Regulatory T Cell-Derived Interleukin-10 Limits Inflammation at Environmental Interfaces

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SUMMARY

The regulatory T (Treg) cells restrain immune responses through suppressor-function elaboration that is dependent upon expression of the transcription factor Foxp3. Despite a critical role for Treg cells in maintaining lympho-myeloid homeostasis, it remains unclear whether a single mechanism or multiple mechanisms of Treg cell-mediated suppression are operating in vivo and how redundant such mechanisms might be. Here we addressed these questions by examining the role of the immunomodulatory cytokine IL-10 in Treg cell-mediated suppression. Analyses of mice in which the Treg cell-specific ablation of a conditional IL-10 allele was induced by Cre recombinase knocked into the Foxp3 gene locus showed that although IL-10 production by Treg cells was not required for the control of systemic autoimmunity, it was essential for keeping immune responses in check at environmental interfaces such as the colon and lungs. Our study suggests that Treg cells utilize multiple means to limit immune responses. Furthermore, these mechanisms are likely to be nonredundant, in that a distinct suppressor mechanism most likely plays a prominent and identifiable role at a particular tissue and inflammatory setting.

INTRODUCTION

A subset of CD4+ T cells known as regulatory T cells is responsible for limiting tissue damage and inflammation associated with both innate and adaptive immune responses (Miyara and Sakaguchi, 2007; Shevach et al., 2001). Treg cells represent a dedicated cell lineage generated in the thymus; their development is dependent upon the expression of the transcription factor Foxp3 (Fontenot et al., 2003; Horii et al., 2003; Khattri et al., 2003). In mice, Foxp3 is restricted in its expression to Treg cells, and it therefore represents the best marker to date for this cell subset (Fontenot et al., 2005). Furthermore, genetic deficiency for Foxp3 leads to a highly aggressive fatal lymphoproliferative autoimmune disorder affecting multiple organs, and drug-induced ablation of Treg cells in neonatal or adult mice results in a similar disease (Fontenot and Rudensky, 2005; Kim et al., 2007). In humans, Foxp3 mutations result in development of an analogous fatal disorder dubbed IPEX (Immune disregulation, Polyendocrinopathy, Enteropathy, X-linked). Autoimmune lesions associated with the Foxp3 deficiency are characterized by lymphadenopathy, splenomegaly, and severe pathology of the skin and gastrointestinal tract, as well as fulminant immune-mediated inflammation of multiple organs, including the liver, pancreas, muscles, and lungs (Brunkow et al., 2001; Wildin and Freitas, 2005). Recent studies indicate that Foxp3 expression is necessary for suppressor function: Genetically marked cells transcribing the Foxp3 locus and lacking expression of the Foxp3 protein are unable to suppress immune responses in vivo and in vitro (Gavin et al., 2007; Lin et al., 2007). Furthermore, ablation of Foxp3 gene expression in mature peripheral Treg cells results in a loss of suppressor function (Williams and Rudensky, 2007).

Despite its major significance, Treg cell-mediated suppression is poorly understood from a mechanistic point of view. Over the last several years, numerous molecular and cellular mechanisms of suppression have been proposed in studies utilizing an antibody-mediated blockade of putative effector molecules or adoptive transfers of Treg cells isolated from mice genetically deficient in these molecules. However, the emerging picture remains confusing because none of the mechanisms have been unequivocally proven to operate in un-manipulated animals. Furthermore, it is not clear whether a single unique mechanism can account for various manifestations of Treg cell suppression or whether multiple mechanisms are needed for suppression to commence. It is also unknown whether distinct suppression mechanisms, either singly or cooperatively, control a particular type of inflammation in a specific location. For example, in vitro studies unequivocally demonstrated a requirement for cell-cell-contact-dependent Treg cell-mediated suppression and excluded a role for IL-10 and TGF-β, two major cytokines...
with pronounced immunomodulatory and immunosuppressive function (Kullberg et al., 2005; Shevach et al., 2001; von Boehmer, 2005). In contrast, some in vivo studies suggested a role for TGF-β1 and IL-10 as effector molecules of Treg cell-mediated suppression of colitis (Asseman et al., 1999; Fahlen et al., 2005; Li et al., 2007; Powrie et al., 1996). However, both cytokines were found to be nonessential for in vivo suppression of autoimmune gastritis (Shevach et al., 2001). The studies implicating IL-10 in Treg-mediated suppression relied on adoptive transfer of CD25+/CD4+ Treg cells isolated from IL-10-deficient donor mice and cotransferred with naïve or immune CD4+ T cells into lymphopenic hosts. A caveat for this experimental approach is that the inflammation induced by transferred T cells is greatly facilitated by their proliferation under lymphopenic conditions. In addition, lymphopenic conditions might not favor IL-10 production by “effector” nonregulatory T cells. The latter cells were shown to serve as a major source of IL-10 in some parasitic infections in mice and humans (Anderson et al., 2007; Jankovic et al., 2007; Nylen et al., 2007). Furthermore, contaminating IL-10-producing Foxp3-negative T cells are present at a low frequency in the splenic and lymph node CD25+CD4+ T cell populations used in a number of transfer experiments (Kamanaka et al., 2006; Maynard et al., 2007). In addition, Foxp3-negative T cell populations, including the IL-10-expressing cell subset, are likely to have inferior proliferative potential compared to that of regulatory Foxp3+ T cells capable of a very robust expansion in lymphopenic hosts (Gavin et al., 2002; Komatsu and Hori, 2007). Together, these considerations make it possible to hypothesize that, in unmanipulated animals, Foxp3-negative T cells might represent a key source of IL-10. Finally, in mice with a targeted insertion of the GFP coding sequence into the Foxp3 gene locus and a concomitant disruption of Foxp3 protein expression, we found that GFP+ T cells expressing this Foxp3null allele express high amounts of IL-10 mRNA and protein, yet lacked suppressor function (Gavin et al., 2007). Thus, the role for IL-10 as a suppressor molecule elaborated by Foxp3+ Treg cells to control immune inflammation remains uncertain.

To directly investigate a role for IL-10 in Treg cell-mediated suppression, we generated Foxp3YFP-Cre mice, capable of Treg cell-specific inactivation of genes of interest, and crossed them with Il10flox/flox mice harboring a conditional Il10 allele. We found that the selective disruption of IL-10 expression in Treg cells led to spontaneous colitis. Furthermore, Il10flox/flox × Foxp3YFP-Cre mice developed substantially augmented immune-response-associated inflammation and pathology in the skin and lungs. Thus, in unmanipulated animals, IL-10 produced by Treg cells plays an important role in suppressing immune inflammation at environmental interfaces. These results strongly suggest that Treg cells utilize multiple suppression mechanisms to control various aspects of inflammation, which is mediated by distinct immune cell types in different settings, and that a deficiency in any single mechanism of suppression is unlikely to be equivalent to a complete lack of regulatory T cells. Furthermore, our data suggest that specific mechanisms of suppression do not operate in a completely redundant fashion and that these mechanisms, upon elucidation of their distinct contribution to Treg cell-mediated suppression, can be harnessed for potential therapeutic interventions.

RESULTS

Generation and Characterization of Foxp3YFP-Cre Mice

To generate mice capable of selective gene inactivation in Treg cells, we utilized gene targeting to insert the DNA sequence coding for a viral IRES followed by YFP-Cre recombinase fusion protein into the 3’ UTR of the Foxp3 locus (Figure 1A). Chimeric males harboring the targeted Foxp3YFP-Cre allele were bred with female mice expressing the FLPe recombinase transgene to excise the Neo+ cassette and to confirm germ-line transmission of the targeted allele (Figure 1B). The Neo+-negative progeny was crossed to C57BL/6 mice, and the resulting Foxp3YFP-Cre mice were analyzed for expression of the YFP-Cre and Foxp3 protein (Figure 1C). The YFP-Cre fusion protein expression was limited to Treg cells. To examine the specificity of Cre-mediated recombination in Foxp3YFP-Cre mice, we introduced the ROSA26YFP (R26Y) recombination reporter allele into these mice. This allele allowed for the detection of Cre-mediated recombination at a single-cell level through flow-cytometric analyses of YFP expression induced upon Cre-mediated excision of a “floxed” transcriptional stop cassette preceding the YFP coding sequence inserted into the ubiquitously expressed ROSA26 locus. Because the YFP reporter expression was markedly higher than that of the YFP-Cre fusion protein, essentially 100% efficient Cre-mediated recombination in Foxp3YFP-Cre Treg cells was readily ascertained upon staining for CD4, CD8, and Foxp3. In addition, individual Foxp3YFP-Cre mice exhibited varying degrees (2%–10%) of R26Y recombination in different hematopoietic lineage cells that lacked detectable YFP-Cre and Foxp3 expression; such cells included immature and mature B cells, T cells, myeloid cells, and bone marrow precursor cells (Figure S1; data not shown). This was probably due to stochastic recombination events resulting from a basal level of Foxp3YFP-Cre transcription, consistent with the emerging data on the pervasive transcription of genes, including “silent” genes and non-coding sequences in addition to actively transcribed genes, involved in developmental processes within the genome (Guenter et al., 2007). However, the “off-target” recombination in Foxp3YFP-Cre mice was probably negligible in light of the fact that a recombination event affecting a single R26Y allele resulted in YFP expression, whereas ablation of a “floxed” gene of interest in homozygous configuration required deletion of both alleles.

Il10 Ablation in Treg Cells Does Not Perturb Their Development or Result in Systemic Autoimmunity

To generate mice in which the IL-10 deficiency was limited to Treg cells, we bred Il10flox/flox mice harboring loxP sites flanking the first exon of the Il10 gene with Foxp3YFP-Cre mice. Genomic PCR-based examination of the wild-type (Il10WT) as well as “floxed” intact (Il10flo) and recombinant (Il10+) alleles of the Il10 gene in FACS-purified YFP+CD4+ and YFP CD4+ T cells showed a highly efficient deletion in YFP+CD4+ T cells, i.e., Foxp3+ Treg cells, in male Il10flox/flox × Foxp3YFP-Cre hemizygous mice, whereas the Il10Δ allele was undetectable in YFP- “non-Treg” CD4+ T cells (see sorting gate in Figure S2). In addition, similar analysis of heterozygote Il10flox/WT Foxp3YFP-Cre mice reveals both the intact Il10flo and Il10WT alleles in YFP-Cre+ non-Treg cells, whereas YFP-Cre+ Treg cells contained...
Figure 1. Generation and Characterization of Knock-In Mice Harboring the Foxp3^{YFP-Cre} Allele

(A) The targeting strategy and map of the Foxp3^{YFP-Cre} construct.
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*Il10*<sup>WT</sup> and *Il10*<sup>-/-</sup> (Figure 2A). We estimated the degree of *Il10*<sup>fox</sup> allele deletion to be at least 90% in YFP<sup>+</sup> cells and undetectable in YFP<sup>-</sup> cells (Figure 2B). To test the specificity of *Il10*<sup>fox</sup> allele deletion at the protein level, we stimulated splenocytes from *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> and littermate control *Il10*<sup>WT/WT</sup> × Foxp3<sup>YFP-Cre</sup> mice in vitro and stained them intracellularly with Foxp3 and IL-10 antibodies (Figure S3A). We observed that IL-10-producing cells were greatly diminished within the Foxp3-expressing, but not Foxp3-negative, CD4<sup>+</sup> T cell subset. Male *Il10*<sup>fl/fl</sup> × Foxp3<sup>YFP-Cre</sup> and female *Il10*<sup>fl/fl</sup> × Foxp3<sup>YFP-Cre</sup> YFP<sup>+</sup>-mice were born at a Mendelian frequency and lacked splenomegaly, lymphadenopathy or other detectable signs of autoimmune pathology up to 8 weeks of age (Figure 2C). Flow-cytometric analysis of mutant mice revealed numerically unaltered thymic and peripheral CD8<sup>+</sup> and CD4<sup>+</sup> T cell subsets, including Foxp3<sup>+</sup> T cell subsets (Figure 2D). Further examination of the expression of several T cell activation markers on the surface of CD4<sup>+</sup> T cells isolated from the lymph nodes, spleens, and mesenteric lymph nodes of mutant and control mice did not reveal noticeable differences (Figure 2E). IL-2, IL-4, IFN-γ, and TNF-α production by CD4<sup>+</sup> T cells was also unaffected in 6- to 8-week-old *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> mice (Figure 2F). These observations were consistent with the largely intact suppressor capacity of Treg cells in these mice; a complete Treg cell deficiency due to loss-of-function or null Foxp3 mutations or due to drug-induced ablation of the Treg cell subset results in a massive boost of cytokine production by T cells and lymphoproliferation. In agreement with these results, we failed to observe major signs of tissue pathology upon histologic examination of various organs of un-manipulated *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> mice, except for colon inflammation in older animals (see below). These results suggest that IL-10 expression by Treg cells is not essential for their requisite role in restraining early-onset systemic autoimmune.

**Il10<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> Mice Develop Spontaneous Colitis**

It has been shown that mice with the germ-line (*Il10*<sup>-/-</sup>) or T cell-specific ablation of the *Il10* gene (*Il10*<sup>fox/fox</sup> × CD4-Cre) develop spontaneous inflammation in the intestine, in which *Helicobacter* sp. plays a prominent role. In one study, up to 40% of *Il10*<sup>-/-</sup>-mice and a slightly smaller percentage of *Il10*<sup>WT</sup>-mice develop spontaneous colitis at age 6 months or older (Roers et al., 2004). To examine whether Treg cell-specific deficiency in IL-10 results in spontaneous colitis, we monitored a cohort of *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> and littermate control *Il10*<sup>WT/WT</sup> × Foxp3<sup>YFP-Cre</sup> mice for clinical signs of colitis, including rectal prolapse and diarrhea. As a positive control in these experiments, we used a cohort of *Il10*<sup>-/-</sup>-mice. We found that starting at 10 weeks of age, *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> animals, but not the control group, presented with gross manifestations of colitis. Upon further examination of the affected animals, we found diffuse colonic thickening and a substantial increase in the size of the mesenteric lymph nodes and the spleen (data not shown). Blinded histological evaluation of sections of the stomach and the small and large intestine from the *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> mice showed that although the stomach and small intestine were not affected by IL-10 deficiency in Treg cells when these mice were compared to control mice, the large intestine, especially the cecum, showed prominent mononuclear infiltration of epithelial tissue as well as tissue destruction (Figures 3A and 3B). Colonic inflammation observed in *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> mice was histologically similar to that observed in *Il10*<sup>-/-</sup>-mice, albeit that in the latter experimental group, the inflammation was more profuse and affected the entire length of the colon (Figures 3A and 3B). In agreement with microscopic observations, the onset of the disease was slightly delayed and the incidence was somewhat lower in *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> mice as compared to *Il10*<sup>-/-</sup>-mice (Figure 3C). It seems unlikely that the observed differences were due to distinct flora in mice of different genotypes because they were cohoused and fecal samples from every experimental group tested positive for *H. hepaticus*, *H. rodentum* and *H. trogonum* by PCR, whereas *H. bilis* and *H. typhlonius* were not detected. Colitis observed in *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> mice was not due to a paucity of Treg cells in the colon; flow-cytometric analysis of the colonic T cell population comprised of both intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) showed an increased size of Foxp3<sup>+</sup> Treg cell subset with unaltered Foxp3 expression on a per-cell basis in *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> mice, as compared to the control *Il10*<sup>WT/WT</sup> × Foxp3<sup>YFP-Cre</sup> mice (Figure 3D). Thus, IL-10 deficient Treg cells are abundant in the colonic tissue but are unable to restrain inflammation. It was also possible that inability of Foxp3-expressing cells to produce IL-10 in the gut might prevent IL-10 secretion by Foxp3<sup>+</sup> T cells. To examine this possibility, we isolated LPL from *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> and control *Il10*<sup>WT/WT</sup> × Foxp3<sup>YFP-Cre</sup> mice and assessed their ability to produce IL-10 upon in vitro stimulation. IL-10 producing Foxp3<sup>+</sup> LPL were readily detectable, whereas Foxp3<sup>+</sup> LPL were lacking (Figure S3B in the Supplemental Data). A moderate, less-than-two-fold reduction in the proportion of IL-10-producing cells could be a result of the inflammatory environment and overall increased T cell infiltration of the tissue. These data together suggest that IL-10 produced by Treg cells plays an important role in restraining local inflammation in the colon.

**Heightened Immune-Mediated Lung Hyperreactivity in *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> Mice**

Although the aforementioned histological examination of multiple organs and tissues (heart, skeletal muscle, stomach, pancreas, small intestine, thyroid, and brain) isolated from *Il10*<sup>fox/fox</sup> × Foxp3<sup>YFP-Cre</sup> mice did not reveal noticeable pathology, we observed mild perivasculitis and moderate (B) Southern-blot analysis of DNA isolated from an ES cell clone with the targeted Foxp3<sup>YFP-Cre</sup> allele. Genomic DNA was digested with BspHI restriction enzyme, separated in the agarose gel, and hybridized with a<sup>32</sup>P-labeled probe specific for the region indicated in (A). Lane 1, untargeted control ES cell clone; lane 2, targeted ES cell clone used for generation of the knock-in mice.

(C) Flow-cytometric analysis of T cell subsets in Foxp3<sup>YFP-Cre</sup> mice. Thymocytes, splenocytes, and lymph node cells isolated from 4- to 6-week-old mice were stained for CD4, CD8, CD25, and Foxp3. Twelve mice were analyzed in three independent experiments.
Figure 2. Lack of Systemic Autoimmunity and Normal Development of Treg Cells in Mice with the IL-10 Deficiency Limited to Treg Cells

(A) Deletion of the Il10 gene in Il10^{flox/flox} × Foxp3^{YFP-Cre} mice is restricted to the Foxp3^+ Treg cell subset. PCR-based analysis of YFP-Cre^+ and YFP-Cre^-/^-CD4^+ T subsets for the presence of wild-type (Il10^{WT}) and conditional undeleted (Il10^{flox}) or deleted (Il10^{D}) Il10 alleles. Genomic DNA was isolated from the

mononuclear infiltration around large airways in the lungs from 

Il10<sup>fl<sup>ox/f<sup>ox</sup> × Foxp<sup>3<sup>C<sup>YFP-Cre</sup></sup></sup> but not from the littermate control 

Il10<sup>WT/</sup><sup>ox/</sup><sup>ox</sup> × Foxp3<sup>C<sup>YFP-Cre</sup></sup> mice (Figure 4A). Flow-cytometric analysis of T cell populations isolated from perfused lungs showed an unaltered Foxp3<sup>+</sup> T cell subset in Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>YFP-Cre</sup></sup> as compared to Il10<sup>WT/</sup><sup>ox/</sup><sup>ox</sup> × Foxp3<sup>C<sup>YFP-Cre</sup></sup> (Figure 4B). Importantly, approximately 12% of Foxp3<sup>+</sup> T cells in the lungs express IL-10 based on analysis of IL-10 reporter mice (Maynard and Weaver, personal communication). These initial observations suggested that IL-10 produced by Treg cells might play a role in restraining lung inflammation. However, previous studies utilizing germ-line IL-10-deficient mice in an experimental model of asthma implicated this cytokine in accentuating the lung pathology downstream of the inflammatory reaction. In these experiments, IL10<sup>−/−</sup> mice sensitized with ovalbumin (OVA) presented with greatly diminished airway hyperreactivity despite lung lymphocyte infiltration and eosinophilia comparable to those observed in wild-type mice upon intranasal OVA challenge (Makela et al., 2000). Therefore, we tested the role for IL-10 produced by Treg cells in OVA-induced lung inflammation in Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>YFP-Cre</sup></sup> and in littermate control Il10<sup>WT/</sup><sup>ox/</sup><sup>ox</sup> × Foxp3<sup>C<sup>YFP-Cre</sup></sup> mice. To better account for the potential exacerbation of lung hyperreactivity due to IL-10 deficiency in Treg cells, we utilized the previously described suboptimal regimen of OVA immunization to minimize the induced lung inflammation in the wild-type control group of mice (Henderson et al., 1996; Zhang et al., 1997). Twenty-four hours after the final intranasal challenge, we found augmented inflammation manifested by an approximately 2.7-fold increase in leukocyte numbers in the bronchoalveolar lavage (BAL) fluid recovered from OVA-treated Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>YFP-Cre</sup></sup> as compared to littermate control Il10<sup>WT/</sup><sup>ox/</sup><sup>ox</sup> × Foxp3<sup>C<sup>YFP-Cre</sup></sup> mice (Figure 5A). In addition, histological examination, combined with the morphometry of the lung tissue of the mutant mice, showed markedly increased mucus production, goblet cell expansion, and edema and an increased mass of the cellular infiltrates associated with the increased eosinophilic infiltration around major airways (Figure 5B and Table 1). In agreement with these results, quantitative real-time PCR analysis of lung tissue mRNA isolated from OVA-treated Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>YFP-Cre</sup></sup> animals revealed no marked changes in IL-4 but increased amounts of IL-5, IL-13, and IFN-γ messages when these mice were compared to OVA-sensitized control mice (Figure S4; data not shown). These results were also consistent with the ELISA analysis of the corresponding cytokines in the lung-tissue extracts of OVA-immunized and challenged Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>YFP-Cre</sup></sup> (data not shown). To further evaluate functional consequences of lung inflammation observed by histological means and analyses of BAL cell content, we measured lung resistance by using invasive plethysmography. Consistent with the utilization of the OVA sensitization and challenge protocol for induction of suboptimal lung inflammation in the wild-type mice, neither the OVA- nor the saline-treated Il10<sup>WT/</sup><sup>ox/</sup><sup>ox</sup> × Foxp3<sup>C<sup>YFP-Cre</sup></sup> control mice showed a marked increase in airway hyperreactivity to aerosolized methacholine. However, we found greatly augmented airway hyperreactivity in response to inhaled methacholine in OVA-treated mice but not in saline-treated Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>YFP-Cre</sup></sup> mice (Figure 5C). The increased inflammation in the lungs of the mutant mice was not a consequence of decreased numbers of IL-10-deficient Treg cells in the inflamed lung tissue because increased size of the lung Foxp3<sup>+</sup> Treg cell subset was found in mutant OVA-challenged mice (Figure S5). Thus, as in the inflamed colonic tissue, IL-10-deficient Treg cells are present in the lungs in increased numbers; however, they fail to provide complete protection against excessive inflammatory responses initiated by the airborne antigen. Taken together, our data show that IL-10 secretion by Treg cells, in addition to its role in restraining immune-mediated inflammation in the colon, is contributing to Treg cell-mediated suppression of immunological reactivity in the airways.

**Increased Skin Hypersensitivity in Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> Mice**

Because we found that the IL-10 production by Treg cells plays a substantial anti-inflammatory role in the lungs and colon, we decided to examine whether immune-mediated inflammation is also affected by the IL-10 deficiency in Treg cells at a third major environmental interface, the skin. Initial examination of skin sections from Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>YFP-Cre</sup></sup> mice failed to reveal noticeable mononuclear infiltration or skin thickening typical of previously described Foxp3<sup>null</sup> mice lacking Treg cells and of Foxp3<sup>DTR</sup> mice subjected to the diphertheria-toxin-mediated Treg cell ablation (Fontenot et al., 2003; Kim et al., 2007). To further explore the question, we sensitized Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> and control Il10<sup>WT/</sup><sup>ox/</sup><sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> mice by painting the skin at the shaved abdomen region with dinitrofluorobenzene (DNFB) and challenged them by application of DNFB on one ear; the other ear was treated with the vehicle as a control. The indicated subsets of purified CD4 T cells from Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> (fl/fl) and Il10<sup>WT/</sup><sup>ox/</sup><sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> mice (fl/wt) (results of one representative experiment are shown).

(B) Bar graphs show relative intensity of the indicated PCR products normalized to the intensity of the Foxp3 band used as a control PCR product. The results represent one of two identical experiments.

(C) Cellularity of the thymus and secondary lymphoid organs in 8- to 10-week-old mice is unaffected by the IL-10 ablation in Treg cells. Each symbol represents mononuclear cell number in the thymus (Thy), spleen (Spl), lymph nodes (LN), and colon-draining mesenteric lymph nodes (MLN) in individual Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> (open symbols) and Il10<sup>WT/</sup><sup>ox/</sup><sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> mice (closed symbols). LN cell counts represent pooled lymph nodes excluding MLN.

(D) Flow-cytometric analysis of splenic and lymph node T cell subsets in Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> and Il10<sup>WT/</sup><sup>ox/</sup><sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> mice. Twelve mice were analyzed in three independent experiments.

(E) Flow-cytometric analysis of expression of activation markers by splenic and lymph node T cells in 5- to 8-week-old Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> and Il10<sup>WT/</sup><sup>ox/</sup><sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> mice. Cells were stained for CD4, CD8, CD25, CD62L, and CD44. Gate for YFP-CD4<sup>+</sup> cells is shown. Twelve mice were analyzed in three independent experiments.

(F) Analysis of intracellular cytokine expression in total splenocytes stimulated with plate-bound CD3 and CD28 antibodies. Splenocytes were isolated from 6- to 8-week-old Il10<sup>fl<sup>ox/</sup>fl<sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> and Il10<sup>WT/</sup><sup>ox/</sup><sup>ox</sup> × Foxp3<sup>C<sup>C<sup>YFP-Cre</sup></sup></sup> mice and after 5 hr activation were stained for CD4, CD8, IL-2, IL-4, IFN-γ, and TNF-α. The data are shown as mean percentages (± standard error) of cytokine-positive cells in CD4<sup>+</sup> or CD8<sup>+</sup> populations in individual mice. Unstimulated cell samples cultured in the absence of antibodies are shown as a control. Eight animals per group were analyzed in two separate experiments.
ensuing skin hypersensitivity reaction was assessed by ear thickness measured before and after the challenge. We found a substantial increase in ear thickness and heightened inflammation in $\text{Il10}^{\text{flox/flox}}$ $\text{Foxp3}^{\text{YFP-Cre}}$ mice as compared to the control animals (Figures 6A and 6B). To examine whether the increased skin hypersensitivity in $\text{Il10}^{\text{flox/flox}}$ $\text{Foxp3}^{\text{YFP-Cre}}$ mice was due to Treg cell homing deficiency, i.e., the inability to home to the skin, or due to their impaired suppressor capacity, we examined CD4+ T cell subsets in the ears of the mutant mice. As in analogous analyses of lung and colon tissue, we found the same proportion of the FoxP3+ CD4+ T cells in the skin of mutant and control mice (Figure 6C). It is noteworthy that the expression of the T cell activation marker CD69 on non-Treg cells was higher in mutant mice than in control mice (data not shown). Thus, Treg cells lacking IL-10 production are not as effective in controlling inflammation in skin as their IL-10-sufficient counterparts. Together, our studies suggest that the production of IL-10 by Treg cells is limiting inflammation at environmental interfaces and is at the same time dispensable for control of systemic autoimmunity.

**DISCUSSION**

Foxp3-expressing Treg cells play a central role in immunological tolerance to self and environmental antigens and in limiting tissue damage associated with the immune response to pathogens. Recent reports have suggested that Treg cells are capable of controlling the activation of a variety of immune cell types, including CD4+ and CD8+ T cells, B cells, NK cells, dendritic cells,
and macrophages. Furthermore, numerous mechanisms were proposed to be responsible for Treg cell-mediated suppression; these included production of immunomodulatory cytokines IL-10 and TGF-β (Asseman et al., 1999; Chen et al., 2005), CTLA-4-mediated cross-linking of B7 molecules on the surface of activated T cells or APC (Oderup et al., 2006; Paust et al., 2004; Tang et al., 2004), elaboration of extracellular adenosine through the cooperative action of ectonucleotidases CD39 and CD73 (Deaglio et al., 2007), extrusion of cAMP (Bopp et al., 2007), and cytolytic activity mediated by granzymes (Gondek et al., 2005) as well as several additional hypothetical mechanisms (Mahic et al., 2006). However, an unequivocal proof of a unique or non-redundant role for any of the above mechanisms in regulatory T cell-mediated suppression in un-manipulated mice has been lacking.

Recent studies have established that the suppressor function of regulatory T cells is critically dependent upon expression of the Foxp3 protein. This conclusion is supported by a loss of suppressor function by genetically marked T cells transcribing the Foxp3 locus yet lacking in functional Foxp3 protein expression in mice with the targeted insertion of GFP into the Foxp3 locus (Gavin et al., 2007; Lin et al., 2007). Furthermore, a decreased Foxp3 protein expression in Treg cells as a result of induced dysregulation of Foxp3 gene transcription resulted in a marked attenuation of the suppressor function (Wan and Flavell, 2007). In agreement with these results, the Foxp3 ablation in mature Treg cells through the Cre-mediated deletion of a conditional Foxp3 allele results in a loss of suppressor function (Williams and Rudensky, 2007). Genome-wide analyses of Foxp3 binding sites utilizing ex vivo-isolated mouse Treg cells or the Foxp3 transfected hybridoma cell line revealed a relatively small number of directly Foxp3-regulated genes, including few genes encoding putative effector molecules, e.g., CTLA4 and Nt5e (Marson et al., 2007; Zheng et al., 2007). In light of these results, it is reasonable to speculate that Foxp3 induces expression of a unique gene mediating suppressor function of Treg cells. Mice lacking such a gene are expected to develop a rapid and fatal autoimmune syndrome analogous to that described in Foxp3DTR mice subjected to chronic Treg cell ablation (Kim et al., 2007). Alternatively, it is possible that Treg cells employ multiple immunomodulatory or suppressive molecules, not necessarily unique to Treg cells, that are employed by these cells with a great deal of efficiency as a result of a superior proliferative and metabolic potential, as well as a migratory capacity bestowed by Foxp3 (Gavin et al., 2002; Gavin et al., 2007; Kim et al., 2007; Komatsu and Hori, 2007; Zheng et al., 2007). It is reasonable to speculate that a given suppressive mechanism might operate in a particular inflammatory-tissue setting. A corollary to this scenario is that spontaneous inflammatory lesions resulting from a deficiency in a single effector mechanism will be much less severe and pervasive than those resulting from a lack of the entire Treg cell subset.

As discussed above, pioneering studies by Powrie and coworkers implicated IL-10 in Treg cell-mediated suppression of induction of immune-mediated colitis. These studies relied on adoptive T cell transfer into lymphopenic hosts and antibody-mediated neutralization of IL-10 or IL-10R (Annacker et al., 2003; Asseman et al., 1999; Asseman et al., 2003; Singh et al., 2001). Furthermore, provision of IL-10-sufficient but not IL-10-deficient CD25+CD4+ T cells resolves the established intestinal inflammation (Uhlig et al., 2006). A priori IL-10 cannot be a unique suppressor molecule utilized by Treg cells. Within the immune system, multiple cell types, including B cells, T cells, mast cells, and dendritic cells, produce IL-10 (Moore et al., 2001). Functional significance of IL-10 production by diverse cell types was highlighted by recent observations of an important role for mast cell-derived IL-10 in cutaneous hypersensitivity reactions induced upon immunization or chronic UV irradiation (Grimbaldeston et al., 2007). The germ-line deletion of the Il10 gene

**Figure 4. Minor Peribronchial Inflammation and a Normal Size of the Foxp3+ Treg Cell Subset in the Lungs of Unmanipulated Il10flox/flox × Foxp3YFP-Cre Mice**

(A) Histology sections of lungs from mice with a Treg cell-restricted IL-10 deficiency. Formalin-fixed sections of lungs of approximately 3-month-old Il10flox/flox × Foxp3YFP-Cre and littermate control Il10flox/flox × Foxp3YFP-Cre mice were stained with H&E. Original magnification, 10×.

(B) Flow-cytometric analysis of Foxp3+ Treg cells in the lung tissue of Il10flox/flox × Foxp3YFP-Cre and littermate control Il10flox/flox × Foxp3YFP-Cre mice. A lung-resident population of mononuclear cells was isolated from PBS-perfused lungs. Cells isolated from three mice of each genotype were pooled and stained for CD4, CD8, CD25, and Foxp3. The CD4+CD8- T cell gate is shown. A representative of two identical experiments is shown.
leads to fulminant spontaneous colitis but not to the fatal and rapid disease observed in Foxp3 mutant mice (Berg et al., 1995; Fontenot et al., 2003; Kuhn et al., 1993). Importantly, we previously reported that the ablation of the \( \text{Il10} \) gene in T cell lineage results in a comparably severe colitis, increased skin hypersensitivity, and the fatal immunopathology associated with \text{Toxoplasma gondii} infection (Roers et al., 2004). However, within the T cell lineage, multiple T cell subsets other than Treg cells, including NKT cells, Foxp3- Tr1 cells, and conventional IFN-\( \gamma \)-producing effector CD4+ T cells elicited in the course of immune response to a pathogen such as \text{Toxoplasma gondii} or \text{Leishmania major}, can serve as a major source of IL-10 production (Anderson et al., 2007; Jankovic et al., 2007; Nylen et al., 2007; Roers et al., 2004). Therefore, elucidation of a functional significance of IL-10 production by Treg cells in control of immune inflammation in unmanipulated mice should provide an acid test for the aforementioned opposing models of Treg cell-mediated suppression.

Here, through the ablation of a conditional \( \text{Il10} \) allele in Treg cells, and using Cre recombinase knocked into the 3’ UTR of the Foxp3 locus, we found that IL-10 plays a prominent role in Treg cell suppressive function in the colon, lung, and skin. Consistent with the recent findings that some IL-10-expressing T cells in the colonic lamina propria (and the majority of these cells in the small intestine) do not express Foxp3, spontaneous intestinal inflammation was largely limited to the colon, and its severity was less pronounced in Foxp3\(^{\text{YFP-Cre}}\) mice than in mice with the germ-line or T cell-specific IL-10 deficiency
(Kamanaka et al., 2006; Maynard et al., 2007; Roers et al., 2004). In contrast to spontaneous colonic inflammation, unchallenged \textit{Il10}\textsuperscript{floxed/floxed} × \textit{Foxp3}\textsuperscript{YFP-Cre} mice maintained under SPF conditions showed very little immune-mediated inflammation in the skin or lungs (data not shown). Nevertheless, upon induction of local antigen-specific immune responses, a markedly enhanced inflammation and tissue pathology was observed in the absence of Treg cell-derived IL-10 in the experimental models of the skin and lung hypersensitivity. Interestingly, in contrast to a sharp increase in airway resistance in OVA-challenged \textit{Il10}\textsuperscript{floxed/floxed} × \textit{Foxp3}\textsuperscript{YFP-Cre} mice, the airway resistance was not markedly augmented in \textit{Il10}\textsuperscript{-/-} mice in comparison to wild-type mice (data not shown). The latter observation is in agreement with another study of \textit{Il10}\textsuperscript{-/-} mice (MakeI et al., 2000); this study also utilized OVA-induced lung inflammation. Lack of lung hyper-reactivity in \textit{Il10}\textsuperscript{-/-} mice can be explained by previously proposed regulation of smooth-muscle contraction by IL-10 originating from cells other than Treg cells (MakeI et al., 2000). In addition, it is possible that a more prominent profuse colitis in mice with the germ-line IL-10 deficiency than in mice in which the IL-10 deficiency limited to Treg cells results in a massive focus of intestinal inflammation, “detracting” pro-inflammatory cells from the lung. We further speculate that this observation of “immune detraction” in the model of induced lung hypersensitivity might be applicable to other types of immune inflammation.

It is noteworthy that the acquisition of IL-10 production by Treg cells, a particular, functionally important suppressor modality, is likely to be facilitated by the environment at mucosal interfaces. This notion comes from the observations that despite the fact that IL-10 expression can be found in a subset of Foxp3\textsuperscript{+} Treg cells present in tissues such as colon or lung, Foxp3-expressing thymocytes lack IL-10 expression. Our studies demonstrate that IL-10 produced by Treg cells is not required for limiting systemic autoimmunity but is needed for restraining immunological hyperreactivity at environmental interfaces. In contrast, CTLA-4 ablation in Treg cells resulted in systemic lymphoproliferative disorder and severe pancreatic lesions, whereas the colon and skin remained largely unaffected (Y.P.R., A. Paterson, A. Sharpe, and A.Y.R., unpublished observations). These results strongly suggest that Treg cells utilize multiple means to limit the immune response and that suppressive mechanisms utilized by Treg cells are likely to be nonredundant, or only partially redundant, with individual suppressor mechanisms operating in a particular tissue and inflammatory setting.

### EXPERIMENTAL PROCEDURES

#### Generation of the Targeted Foxp3\textsuperscript{YFP-Cre} Allele

A 7.9 kilobase (kb) XbaI fragment of the Foxp3 locus containing exons 6 through 11 was subcloned from a 30.8 kb cosmid containing the complete Foxp3 gene into a pBlueScript vector containing a PGK-DTA-negative selection cassette. A Sail restriction site was engineered in place of the Bael site present in the 3' UTR region of the Foxp3 gene, upstream of the polyadenylation signal. To generate the targeting construct, we cloned IRES-YFP-Cre-BGHyP-AK-FRT-Neo-FRT cassette provided by Richard Locksley (UCSF) into the Sail site. The linearized targeting construct was electroporated into R1 ES cells, and neo-resistance clones were screened by PCR across the 3' arm for evidence of the homologous recombination. Positive clones were further screened by Southern blot analysis of BspHI-digested genomic DNA. ES cells harboring the correctly targeted Foxp3 locus were injected into C57BL/6 blastocysts, and chimeric male offspring were mated to the FLP-deleter transgenic strain of mice so that the PGK-Neo cassette would be excised. Germ-line transmission of the targeted allele was confirmed by PCR with genomic tail DNA.

#### PCR Confirmation of the Deletion of the Il10flx Allele

Genomic DNA from sorted T cells was isolated with DNeasy Blood & Tissue Kit (Qiagen) and diluted to a final concentration 5 ng/µl in Tris-EDTA buffer (pH 7.4). Serial dilutions of template were used in PCR with two pairs of Il10-specific primers: 5'-ACTGGCTCAGCACTGCTATGC-3' and 5'-GCTTCTTTGGACCTTCCATACCCAG-3' for detecting Il10\textsuperscript{flx/flx} or Il10\textsuperscript{flx/+} alleles; 5'-CAGGATTGGACAGTGCTAGAGC-3' and 5'-GAACCTGCAGCTAAATCTCTCGTGC-3' for detecting the Il10 allele lacking excised floxed fragment. To normalize the amount of input DNA, we used PCR with primers to the Foxp3 gene: 5'-AGAGACAGCAGAGGGTGATG-3' and 5'-TGCTCAGGCGCTGAGGCA-3'.
Lymphocyte-containing cell populations were isolated from the ear skin as described (Ohl et al., 2004).

Cell Purification and Flow-Cytometric Analysis of T Cell Subsets and Cytokine Production

Cell populations were purified with magnetic beads and an AutoMACS magnetic cell sorter (Miltenyi Biotec) or with a FACSARia fluorescent cell sorter (BD Biosciences). The purity of all cell preparations was >90%. Flow-cytometric analyses were carried out with a FACSARia flow cytometer (BD Biosciences). Fluorochrome-conjugated mouse CD4, CD8a, CD44, CD69, CD62L, CD25, and Foxp3 antibodies (BD Biosciences or eBioscience) were used. Flow-cytometric analysis of cytokine production was performed as described elsewhere (Williams and Rudensky, 2007). For IL-10 secretion analysis, cells were plated in round-bottom plates at 5 x 10^5/ml in 0.2 ml of RPMI/10% FCS. LPL cells were stimulated in vitro with PMA (100 ng/ml) and ionomycin (1 μg/ml) for 4 hr in the presence of Golgi Plug (BD PharMingen). Splenocytes were stimulated with plate-bound CD3 and CD28 antibodies (1 μg/ml each) for 72 hr in the presence of rhTGF-β1 (2 ng/ml) and IL-2 (100 U/ml) and restimulated for 4 hr with PMA and ionomycin (50 ng/ml and 500 ng/ml, respectively) in the presence of Golgi Plug. Stimulated and unstimulated control cells were stained for surface expression of CD4, FoxP3, and IL-10 antibodies according to the manufacturer’s (eBioscience’s) protocol.

Histopathological Evaluation of Spontaneous Colitis

Samples of small intestine, cecum, and colon from 3–4 animals per group were fixed in 10% neutral buffered formalin and processed routinely for hematoxylin.
and eosin staining. Histological evaluation of colitis within the cecum and proximal and mid-colon was performed as described previously (Burich et al., 2001), except that the small intestine was included. In brief, a pathologist blinded to genotype and using a 0–4 scale scored mucosal changes (erosion, ulceration, and/or hyperplasia), inflammation, and extent of section involvement. An inflammatory bowel disease (IBD) score was generated from the sum of the individual section scores.

Induction of Allergic Lung Inflammation and Evaluation of Lung Function and Lung Histopathology

Mice received two i.p. injections of 100 μg OVA (Pierce Chemical Co.) complexed with aluminum potassium sulfate (alum; Sigma-Aldrich) in 0.2 ml on days 1 and 14. Mice were anesthetized by administration of 130 mg/kg ketamine and 8.8 mg/kg xylazine in saline prior to intranasal (i.n.) challenge with 100 μg OVA (0.05 ml; 2 mg/ml) on day 14 and with 50 μg OVA (0.05 ml; 1 mg/ml) on day 25 (Henderson et al., 1996; Zhang et al., 1997). Control groups of mice received 0.2 ml saline with alum i.p. on days 1 and 14 and 0.05 ml saline without alum i.n. on days 14 and 25. For BAL cell isolation, the right lung was lavaged three times with 0.5 ml of normal saline 24 hr after the final OVA or saline treatment, and cells were analyzed as described (Henderson et al., 1996).

To evaluate pulmonary function, we measured invasive pulmonary mechanics in mice in response to methacholine as previously described (Henderson et al., 2007; Henderson et al., 1996), except that the thorax was not opened. Lung pathology evaluation was performed in a blinded fashion as described elsewhere (Henderson et al., 2005) (for a detailed description, see Supplemental Data).

Real-Time PCR

Total RNA was isolated from the right lung with an RNeasy mini kit (QIAGEN), and mRNA amounts for IL-4, IL-5, IL-13, IFN-γ, and GAPDH were determined by quantitative PCR on a 7900HT instrument (Applied Biosystems). For design of all primers, Primer3 software was used across the intronic sequences (Table S1). PCR product sizes of ~100 bp were confirmed by gel electrophoresis.

Skin-Contact Hypersensitivity Reaction

We induced skin hypersensitivity by painting the shaved abdomen with 100 μl of 0.2% (wt/vol) dinitrofluorobenzene (DNFB; Sigma-Aldrich) in a 4:1 mixture of acetone and olive oil for sensitization. The mice were challenged 6 days later by application of 10 μl 0.2% (wt/vol) DNFB in olive oil to each side of one ear. Ear thickness was determined with an engineer’s caliper (Mitutoyo) before challenge and at 48 hr after treatment. The percent increase of ear thickness was compared for the different experimental groups with the Student’s t test.

SUPPLEMENTAL DATA

One table, five figures, and additional Experimental Procedures are available online at http://www.immunity.com/cgi/content/full/28/4/546/DC1/.

ACKNOWLEDGMENTS

We thank C. Maynard and C. Weaver (University of Alabama-Birmingham) for sharing unpublished data; D. Liggett for assistance with the analysis of histopathology; K. Fortbush, L. Karpik, and T. Chu for assistance, and members of the Rudensky lab for discussions. This work was supported by the Howard Hughes Medical Institute (A.Y.R.) and US National Institutes of Health (A.Y.R. and W.R.H.).

Received: September 21, 2007
Revised: January 11, 2008
Accepted: February 13, 2008
Published online: April 3, 2008

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