

Adaptive Foxp3⁺ Regulatory T Cell-Dependent and -Independent Control of Allergic Inflammation

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SUMMARY

Adaptive Foxp3⁺ regulatory T (Treg) cells develop during induction of mucosal tolerance and after immunization. Large numbers of Foxp3⁺ T cells have been found in inflamed tissues. We investigated the role of adaptive Foxp3⁺ Treg cells in mucosal tolerance and in chronic allergic lung inflammation. We used two strains of mice that are devoid of naturally occurring Treg cells; one is capable of generating adaptive Foxp3⁺ Treg cells upon exposure to antigen, whereas the other is deficient in both naturally occurring and adaptive Foxp3⁺ Treg cells. We found that adaptive Foxp3⁺ Treg cells were essential for establishing mucosal tolerance and for suppressing IL-4 production and lymphoid neogenesis in chronic inflammation, whereas IL-5 production and eosinophilia could be controlled by Foxp3-independent, IFN- γ -dependent mechanisms. Thus, whereas adaptive Foxp3⁺ Treg cells regulate sensitization to allergens and the severity of chronic inflammation, IFN- γ -producing cells can play a beneficial role in inflammatory conditions involving eosinophils.

INTRODUCTION

Immunological tolerance to inhaled environmental allergens protects individuals from developing pathological responses in the airways and lungs. Failures in the mechanisms of tolerance lead to the sensitization to allergens and to inflammatory reactions such as allergic asthma. Allergic asthma is characterized by production of T helper 2 (Th2) cytokines, IgE antibodies, eosinophilic lung inflammation, bronchial hyper-responsiveness, and lung remodeling (Wills-Karp, 1999)(Cohn et al., 2004).

Th2 cell-mediated allergic reactions can be prevented by CD4⁺ regulatory T cells (Curotto de Lafaille et al., 2001). Naturally occurring CD4⁺ Treg cells express Foxp3 (Fontenot et al., 2003; Hori et al., 2003; Khattry et al., 2003), develop in the thymus, and migrate to the peripheral lymphoid organs (Shevach et al., 2006). It is now clear that Foxp3⁺ Treg cells phenotypically indistinguishable from naturally occurring Treg cells can also develop in the periphery under a number of conditions (Apostolou and von Boehmer, 2004; Cobbold et al., 2004; Curotto de Lafaille

et al., 2004; Mucida et al., 2005). In vitro, naive T cells can convert to Foxp3⁺ cells by stimulation in the presence of TFG- β and IL-2 (Chen et al., 2003; Zheng et al., 2007). In mice and humans, deficiency in Foxp3 causes spontaneous autoimmunity with multi-organ inflammation and early death (Bennett et al., 2001; Chatila et al., 2000). Foxp3 deficiency also leads to increased atopy (Lin et al., 2005).

Previously, we studied T cell differentiation during oral-tolerance induction in mice that harbor a monoclonal CD4⁺ T population specific for chicken ovalbumin (OVA) and that lack naturally occurring Treg cells. We found that OVA-specific Foxp3⁺ Treg cells were induced upon addition of OVA to the drinking water. Tolerant mice were protected against immunization with OVA in adjuvants, whereas naive mice became sensitized; they developed a Th2 response, IgE antibodies, and allergic inflammation (Mucida et al., 2005). Intriguingly, OVA-specific Foxp3⁺ Treg cells were also induced, alongside Th2 cells, by immunization of naive mice with adjuvants; the role of these immunization-induced Treg cells is not clear because they obviously do not prevent the primary allergic response.

Although the name "adaptive" Treg cells is mostly used in reference to Foxp3-negative regulatory T cells such as Tr1 cells (Levings et al., 2005; Vieira et al., 2004), here we refer to mucosa-induced and immunization-induced Foxp3⁺ Treg cells as adaptive Foxp3⁺ Treg cells to indicate that these cells arise from naive Foxp3⁻ T cells that were not selected in the thymus as naturally occurring Foxp3⁺ Treg cells. Despite the strong correlation between immunological tolerance and allergen-driven induction of Foxp3⁺ Treg cells, it is not known whether adaptive Foxp3⁺ Treg cells are essential for mucosal tolerance or whether other redundant tolerance mechanisms operate as well.

The presence of large numbers of Foxp3⁺ Treg cells in situations of severe inflammation also remains unexplained. Although it has become increasingly clear that Treg cells affect all types of Th-mediated responses, including those against foreign antigens and allergens, there is an apparent paradox, which is that large numbers of Treg cells are found at sites of ongoing chronic inflammation (Ruprecht et al., 2005; Saruta et al., 2007; Sasaki et al., 2007; Smyth et al., 2007; van Amelsfort et al., 2004). It is unclear whether in chronic inflammation these Treg cells are unable to carry out their function, whether they are overwhelmed or neutralized, or even whether they contribute to the pathology, for instance by secreting TGF- β that is important in fibrosis. In acute allergic inflammation, antibody depletion of CD25⁺ cells had a worsening effect in one of two mouse strains tested

(Lewkowich et al., 2005), and a study showed that the number of Treg cells correlated inversely with the severity of allergic inflammation in a rat model of asthma (Strickland et al., 2006), an indication that Treg cells might modulate lung inflammation.

To obtain unambiguous evidence for a role of adaptive Foxp3⁺ Treg cells in mucosal tolerance and chronic allergic inflammation, we compared mice that lack naturally occurring Treg cells but have the capacity to generate adaptive Foxp3⁺ Treg cells with mice that lack naturally occurring Treg cells and also lack the capacity to generate adaptive Treg cells as a result of a deficiency in the *Foxp3* gene. Our results show that the induction of adaptive Foxp3⁺ Treg cells is essential to establish mucosal tolerance by the oral and respiratory routes. Using a model of chronic allergic inflammation, we demonstrate the existence of Foxp3-dependent and -independent suppressive mechanisms. Although overall disease severity and IL-4 mediated pathology was strongly dependent on Foxp3⁺ Treg cells, the suppression of IL-5 production and eosinophilia largely occurred in a Foxp3-independent, IFN- γ -dependent manner. Thus, increased Foxp3⁺ Treg function is expected to benefit all inflammatory conditions, but specific cytokine therapy might apply to particular types of inflammation.

RESULTS

The Role of Adaptive Foxp3⁺ Treg Cells in Mucosa-Induced Immunological Tolerance

T-B monoclonal mice (T-Bmc) have non-self-reactive repertoires of T and B cells. In these mice, all T cells (DO11.10 T cell receptor [TCR] transgenic) recognize chicken ovalbumin (OVA), and all B cells (17/9 IgH and IgL knock-in) recognize a peptide from influenza hemagglutinin (HA). The T and B cell repertoires are monoclonal because of the crossing with Rag-1-deficient mice (Curotto de Lafaille et al., 2001). Like all monoclonal T cell receptor-transgenic mice described thus far, T-Bmc lack naturally occurring Foxp3⁺ Treg cells (Mucida et al., 2005; Shen et al., 2005). We showed previously that the oral administration of OVA to T-Bmc mice resulted in immunological tolerance, which correlated with the induction of antigen-specific adaptive Foxp3⁺CD25⁺ Treg cells. To ensure that the observed conversion of Foxp3[−] into Foxp3⁺ cells in OVA-fed T-Bmc mice was not an artifact caused by the very high frequency of OVA-specific T cells in T-Bmc mice, we transferred bone marrow from T-Bmc mice to wild-type BALB/c recipient mice. In the resulting chimeras, the proportion of OVA-specific Rag-deficient T cells was less than 2% of the T cells, and the vast majority of the T cells were polyclonal wild-type cells of host origin. Importantly, in these chimeras we also observed the “de novo” expression of Foxp3 in donor-derived OVA-specific cells after OVA feeding, whereas no conversion occurred in controls that did not received OVA (Figure S1 in the Supplemental Data).

Foxp3-deficient *scurfy* mice develop a fulminating inflammatory disease and die at about the age of weaning (Kanangat et al., 1996). To study the role of adaptive Foxp3⁺ regulatory T cells without the influence of the imminent inflammatory disease, we crossed *scurfy* mice with T-Bmc mice, whose monoclonal T and B lymphocytes do not recognize self- or environmental antigens. In the absence of deliberate antigen exposure, lymphocytes in the T-Bmc Foxp3^{sf} mice had a naive phenotype, and dendritic cells did not show signs of spontaneous activation (Figure S2).

To determine whether Foxp3⁺ Treg cells were absolutely required for mucosa-induced tolerance, we compared T-Bmc Foxp3^{sf} mice with littermates carrying normal *Foxp3* genes (T-Bmc Foxp3^{wt}). We first tested whether administration of OVA by oral or intranasal mucosal routes to T-Bmc Foxp3^{sf} mice would prevent an allergic response to subsequent immunization with antigen (OVA-HA) in alum (Figure 1A). To determine whether the mice had become sensitized and capable of developing allergic lung inflammation, OVA-HA was subsequently instilled by intranasal route (Figure 1A).

T-Bmc mice carrying the mutated *Foxp3* gene failed to develop mucosa-induced tolerance to OVA, as demonstrated by the high IgE levels in sera of T-Bmc Foxp3^{sf} mice that received oral or intranasal OVA before immunization (Figure 1B). In contrast, IgE concentrations were 99% lower in similarly treated T-Bmc Foxp3^{wt} mice. Furthermore, IgE values were as high in T-Bmc Foxp3^{sf} mice given mucosal OVA as in animals not given mucosal OVA (Figure 1B), suggesting that in this experimental setup mucosal tolerance can be fully accounted for by Foxp3-dependent pathways. Interestingly, immunized T-Bmc Foxp3^{wt} and Foxp3^{sf} mice not previously given OVA through mucosa developed similarly high IgE responses (Figure 1B). Similar conclusions were reached with other inflammation readouts. Cells obtained from broncho-alveolar lavage (BAL) of T-Bmc Foxp3^{sf} mice pretreated with oral or intranasal OVA before immunization produced IL-4, whereas IL-4 production was suppressed in T-Bmc Foxp3^{wt} mice (Figure 1C). Quantification of eosinophils in the BAL revealed intense eosinophilic inflammation in Foxp3^{sf} mice but not in T-Bmc Foxp3^{wt} mice administered oral or nasal OVA before immunization (Figure 1D). The failure of mucosal tolerance in T-Bmc Foxp3^{sf} mice was also illustrated by the unrestrained development of germinal centers and class switching in spleen and lymph nodes (Figure S3).

Foxp3 expression was detected in BAL cells of tolerant and allergic Foxp3^{wt} mice, demonstrating that Foxp3⁺ Treg cells were induced after antigen treatment and localized to the airways after intranasal challenge (Figures 1E and 1F). The induction of OVA-specific adaptive Foxp3⁺ Treg cells and immunological tolerance in this model did not require the thymus in light of the fact that it occurred in thymectomized animals (Figure S4).

To confirm that the phenotype of the T-Bmc Foxp3^{sf} mice was solely the consequence of their T cell defect, we transferred naive OVA-specific T cells from Foxp3^{wt} mice to T-Bmc Foxp3^{sf} mice. The recipient mice were subsequently administered OVA in drinking water for 5 days and then immunized with OVA-HA in alum. Two weeks after immunization, the concentration of IgE antibodies in serum was determined. As anticipated, the transfer of T cells from T-Bmc Foxp3^{wt} mice could complement the tolerance defect of T-Bmc Foxp3^{sf} mice (Figure S5).

Altogether, these experiments demonstrate that Foxp3-expressing T cells are essential for establishing immunological tolerance through the intestine and the airways and that other (Foxp3-independent) tolerance mechanisms play little or no role.

Adaptive Foxp3⁺ Treg Cells Display High Basal Proliferation and a Rapid Increase of Inflammation

The observation that Treg cells are induced by immunization (Mucida et al., 2005), and that they represent a higher proportion of CD4⁺ cells in the inflamed airways than in the lymphoid organs

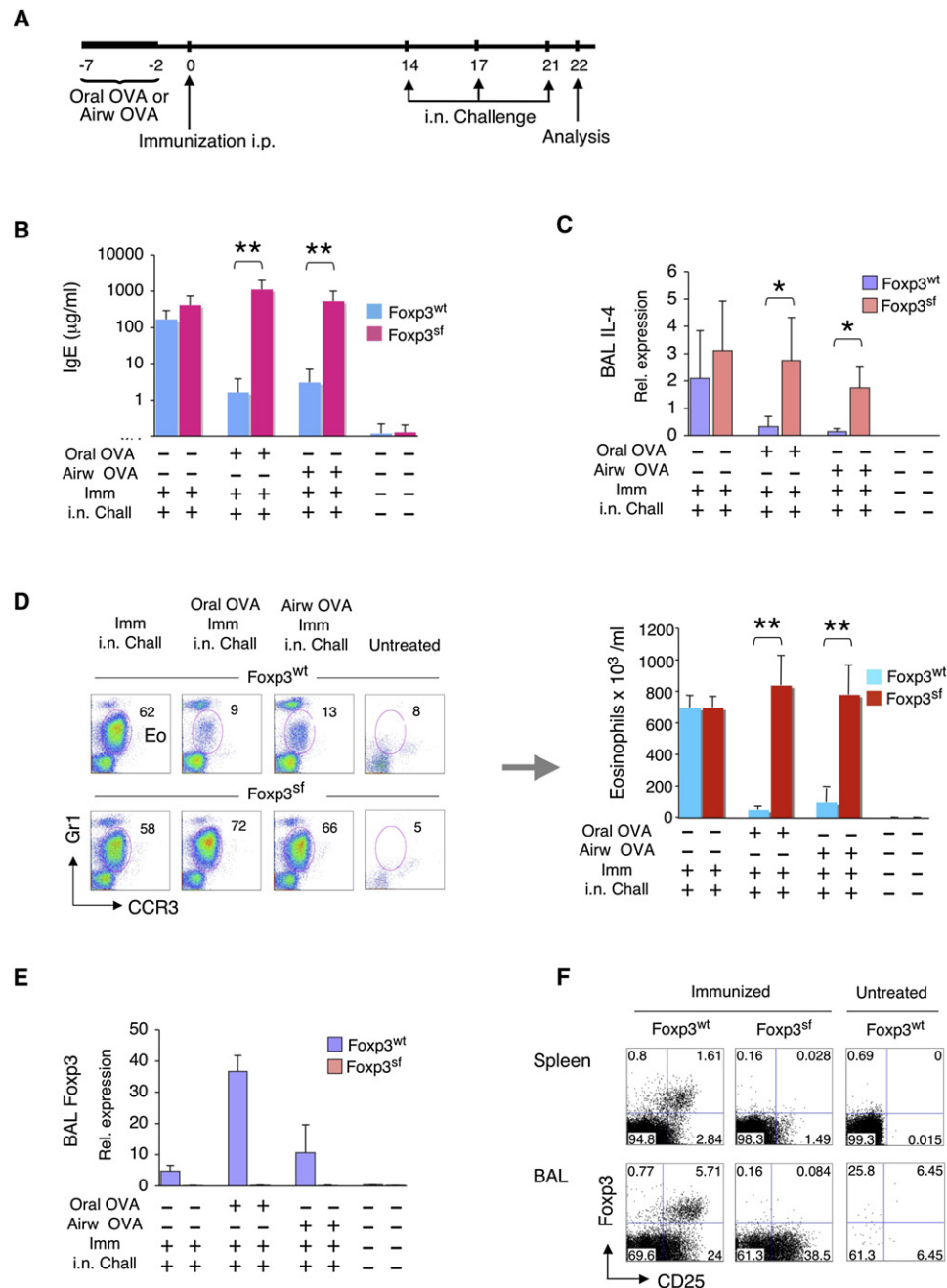


Figure 1. Mucosal Tolerance Is Impaired in Foxp3-Deficient Mice

(A) Protocol for inducing mucosal tolerance to allergic sensitization. T-Bmc mice were pretreated with OVA in the drinking water for 5 days (–7 to –2) or with OVA intranasally for 3 days (–7 to –5). Mice were then immunized by intraperitoneal (i.p.) injection of OVA-HA in alum (day 0). Lung inflammation was induced by intranasal (i.n.) administration of OVA-HA on days 14, 17, and 21. Analysis was performed one day after.

(B) Serum concentration of IgE on day 22. The data are average + SD of 8–16 mice per group.

(C) Expression of *IL-4* mRNA in total BAL cells from day 22 as determined by quantitative PCR (Q-PCR). Average + SD of 3–7 mice per group.

(D) Eosinophils (Eo) in BAL were identified by flow cytometry as MHC II[–], CCR3⁺, Gr1 intermediate cells. The left panel shows representative dot plots of gated MHC II[–] BAL cells. Eosinophil numbers in BAL are shown in the bar graphic on the right. The data are the averages + SD of 3–4 mice per group and are representative of three independent experiments.

(E) Expression of *Foxp3* mRNA in total BAL cells from day 22 as determined by Q-PCR. Shown are averages + SD of 3–7 mice per group.

(F) Identification of Foxp3⁺CD4⁺ cells in immunized and airway-challenged mice. Flow cytometry of BAL and spleen cells from T-Bmc mice that had been immunized and challenged with OVA-HA through the airways and from untreated mice. Representative dot plots show the expression of Foxp3 and CD25 in gated CD4⁺ cells.

**p ≤ 0.01; *p ≤ 0.05.

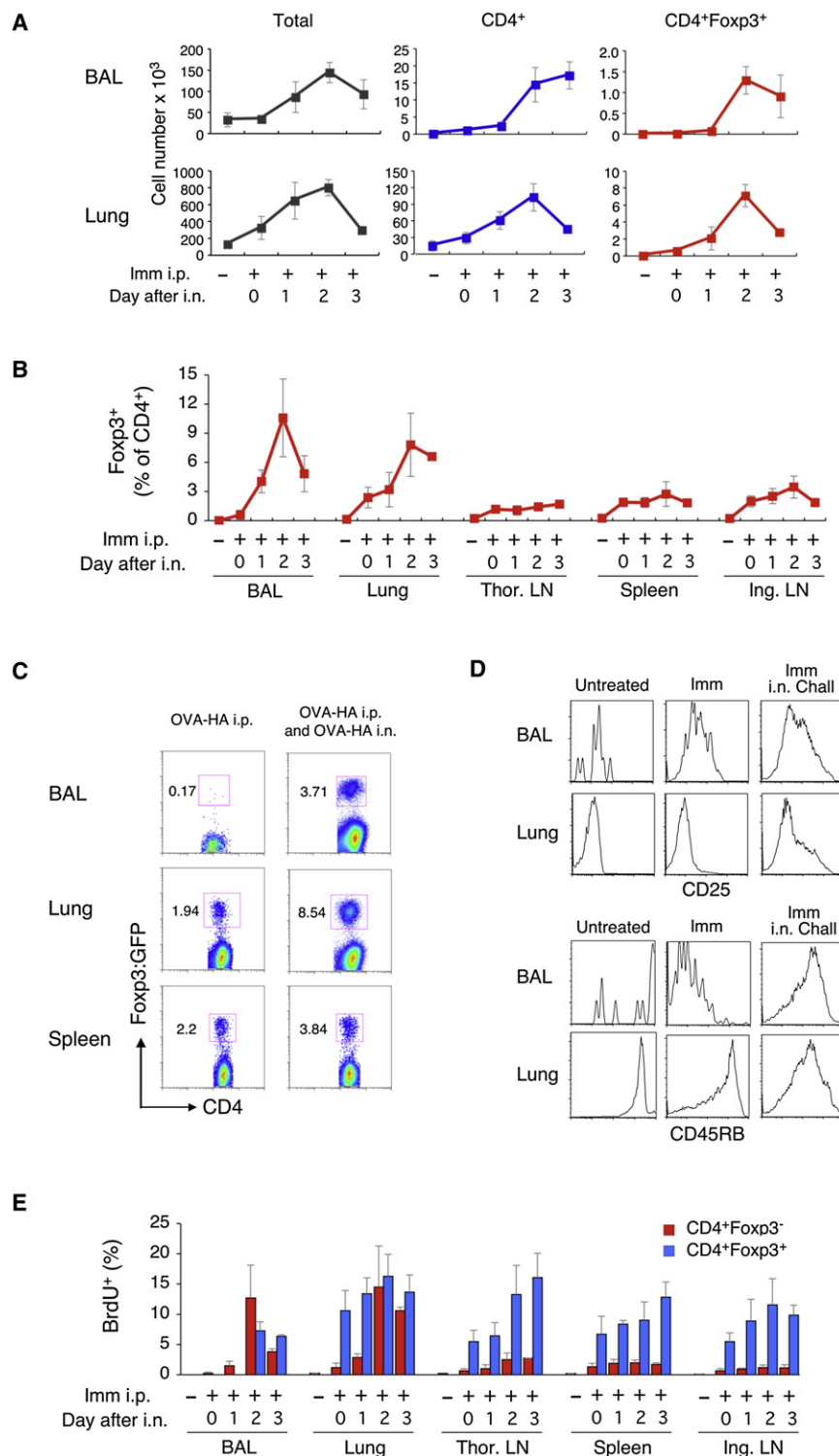


Figure 2. Accumulation of Foxp3⁺ CD4⁺ Cells during the Onset of Allergic Inflammation in the Lung

T-Bmc Foxp3^{wt} mice were immunized intraperitoneally with OVA-HA in alum and 2 weeks later were administered 10 μ g OVA-HA intranasally. Mice were injected with BrdU 6 hr before analysis. BAL, lung, LN, and spleen cells were harvested from untreated mice, immunized but unchallenged (Imm), and immunized and challenged (Imm i.n.) mice 1, 2, and 3 days after i.n. challenge. Values shown in (A), (B), and (E) are average $\pm$ SD of data compiled from four independent experiments ($n = 5$ untreated; $n = 8$ Imm i.n. day 0; $n = 9$ Imm i.n. day 1; $n = 9$ Imm i.n. day 2; $n = 2$ Imm i.n. day 3). (A) Kinetics of inflammation in the lung after intranasal administration of antigen. The numbers of total cells, CD4⁺ cells, and CD4⁺Foxp3⁺ cells in BAL and lungs of mice during the onset of allergic inflammation are shown.

(B) Changes in the percentage of Foxp3⁺ cells among the CD4⁺ population of tissues and lymphoid organs during the onset of lung inflammation. BAL, lung, thoracic LN, spleen, and inguinal LN were analyzed.

(C) Paucity of Foxp3⁺ cells in the airways of immunized mice with no lung inflammation. T-Bmc Foxp3^{gfp}.KI mice were immunized with OVA-HA in alum and were or were not administered OVA-HA intranasally twice. Flow-cytometric analysis was performed 1 day after the last intranasal challenge. Representative plots show Foxp3-GFP expression in gated CD4⁺ cells.

(D) CD4⁺ cells in the BAL of immunized but unchallenged mice have an activated and memory phenotype. Expression of CD25 and CD45RB in CD4⁺ cells from BAL and lungs of untreated, immunized but unchallenged (Imm), and immunized and challenged (Imm i.n. Chall) T-Bmc mice. Representative flow histograms of gated CD4⁺ cells are shown.

(E) BrdU incorporation in CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ cells of tissues and lymphoid organs during the onset of allergic inflammation. Representative flow plots of BrdU staining are shown in Figure 6S.

of allergic mice (Figures 1F), prompted us to analyze Foxp3⁺ Treg cell proliferation and accumulation before and after intranasal challenge. Comparative analysis was carried out for Foxp3⁻CD4⁺ cells.

T-Bmc mice were immunized with OVA-HA in alum by intraperitoneal route, and 2–3 weeks later they received intranasal

cells became more prominent; they had increased 13-fold in the lungs and more than 100-fold in the BAL by the second day after intranasal challenge. In comparison, CD4 cells increased 4-fold in the lungs and 14-fold in the BAL, and the increase in total cells was even lower (Figure 2A). Thus, there was an enrichment of Treg cells in lungs and airways as the inflammation progressed.

Comparison of the frequencies of the different CD4⁺ T cell subsets in lungs, airways, and lymphoid organs showed that, on average, inflamed lungs and airways contained the highest percentage of Foxp3⁺ cells (Figure 2B).

It was interesting to observe the relative paucity of Foxp3⁺ cells within the BAL population from immunized mice that had not been administered intranasal antigen. In these mice, (indicated as "0" in Figure 2), the percentage of Foxp3⁺ within CD4⁺ cells was much lower in the BAL than in the lungs, LN, or spleen. This is further illustrated in Figure 2C by the analysis of BAL, lung, and spleen CD4⁺ cells from *Foxp3gfp*.KI reporter T-Bmc mice. After intranasal challenge, the percentage of GFP⁺ cells increased dramatically in the BAL, consistent with the results shown in Figure 2B.

BAL CD4⁺ cells in immunized but unchallenged mice had an activated and memory CD25⁺CD45RB^{low} phenotype (Figure 2D). In contrast, CD4⁺ cells recovered from the lungs of the same mice showed a resting phenotype containing low numbers of CD25⁺ and CD45RB^{low} cells, and a percentage of Foxp3⁺ cells similar to lymphoid organs. Thus, although conventional memory CD4⁺ T cells were able to cross the lung epithelial barrier under noninflammatory local conditions, adaptive Foxp3⁺ Treg cells were less able to do so and required further antigen stimulation to reach the airway space.

To determine the proliferation rates of Foxp3⁺ and Foxp3[−]CD4⁺ cells in tissues and lymphoid organs, we analyzed BrdU incorporation (Figure 2E and Figure S6). Spleen and LN Foxp3⁺ cells from immunized mice incorporated BrdU at higher rates than Foxp3[−]CD4⁺ before and after intranasal challenge. In lungs of immunized but unchallenged mice, approximately 10% of Foxp3⁺ cells were labeled with BrdU, but less than 2% of Foxp3[−]CD4⁺ cells were thus labeled. Intranasal challenge caused a 12-fold increase in the percentage of BrdU⁺ cells within the Foxp3[−]CD4⁺ cells of the lungs, but less than a 2-fold increase in the percentage of BrdU⁺ Foxp3⁺ cells. In draining thoracic LN, BrdU incorporation increased 3.9-fold in Foxp3[−]CD4⁺ cells and 2.4-fold in Foxp3⁺ cells (Figure 2E). The increase in the proliferation rate of Foxp3⁺CD4⁺ T cells was rather unremarkable at the time in which Treg numbers were increasing dramatically. These data can be explained by an increased conversion of Foxp3[−]CD4⁺ cells into Foxp3⁺ cells in situ within the lung tissue, although other scenarios are also possible.

In sum, we showed that the development of allergic lung inflammation is accompanied by a preferential accumulation of Foxp3⁺ Treg cells in lungs and airways and that adaptive Foxp3⁺CD4⁺ T cells have a higher basal level of proliferation than Foxp3[−]CD4⁺ cells in immunized mice.

Oral OVA Alone Does Not Induce Effector Th Cell Differentiation in T-Bmc Foxp3^{sf} Mice

It has been known for a long time that antigen administration through mucosal routes results in immunological tolerance. We reported that oral tolerance in T-Bmc Foxp3^{wt} mice correlated with the generation of Foxp3⁺ Treg cells (Mucida et al., 2005). We then asked whether the administration of oral OVA per se would induce the differentiation of CD4⁺ Th effector cells when Foxp3⁺ Treg cells could not be generated. To this end, we fed OVA to T-Bmc mice with either wild-type or *scurfy* Foxp3 genes and studied the activation and effector functions of their mesen-

teric LN T cells (Figure 3A). Two days after the oral OVA treatment, the early activation marker CD69 was similarly upregulated in T cells from T-Bmc Foxp3^{wt} or T-Bmc Foxp3^{sf} mice (47% and 44% of CD69⁺ cells among OVA-specific T cells, respectively). Far fewer OVA-specific T cells expressed CD25 or CD103 at the same time point (Figure 3B). A population of OVA-specific T cells coexpressing Foxp3, CD25, and CD103 was induced in Foxp3^{wt} mice but not in Foxp3^{sf} mice (Figure 3B). After OVA treatment, 1%–2% of OVA-specific cells expressed Foxp3 in the mesenteric LN of T-Bmc Foxp3^{wt} mice.

Notably, in spite of the deficiency in Treg development, mesenteric LN T cells of T-Bmc Foxp3^{sf} mice did not become differentiated Th1, Th2, or Th17 cells, as shown by the analysis of *IL-4*, *IFN-γ*, and *IL-17* transcripts (Figure 3C). We analyzed B cell activation and observed that the oral OVA treatment induced a small increase in the percentage of FAS⁺B220⁺ cells in both Foxp3^{wt} and Foxp3^{sf} mice (Figure S7).

These results show that oral OVA treatment induced a population of adaptive Foxp3⁺ T cells in mesenteric LN of T-Bmc mice but that even when it was not possible to generate Foxp3⁺ Treg cells (because of the *scurfy* mutation), there was no induction of effector Th functions. Our data support the view (Iweala and Nagler, 2006) that the default response of the intestinal environment to innocuous antigens is noninflammatory.

Adaptive Foxp3⁺ Treg Cells Control the Severity of Inflammation

The role of Foxp3⁺ Treg cells in sites of chronic inflammation is not clear. Because there are few Treg cells in uninfamed tissue, and many Treg cells in inflamed tissues, it is possible that Treg cells contribute to the pathogenesis of inflammation or that they are neutral because they are turned off in an inflammatory environment. It is also possible that inflammation would be worse if Foxp3⁺ Treg cells were not present at the sites. We sought to take advantage of our experimental system to address these possibilities.

We immunized T-Bmc Foxp3^{sf} and Foxp3^{wt} mice with OVA-HA in alum, a treatment that induces a strong Th2 and IgE response in both types of mice, and subsequently administered OVA-HA intranasally twice a week for eight weeks to create a chronic stimulation (Figure 4A). We observed marked differences in lung inflammation between wild-type mice and those carrying *scurfy* Foxp3 genes. H&E-stained lung sections showed dense perivascular and peribronchial cellular infiltrates in chronically challenged T-Bmc Foxp3^{sf} mice, whereas a greatly reduced inflammatory load was observed in Foxp3-competent T-Bmc mice (Figure 4B). In fact, chronically challenged T-Bmc Foxp3^{wt} mice had less lung inflammation than mice with acute inflammation. In contrast, in T-Bmc Foxp3^{sf} mice the degree of inflammation increased as the number of exposures to antigen increased (Figure 4B).

The analysis of lung-infiltrating cells showed that chronically challenged T-Bmc Foxp3^{sf} mice had three to four times more cells infiltrating the lungs than Foxp3^{wt} mice (Figure 4C). The number of CD4⁺ cells in lungs of Foxp3^{sf} mice was about 5-fold higher than in lungs of chronically challenged Foxp3^{wt} mice, and the number of IL-4-producing cells was about 12-fold higher (Figure 4C). Consistent with the strict IL-4 dependency of IgE, chronically challenged T-Bmc Foxp3^{sf} mice had

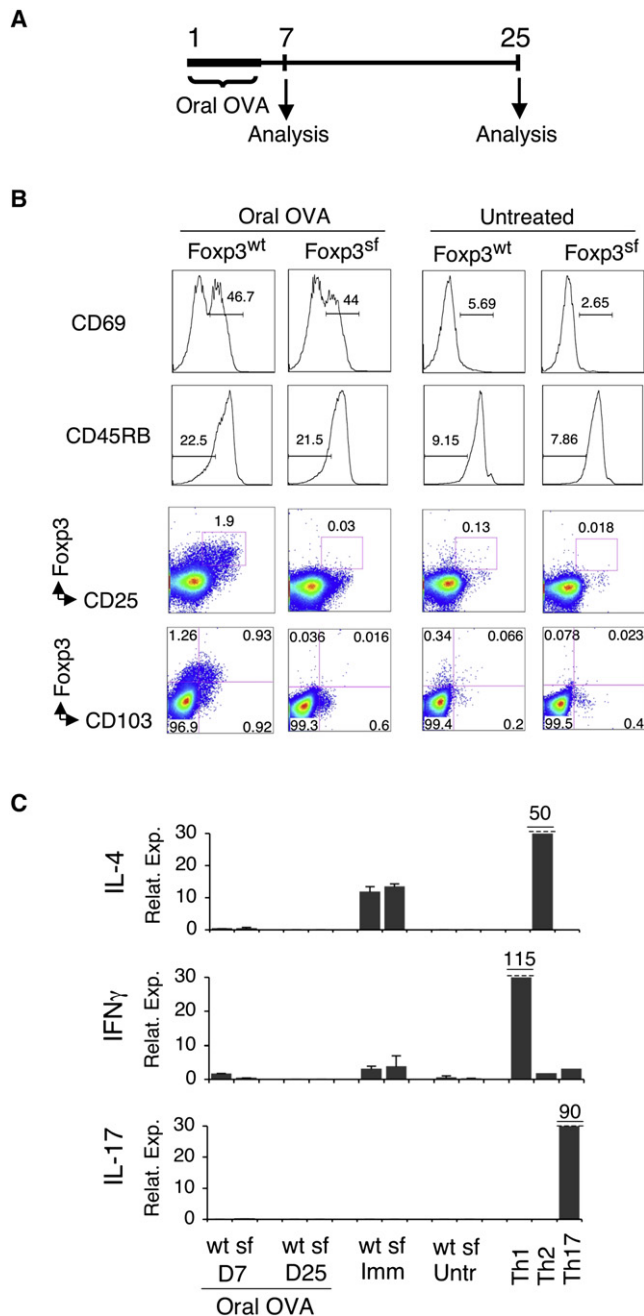


Figure 3. Oral OVA Does Not Induce Th Effector Cells in Foxp3^{wt} or Foxp3^{sf} Mice

(A) Experimental protocol. T-Bmc Foxp3^{wt} and T-Bmc Foxp3^{sf} mice were administered OVA in the drinking water for 5 days. Two and 20 days later the mice were sacrificed, and T and B cell responses were analyzed.

(B) Comparative analysis of CD69, CD45RB, CD25, CD103, and Foxp3 expression in CD4⁺ cells from mesenteric LN cells of T-Bmc Foxp3^{wt} and Foxp3^{sf} mice treated with oral OVA (day 7).

(C) Oral OVA does not induce Th1, Th2, or Th17 cells in mesenteric LN of T-Bmc Foxp3^{sf} mice. Q-PCR analysis of IL-4, IFN-γ, and IL-17 expression in total LN cells from oral OVA-treated mice (days 7 and 25). For comparison, samples from mesenteric LN cells of immunized mice (Imm) and from in vitro differentiated Th1, Th2, and Th17 cells were also included. The results shown are averages + SD of 2–3 mice per group. The data are representative of three independent experiments.

5-fold higher serum IgE than chronically challenged animals able to generate Foxp3⁺Treg cells (Figure 4D), even though both mice strains generated comparable hyper-IgE responses in acute phase.

The differences in IL-4 and IgE production in chronically challenged mice were confirmed through Q-PCR analysis of RNA expression in BAL cells, lungs, and draining LN (Figure 4E). We included samples from immunized mice with acute inflammation to examine the acute-to-chronic progression. We also analyzed the expression of IL-10, a cytokine produced by Th2 and Tr1 cells; IL-33, a cytokine produced by lung epithelial cells that promotes Th2 responses (Schmitz et al., 2005); and FcεRI, which is expressed by mast cells and basophils. In T-Bmc mice with intact Foxp3 genes, the IL-4 mRNA production decreased significantly from the acute to the chronic phase in thoracic LN and less substantially in BAL cells and lungs. In contrast, IL-4 mRNA was upregulated several-fold from the acute to the chronic phase in T-Bmc Foxp3^{sf} mice (Figure 4E). A similar pattern of expression was observed for IL-10. A large increase in mRNA for FcεRI was observed in chronically challenged T-Bmc Foxp3^{sf} mice, indicating an increased number of mast cells and/or basophils. A much lower increase in FcεRI mRNA was observed in chronically challenged mice with adaptive Foxp3 Treg cells (Figure 4E). Analysis of IgE transcripts in lungs and draining LN yielded a rather similar result. Production of IL-33 mRNA was also sustained in Treg-deficient chronically challenged mice, whereas it was suppressed in chronically challenged mice capable of generating adaptive Foxp3⁺ Treg cells (Figure 4E). From these data we conclude that, in situations of chronic antigen exposure, adaptive Foxp3⁺ Treg cells have an important role in reducing IL-4 production, IL-4 mediated pathology, and overall inflammation in the lung.

Adaptive Foxp3⁺ Treg Cells Control Formation of Ectopic Organized Lymphoid Tissue

A feature of many chronic inflammatory diseases is the ectopic development of organized lymphoid tissue with defined T and B cell areas and germinal centers (Aloisi and Pujol-Borrell, 2006; Drayton et al., 2006; Moyron-Quiroz et al., 2007). We therefore investigated whether the dense lymphoid aggregates found in the lungs of chronically challenged T-Bmc Foxp3^{sf} mice showed lymphoid tissue-organization. Indeed, lymphoid aggregates with distinct T and B cell areas, including GL7⁺ germinal centers, could be clearly identified in frozen lung sections from T-Bmc Foxp3^{sf} mice (Figure 5A). Furthermore, large numbers of IgE⁺ and IgG1⁺ cells localized to the lymphoid aggregates, suggesting in situ class switching in the lungs (Figure S8). Thus, in mice that lack naturally occurring as well as adaptive Foxp3⁺ Treg cells, chronic antigen stimulation through the airways leads to an abundance of organized lymphoid tissue in the lungs. Ectopic lymphoid tissue in the lungs was much less abundant in mice able to generate adaptive Foxp3⁺ Treg cells.

Given the amplified inflammation observed in T-Bmc Foxp3^{sf} mice compared to T-Bmc Foxp3^{wt} mice, we studied whether lung remodeling, a feature of chronic asthma, was increased in mice deficient in Foxp3. We observed severe smooth-muscle hypertrophy in blood vessels and, to a lesser degree, in the bronchia in T-Bmc Foxp3^{sf} mice but observed much-less-intense

remodeling in T-Bmc *Foxp3*^{wt} mice (Figure 5B). In sum, the severity of chronic allergic inflammation is greatly reduced by the presence of adaptive Foxp3⁺ Treg cells.

Adaptive Foxp3⁺ Treg Cells Control the Spreading of IL-4-Producing Cells

We next assessed whether the lack of adaptive Foxp3⁺ Treg cells could result in spreading of the Th2 response from the airways and lungs to other tissues during continuous exposure to OVA via the nasal route. In fact, as the number of OVA instillations increased, we noticed inflammation in the ears and other parts of the skin only in T-Bmc *Foxp3*^{sf} mice. We thus analyzed IL-4 production by cells from the lungs, thoracic LN, mesenteric LN, and spleen. Whereas in chronically exposed T-Bmc *Foxp3*^{wt} mice we could only detect large numbers of IL-4-producing cells in the lungs, in equally treated T-Bmc *Foxp3*^{sf} mice we found OVA-specific Th2 cells in distal LN as well as the spleen (Figure 6A). It is worth noting that because the first immunization was intraperitoneal (Figure 4A), during the acute response we also found OVA-specific Th2 cells in spleen and mLN of T-Bmc *Foxp3*^{wt} mice; however, upon repeated intranasal challenge the presence of OVA-specific Th2 cells became restricted to the lungs.

The control of the spreading of Th2 cells in the chronic phase was paralleled by an increase in Foxp3⁺ Treg cells upon chronic antigen stimulation. Q-PCR analysis of lung tissue and draining LN showed that *Foxp3* expression increased about 4- and 5-fold, respectively, from the acute to the chronic phase. FACS analysis of cells from the BAL showed a 2-fold increase in the percentage of Foxp3⁺ cells within the T cell population (Figure 6B). In sum, adaptive Foxp3 Treg expansion in chronic inflammation mediates a profound decrease in IL-4-producing cells in proximal and distal lymphoid organs.

Foxp3-Independent Control of Eosinophilia

We next assessed IL-5 production and eosinophilia during the acute and chronic phases in mice able or unable to generate adaptive Foxp3⁺ Treg cells. We observed more than a 90% reduction of eosinophil numbers in the BAL of chronically challenged T-Bmc *Foxp3*^{wt} mice compared with BAL from acute-phase mice (Figure 7A). Chronically challenged T-Bmc *Foxp3*^{sf} mice also showed a significant reduction in eosinophilia compared to acute-phase mice, although this reduction (75%) was less pronounced than in T-Bmc *Foxp3*^{wt} mice. Essentially the same results were obtained by Q-PCR analysis with eosinophil peroxidase (Figure 7B). Thus, eosinophilia can be controlled to a significant extent in the absence of both naturally occurring and adaptive Foxp3⁺ Treg cells.

Consistent with the reduced eosinophilia, we found that *IL-5* mRNA expression was greatly reduced in BAL and thoracic LN cells of chronically challenged mice irrespective of the presence or absence of adaptive Foxp3⁺ T cells (Figure 7B). Intracellular staining of IL-5 in lung-infiltrating T cells also showed marked reduction in the chronic phase compared to the acute phase (Figure 7C). Thus, suppression of eosinophilia in chronically challenged mice is probably secondary to the suppression of IL-5 production and occurs even in the complete absence of Foxp3⁺ Treg cells.

In order to investigate the mechanism of Foxp3⁺ Treg-independent reduction in eosinophilia, we focused on IFN- γ . We

showed previously that the presence of small amounts of IFN- γ were important in reducing the amount of IL-5 production by Th2 cells (Wensky et al., 2001). It has also been shown that IFN- γ suppressed eosinophilia during acute allergic inflammation in sensitized BALB/c mice (Iwamoto et al., 1993). If IFN- γ is critical for the reduction in eosinophils during chronic lung inflammation in a Foxp3⁺Treg-independent fashion, neutralization of IFN- γ should maintain the eosinophilia in *Foxp3*^{sf} and *Foxp3*^{wt} mice. Indeed, injection of anti-IFN- γ antibody weekly into T-Bmc *Foxp3*^{wt} or T-Bmc *Foxp3*^{sf} mice exposed to chronic intranasal antigen led to a 600% increase in the number of eosinophils in the BAL (Figure 7D). These results support a role for IFN- γ in the Foxp3-independent control of eosinophilia.

DISCUSSION

We analyzed the importance of adaptive Foxp3⁺ Treg cells in mucosal tolerance and in chronic allergic inflammation. We found that the induction of Foxp3⁺ Treg cells was essential for establishing tolerance through the respiratory and gut mucosa. In chronic allergic inflammation, adaptive Foxp3⁺ Treg cells controlled the severity of inflammation, IL-4-mediated pathology, and the formation of organized lymphoid structures in the lungs. In contrast, we found that IL-5 production and eosinophilia in chronic inflammation were suppressed by IFN- γ independently of Foxp3.

To study the function of adaptive Foxp3⁺ Treg cells, we needed to build an experimental mouse system in which the effect of adaptive Foxp3⁺ Treg cells could be distinguished from that of naturally occurring Foxp3⁺ Treg cells. These mice would have conventional T cells but lack naturally occurring Treg cells. All TCR transgenic *Rag*^{-/-} mice described so far have conventional T cells but lack natural Treg cells. We first demonstrated the functional absence of Treg cells in MBP TCR Tg *Rag*^{-/-} mice that develop spontaneous autoimmune disease (Lafaille et al., 1994; Olivares-Villagomez et al., 1998) and, much later, described the absence of Foxp3⁺ T cells in these mice (Shen et al., 2005). The lack of thymic-derived Foxp3⁺ T cells in TCR Tg *Rag*^{-/-} mice was fundamental to the demonstration of in vivo peripheral conversion of CD4⁺Foxp3⁻ cells into functional CD4⁺Foxp3⁺ Treg cells (Apostolou and von Boehmer, 2004) (Cobbold et al., 2004) (Curotto de Lafaille et al., 2004; Mucida et al., 2005).

Although they solved one problem, the TCR Tg *Rag*^{-/-} experimental systems could be criticized because, by necessity, they harbored a monoclonal T cell repertoire. It was thus important that we establish that the same principles of adaptive Foxp3⁺ Treg induction applied when the T cells derived from TCR Tg *Rag*^{-/-} mice were a small minority in the context of wild-type mice. Therefore, the experimental system used in this manuscript reproduces the mucosal-tolerance-inductive mechanisms that operate in wild-type mice with full lymphocyte repertoires.

A second issue in the development of the experimental system was to specifically eliminate adaptive Foxp3⁺ regulatory T cells. By using the *scurfy* null allele of the *Foxp3* gene crossed into TCR Tg *Rag*^{-/-} mice, we established mice that did not have naturally occurring Treg cells (because they are TCR Tg *Rag*^{-/-}) and that could not make adaptive Foxp3⁺ cells (because of the *scurfy* mutation). Comparing these mice to TCR Tg *Rag*^{-/-} mice that had

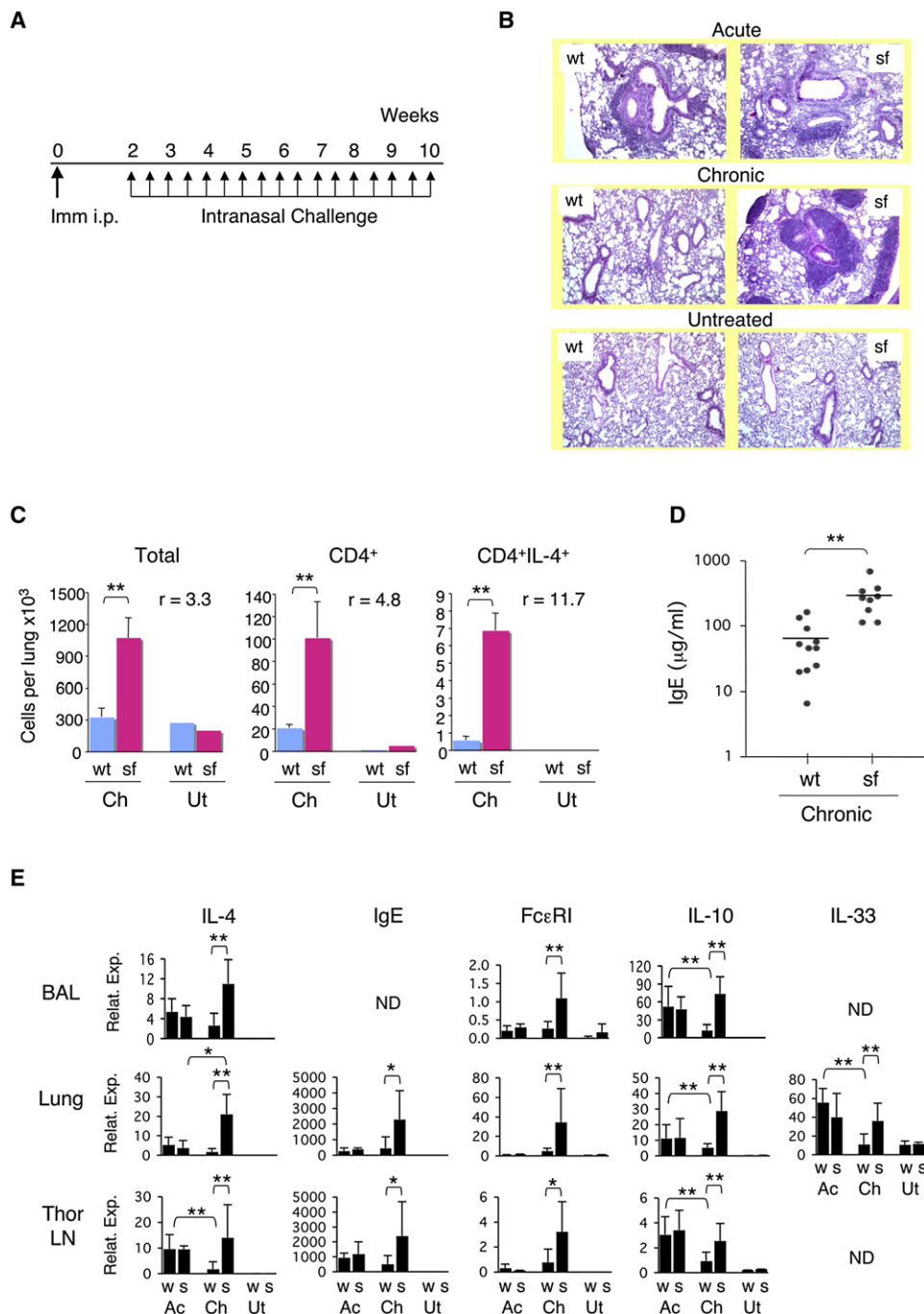


Figure 4. Immunized T-Bmc Mice with Deficient *Foxp3* Genes Develop Severe Lung Inflammation upon Chronic Exposure to Antigen through the Airways

(A) Experimental protocol for chronic antigen exposure. T-Bmc mice carrying wild-type (wt) or *scurfy* (sf) *Foxp3* genes were immunized with OVA-HA in alum and were subsequently administered intranasal OVA-HA twice a week for 8 weeks (chronic group). For acute inflammation, immunized mice were administered OVA-HA intranasally three times.

(B) Representative H&E sections of lungs from immunized mice administered intranasal OVA-HA three times (acute) or during 8 weeks (chronic) and from untreated mice. Original magnification: 40 $\times$.

(C) Cellular infiltrates in lungs of immunized mice chronically exposed to OVA-HA. The graphics show the average and SD of total, CD4⁺, and CD4⁺IL-4⁺ lung-infiltrating cells. The data are representative of three independent experiments. r: ratio between average of chronically challenged *Foxp3*^{sf} and chronically challenged *Foxp3*^{wt} mice.

(D) IgE levels in sera from immunized mice administered OVA-HA intranasally for 8 weeks. Each dot corresponds to one mouse.

(E) Q-PCR analysis of *IL-4*, *IgE*, *Fc ϵ RI*, *IL-10*, and *IL-33* mRNA expression in mice with acute (Ac) or chronic (Ch) lung inflammation and in untreated (Ut) mice carrying wild-type (w) or *scurfy* (s) *Foxp3* genes. The results are averages + SD. n = 11 for Ch wt; n = 8 for Ch sf; n = 4–5 for Ac and Ut groups. ND: not done.

**p $\leq$ 0.01; *p $\leq$ 0.05.

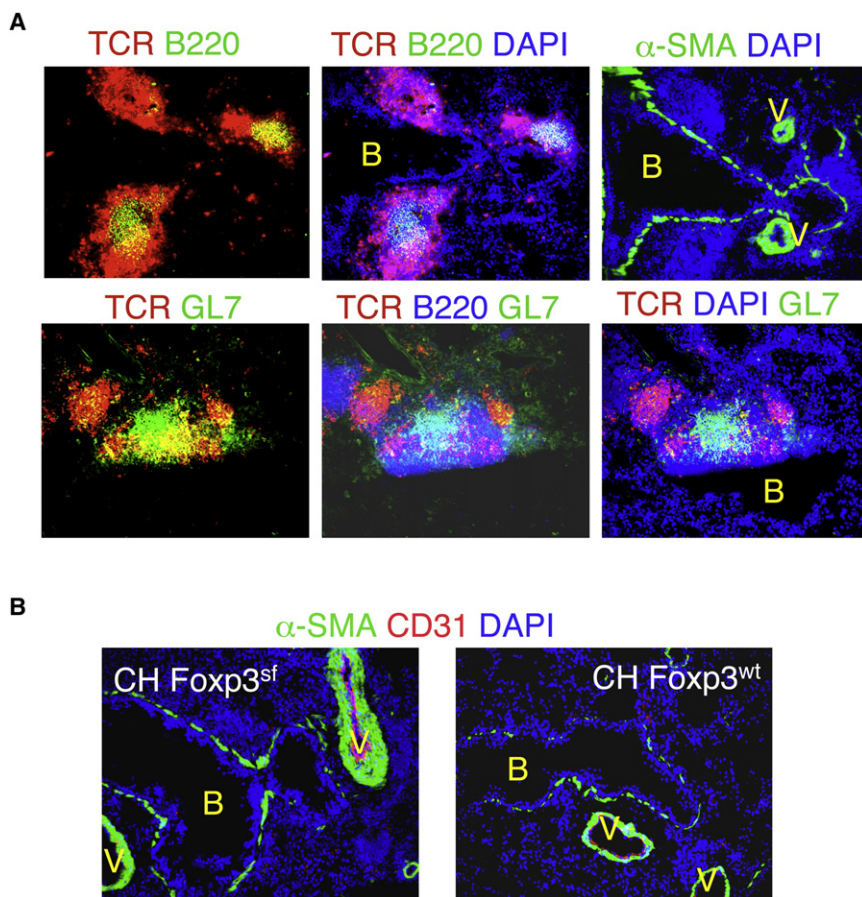


Figure 5. Lymphoid Neogenesis and Smooth-Muscle Remodeling in Lungs of Chronically Challenged T-Bmc Foxp3^{sf} Mice

(A) Frozen lung sections from T-Bmc Foxp3^{sf} mice chronically exposed to OVA-HA were stained with antibodies to the DO11.10 TCR (KJ1-26), to B cells (B220), and to germinal-center cells (GL7). Nuclei were stained with DAPI.

(B) Smooth-muscle hypertrophy in chronically challenged T-Bmc mice. Frozen lung sections were stained with antibodies against α -smooth muscle actin (α -SMA) for visualization of smooth muscle and with anti-CD31 (PECAN-1) for visualization of vascular endothelium. Nuclei were stained with DAPI.

Representative sections are shown. Magnification: 100 $\times$. B: bronchia; V: vessels.

Although oral OVA per se did not induce tolerance in Foxp3-deficient T-Bmc mice, it did not induce effector functions either; no increase in the Th1, Th2, or Th17 cytokines was observed. This was striking in consideration of the fact that the mesenteric LN T cells upregulated CD69, an indication of antigen-mediated activation. Because TGF- β is required for the development of both adaptive Treg and Th17 cells, it was postulated that there is a reciprocal pathway leading to either Th17 or Treg cells (Bettelli et al., 2006). Thus, a higher percentage of Th17

wild-type Foxp3 genes allowed us to specifically study the role of adaptive Treg cells. Using these paired mouse strains, we showed that adaptive Foxp3⁺ Treg cells are essential for mucosa-induced tolerance. Pretreatment of mice with OVA through the gut or the airways induces immunological tolerance by means of the TGF- β -dependent generation of adaptive Foxp3⁺ Treg cells. The adaptive Foxp3⁺ Treg cells that arise without a concomitant development of effector Th cells (see below) are extremely effective at preventing T and B cell responses to immunization. As a consequence, lung inflammation due to OVA administered through the airways is also prevented. Thus, the lack of pre-existing adaptive Foxp3⁺ Treg cells at the time of immunization in mucosal-OVA-treated T-Bmc Foxp3^{sf} mice results in their allergic sensitization.

In the absence of adaptive Foxp3⁺ Treg cells, the Th2 and IgE responses of orally treated T-Bmc Foxp3^{sf} mice were of the same magnitude as the responses in T-Bmc Foxp3^{wt} mice to which no mucosal tolerance protocol was applied. Thus, in the absence of adaptive Foxp3⁺ Treg cells, Foxp3-independent mechanisms of mucosal tolerance did not contribute to the reduction of Th2-mediated responses. Given the fact that other T cells, such as Th3 cells (Fukauro et al., 1996), have been shown to be important in oral tolerance, it is likely that these cells operate in the same pathway as adaptive Foxp3⁺ Treg cells; for instance, Th3 cells could supply the TGF- β that is necessary for the in vivo generation of oral-tolerance-derived Foxp3⁺ Treg cells (Carrier et al., 2007; Mucida et al., 2005).

cells could have been expected in the absence of Foxp3, but this was not the case. Our data are consistent with the idea that different intestinal microenvironments give rise to Th17 cells and Treg cells (Coombes et al., 2007; Denning et al., 2007; Mucida et al., 2007; Sun et al., 2007) and support the view that, in the absence of adjuvant factors, oral antigen is presented in a microenvironment that can only give rise to Treg cells.

We studied the proliferation and accumulation of Treg cells during the onset of lung inflammation. We observed that adaptive Foxp3⁺ T cells had a higher proliferation rate in vivo than non-Treg CD4⁺ cells of identical specificity. Other laboratories have observed robust proliferation of Treg cells in vivo (Annacker et al., 2001; Klein et al., 2003; Walker et al., 2003). However these preceding studies did not focus on Treg cells in inflamed tissues, where Treg accumulation is the highest.

We also found a different homing pattern of Treg cells and conventional CD4⁺ T cells into the airways. In non-immunized T-Bmc mice the vast majority of T cells display a naive phenotype, and very few CD4⁺ T cells are found in the BAL. Upon intraperitoneal immunization, T cells with activated and memory phenotypes, but not Foxp3⁺ T cells, are found in the BAL. Only after intranasal challenge with antigen is it that a substantial proportion of Foxp3⁺ Treg cells make their way across the lung epithelium into the airways.

During chronic allergic exposure through the airways, adaptive Foxp3⁺ Treg cells became overrepresented in the CD4⁺ population of the inflamed tissue and affected the evolution of

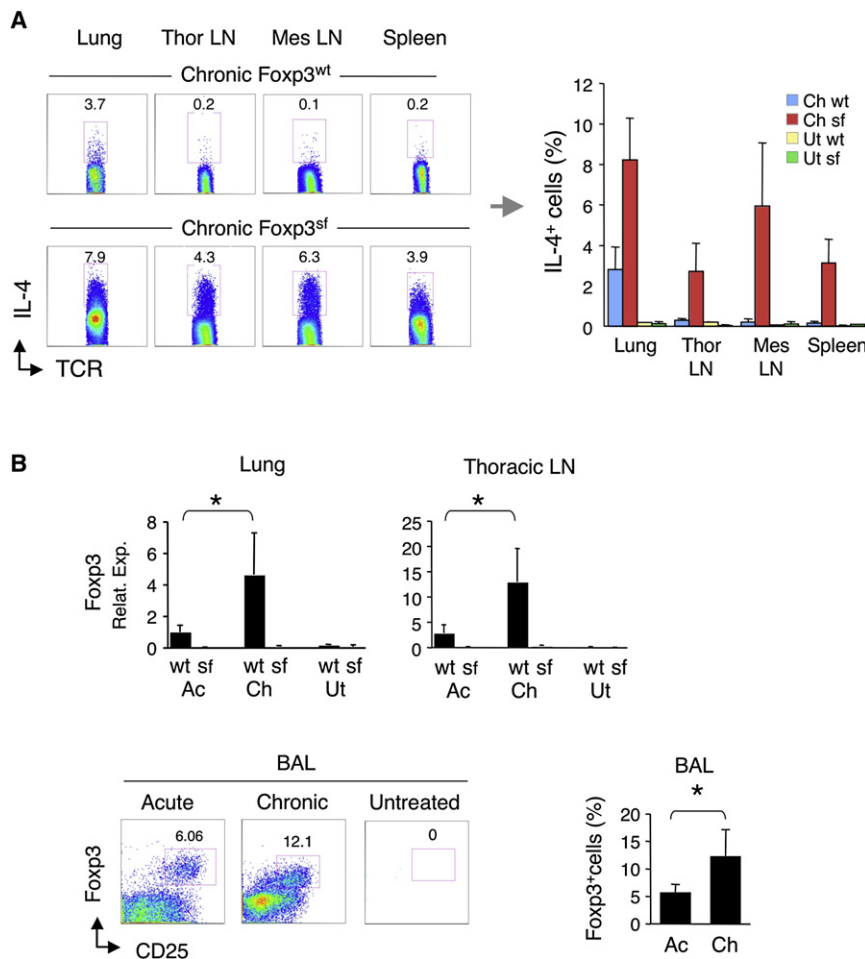


Figure 6. Disseminated IL-4 Production in Lymphoid Organs of Chronically Challenged Mice with No Adaptive Treg Cells

(A) Widespread presence of IL-4-producing cells in secondary lymphoid organs of chronically challenged T-Bmc *Foxp3^{sf}* mice. IL-4 production was analyzed by flow cytometry in cells from lung, thoracic LN, mesenteric LN, and spleen from chronically challenged (Ch) and from untreated (Ut) T-Bmc mice. The figure shows representative plots of gated TCR⁺ (KJ1-26⁺) cells. The bar graphic on the right shows averages + SD of IL-4-producing cells within the KJ1-26⁺ population (two to four mice per group).

(B) Upper section: Q-PCR analysis of *Foxp3* expression in lung and thoracic LN of mice with acute (Ac) or chronic (Ch) inflammation and from untreated (Ut) mice. Values are averages + SD. Numbers of mice per group are as in Figure 4E. Lower section: Representative dot plots show expression of *Foxp3* and CD25 in CD4⁺ BAL cells from mice with acute and chronic inflammation. The bar graphic on the right shows the average + SD values ($n = 4$ mice per group). * $p \leq 0.05$.

inflammation quite dramatically. Adaptive Foxp3⁺ Treg cells mediated a marked reduction of cytokine production in lungs and lymphoid organs and a reduction of cellular inflammation in the lungs. Interestingly, the resolution of lung inflammation in immunized wild-type BALB/c mice chronically exposed to antigen follows a similar pattern (Shinagawa and Kojima, 2003; also data not shown). We called this phenomenon “post-sensitization regulation” because it occurs after the onset of Th2 inflammation. In contrast, mucosal tolerance prevents the development of effector cytokine-producing Th cells altogether.

Post-sensitization regulation in the chronic phases also involved decreased IL-5 production and eosinophilia, but this occurred in mice that developed Foxp3⁺ Treg cells as well as in Foxp3-deficient mice. We found that the decreased eosinophilia was dependent on IFN- γ ; antibody neutralization of IFN- γ restored high eosinophil numbers in both mouse strains.

Our laboratory showed that IFN- γ selectively regulates IL-5 production in differentiating Th2 cells without interfering with IL-4 production (Wensky et al., 2001). Thus, in chronically challenged mice with no Foxp3⁺ Treg cells, IFN- γ might affect newly differentiating Th2 cells by suppressing IL-5 production while maintaining IL-4 production. Another possible explanation for the dissociation between IL-4 and IL-5 production in the chronically challenged T-Bmc *Foxp3^{sf}* mice is that many of the IL-4-producing cells in inflamed tissue are cells that would have

become Foxp3⁺ if they had a normal *Foxp3* gene (Williams and Rudensky, 2007). Finally, it is also possible that the cellular sources of IL-4 and IL-5 change from the acute to the chronic phases and that cells other than Th2 cells, such as mast cells and basophils, make a greater contribution to IL-4 production at the chronic stages.

The issue of whether adaptive Foxp3⁺ Treg cells and naturally occurring Foxp3⁺ Treg cells have overlapping or distinct functions remains unresolved. It is tempting to speculate that there is a division of labor between both types of Foxp3⁺ Treg cells. It is believed that naturally occurring Treg cells are selected in the thymus by high-avidity ligands. The T cell receptor repertoire of naturally occurring Treg cells is likely to be biased toward ligands that are present in the thymus, and naturally occurring Treg cells are crucial in self-tolerance. In contrast, adaptive Foxp3⁺ Treg cells are selected in the thymus in the same avidity window as naive CD4⁺ T cells and share the TCR repertoire with all other peripheral Foxp3⁺ T cells that respond to antigens not present in the thymus. Such adaptive Foxp3⁺ Treg cells are expected to play a key role in limiting immune-mediated tissue damage that arises upon chronic exposure to non-self antigens, such as environmental allergens or chronic infections. In this manuscript we have shown that this role can, indeed, be played by adaptive Foxp3⁺ Treg cells.

EXPERIMENTAL PROCEDURES

Mice and Antigen Treatment

T-B monoclonal mice (T-Bmc) (Curotto de Lafaille et al., 2001) carrying wild-type and *scurfy* *Foxp3* genes in a BALB/c background were bred in the SPF animal facility at the Skirball Institute, NYUSM. Scurfy and BALB/c mice were purchased from The Jackson Laboratories and Taconic. *Foxp3gfp.KI*

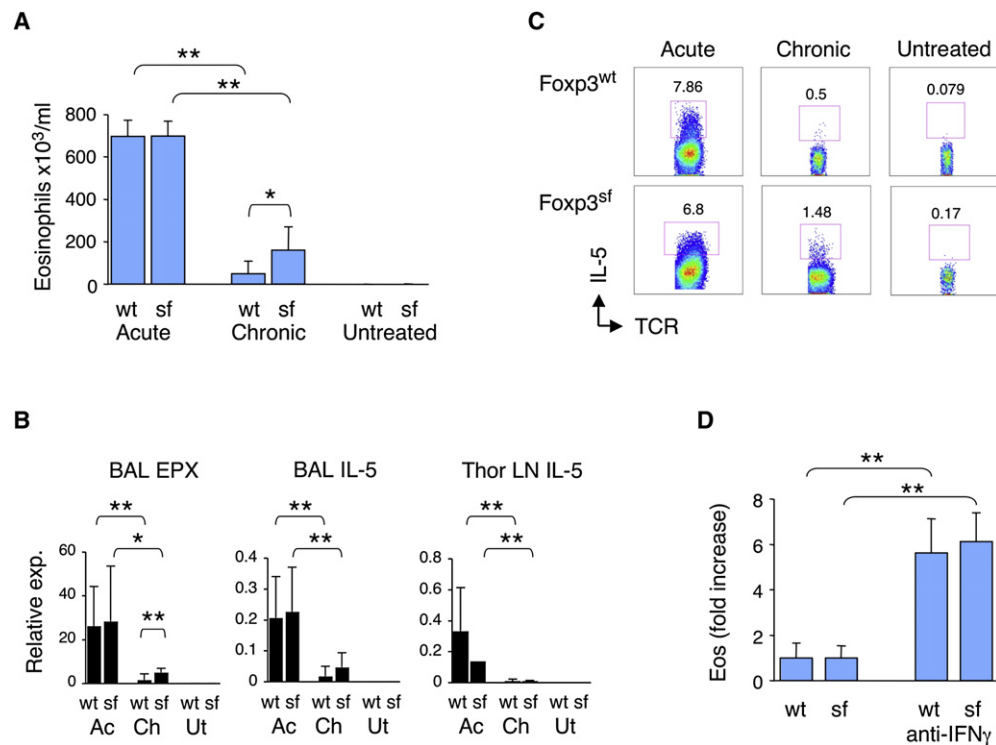


Figure 7. Treg-Independent Suppression of Eosinophilic Inflammation and IL-5 Production in Mice with Chronic Inflammation

(A) Eosinophil numbers in the BAL of immunized mice administered OVA-HA intranasally three times (acute) or for eight weeks (chronic) as described in Figure 4A. (B) Q-PCR analysis of eosinophil peroxidase (EPX) and IL-5 mRNA expression in mice with acute (Ac) or chronic (Ch) lung inflammation and in untreated mice (Ut). Numbers of mice per group are as in Figure 4E.

(C) Decreased IL-5 production in mice with chronic lung inflammation. IL-5 production was analyzed ex vivo in lung-infiltrating cells from mice with acute or chronic inflammation and from untreated mice. The figure shows representative plots of IL-5 staining in gated TCR⁺ (KJ1-26⁺) cells.

(D) IFN- γ suppresses eosinophilic inflammation in chronically challenged mice. Immunized mice were treated for 5 weeks with intranasal OVA-HA, and during that period they were injected with anti-IFN- γ antibodies. The figure shows the n-fold change in BAL eosinophil numbers in antibody-treated mice. n = 3 for Ch wt and Ch wt IFN- γ ; n = 4 for Ch sf and Ch sf IFN- γ .

(A, B, and D) Data are averages $\pm$ SD. **p $\leq$ 0.01; *p $\leq$ 0.05.

reporter (Korn et al., 2007) was backcrossed to the T-Bmc mice in a BALB/c background for four generations. Oral and airway tolerance to chicken ovalbumin (OVA) were induced as described (Mucida et al., 2005). For immunization, mice were injected with 100 μ g OVA-HA in 1 mg of alum by intraperitoneal route. To induce acute lung inflammation, we administered 10 μ g of OVA-HA by intranasal route three times. To determine T cell proliferation rates, we injected mice with 3 mg of BrdU by combined intraperitoneal and intravenous routes 6 hr before analysis. To induce chronic lung inflammation, we administered 10 μ g of OVA-HA twice a week for 8 weeks. To neutralize IFN- γ , we injected immunized mice with 1 mg of the antibody XMG.1 by intraperitoneal route once a week, beginning one day before the first intranasal treatment. BAL collection and analysis and lung histology were performed as described (Mucida et al., 2005). All mouse procedures were approved by the New York University Institutional Animal Care Use Committee.

Mononuclear Cell Isolation and Flow Cytometry

BAL, LN, and spleen mononuclear cell suspensions were prepared as described (Curotto de Lafaille et al., 2001; Mucida et al., 2005). To obtain infiltrating lung cells, we digested lungs from perfused mice with liberase blendzyme (Roche) and purified the infiltrating cells over 38% Percoll (Amersham Biosciences). Cytokine-producing cells were detected by flow cytometry after ex vivo stimulation with PMA and ionomycin. Staining of Foxp3, BrdU, and cytokines was performed on fixed and permeabilized cells. Data were acquired in a FACS Calibur (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Quantitative PCR Analysis

RNA was extracted with TRIzol (Invitrogen). cDNA synthesis and real-time PCR were performed by standard procedures. Expression values were normalized to β -actin. Most oligonucleotide sequences have been described previously (Curotto de Lafaille et al., 2004; Erazo et al., 2007; Ivanov et al., 2006; Mucida et al., 2005). Additional sequences are provided in the Supplemental Data.

Lung Immunohistology

Frozen lung sections were fixed with acetone, blocked, stained, and analyzed as described (Erazo et al., 2007).

Statistical Analysis

A two-tailed Student's t test was used for comparisons. p values $\leq$ 0.05 were considered statistically significant.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and eight figures and can be found with this article online at <http://www.immunity.com/cgi/content/full/29/1/114/DC1/>.

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