclosures**

The authors have no financial conflict of interest.

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