Regulatory T cells inhibit acute IFN- γ synthesis without blocking T-helper cell type 1 (Th1) differentiation via a compartmentalized requirement for IL-10

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CD4⁺CD25⁺Forkhead box P3 (Foxp3)⁺ regulatory T cells (Tregs) control immune responses to self and foreign antigens in secondary lymphoid organs and at tissue sites of inflammation. Tregs can modify the function of many immune cells and have been proposed to block early proliferation, differentiation, and effector function. Acute ablation of Tregs has revealed rapid cytokine production immediately after Treg removal, suggesting that Tregs may regulate effector function acutely rather than regulating the programming for immune function. We developed *in vitro* and *in vivo* models that enabled the direct test of Treg regulation of T-helper cell type 1 (Th1) differentiation. CD28 signaling is known to abrogate Treg suppression of IL-2 secretion and proliferation, but our studies show that Treg suppression of IFN- γ during Th1 priming proceeds despite enhanced CD28 signaling. Importantly, during Th1 differentiation, Tregs inhibited early IFN- γ transcription without disrupting expression of Th1-specific T-box transcription factor (Tbet) and Th1 programming. Acute shutoff of effector cytokine production by Tregs was selective for IFN- γ but not TNF- α and was independent of TGF- β and Epstein-Barr virus-induced gene 3. *In vivo*, Tregs potently controlled CD4 IFN- γ and CD4 effector cell expansion in the lymph node (four- to fivefold reduction) but not Th1 programming, independent of IL-10. Tregs additionally reduced CD4 IFN- γ in the inflamed dermis (twofold reduction) dependent on their production of IL-10. We propose a model for Treg inhibition of effector function based on acute cytokine regulation. Interestingly, Tregs used different regulatory mechanisms to regulate IFN- γ (IL-10-dependent or -independent) subject to the target T-cell stage of activation and its tissue location.

The suppressive activities of CD4⁺CD25⁺ forkhead box P3 (Foxp3)⁺ regulatory T cells (Tregs) extend beyond their maintenance of self-tolerance (1) to include suppression of immunity to infection and tumors (2, 3). The variety of mechanisms Tregs can use provides the potential to disable a CD4⁺ T-cell during initial activation, proliferation, differentiation, and effector function (4–6). Full regulatory function *in vivo* also requires the presence of Tregs in both the draining lymphoid nodes and the inflamed tissue (7–10). Specific IL-10 ablation in Tregs led to dysregulated immunity at barrier surfaces such as the lung, gut, and skin (11), suggesting Treg effector mechanisms may be context dependent. Indeed Tregs can inhibit CD8 lytic granule release by TGF- β but leave IFN- γ secretion intact, demonstrating the specificity and context dependency of Treg-mediated suppressive mechanisms (12).

Although Tregs can potently suppress immune responses, the series of events that contribute to such regulation is unclear. A number of *in vivo* studies have suggested that Tregs block T-helper cell differentiation of naïve T cells into effector T cells (13, 14). The reports focus on the downstream diminution in effector T-cell number, an outcome that also could be reached through Treg effects on expansion, survival, or acute regulation of effector function. Therefore, the role that Tregs play in modifying the CD4⁺ T-cell differentiation program has not been defined. The ability of Tregs to regulate proliferation makes it difficult to dissociate an active role in modulating the differentiation program from a passive one resulting from a lack of division.

To examine the role of Tregs in early stages of CD4⁺ T-helper cell type 1 (Th1) differentiation, we established models for detecting the initial IFN- γ -producing T-cell pool *in vivo* and *in vitro*. CD28 signaling abrogates Treg-mediated suppression of proliferation by providing a direct signal to the CD4⁺ target T-cell, making the population resistant to Treg activity (15). We took advantage of this system in which proliferative suppression can be bypassed to investigate whether Tregs are able to impose suppressive activities on the Th1 differentiation program. Early during Th1 priming, Tregs suppressed secretion of IFN- γ by CD4⁺ target T cells but did not inhibit Th1 differentiation. Surprisingly, Tregs specifically modulated IFN- γ transcription but not transcription of other Th1 cytokines, TNF- α , or the lineage-specific transcription factor T-box transcription factor (Tbet). Consequently, despite acute shutoff of IFN- γ production during early priming, Tregs failed to block Th1 lineage commitment. *In vivo*, acute regulation of IFN- γ in the lymph node was not dependent on the presence of IL-10, but loss of IL-10 in Tregs significantly attenuated their ability to regulate IFN- γ in the inflamed tissue.

Results

CD28 Uncouples Suppression of Proliferation and Effector Cytokines.

The study of Treg effects on differentiation has been hindered by their potent inhibition of T-cell proliferation. CD28 signaling in target T cells can override Treg suppression of T-cell proliferation (15–17) and may facilitate the study of proliferation-independent Treg effects. To test this notion, we measured cytokine secretion by CD4 target T cells early during Th1 priming with anti-CD3 and anti-CD28 mAbs in the presence Tregs. As previously reported (15), CD28 blocks Treg suppression of target T-cell IL-2 early before division (10 h; Fig. 1*A* and *B*) enabling subsequent division (36 h; Fig. 1*A*). At 36 h, however, target T cells secreted IFN- γ when cultured alone but failed to secrete IFN- γ when cultured with Tregs (Fig. 1*A* and *B*) despite robust proliferation (Fig. 1*A*) over a range of Treg:target T cell ratios (Fig. 1*C*). Tregs led to a decrease in IFN- γ frequency and amount per cell (Fig. 1*B*). Addition of nonregulatory T cells, to control for cell density, did not reduce IFN- γ (Fig. 1*A* and *B*). Hence, CD4⁺ T cells remain susceptible to Treg suppression of IFN- γ despite provision of CD28 signals (Fig. 1*A*) with a selective loss in the number of IFN- γ producers (Fig. 1*D*). Thus, regulation of IFN- γ production is independent of regulation of proliferation.

Early Treg Blockade of IFN- γ Is Independent of Cytokine Consumption or Production of Suppressive Cytokines. To determine when and how Tregs suppress IFN- γ , we looked at the kinetics of IFN- γ

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Fig. 1. CD28 uncouples suppression of proliferation and effector cytokines. (A) CFSE-labeled CD25⁺Thy1.1⁺CD4⁺ target T cells were Th1-primed with APC, anti-CD3, anti-CD28, rIL-2, and rIL-12 alone or with CD25⁺Thy1.2⁺CD4⁺ Tregs (plus CD25⁺) or CD25⁺Thy1.2⁺CD4⁺ control cells (plus Ctrl) at a 1:1 ratio. Cytokine secretion was measured at 10 h for IL-2 and 36 h for IFN- γ using the cytokine capture assay, gated on live Thy1.1⁺7AAD[−] target T cells. Numbers in quadrants are percent of gated. (B) Percentage of live Thy1.1⁺7AAD[−] IL-2 (Left) and IFN- γ secreting cells (Center) and mean fluorescence intensity (MFI) of IFN- γ (Right) from A. (C) Naive target T cells were stimulated as in A, alone or with varying numbers of Thy1.2⁺ Tregs. (D) Total target T-cell numbers, cultured alone or with Tregs, at 36 h of Th1 priming. (E) Frequency of IFN- γ -producing target T cells at various time points. (F) Target T cells were stimulated as in A with WT, IFN- γ receptor KO (IFN- γ R^{−/−}), IL-12 receptor β 1 KO (IL-12R β 1^{−/−}), or IL-12 receptor β 2 KO (IL-12R β 2^{−/−}) Tregs. Bars show percentage of IFN- γ secretors at 36 h. (G) Bcl-2 expression in the target T cells at 36 h. (H) Target T cells stimulated as in A alone or with Tregs in the top and/or bottom chamber of a transwell plate (0.4- μ m pore size). Bars show percentage of IFN- γ -secreting target T cells in the bottom chamber at 36 h. Data are from at least three individual experiments. * P < 0.05, ** P < 0.005, *** P < 0.0005.

production. Secretion of IFN- γ by naive target T cells was observed as early as 6–12 h poststimulation and peaked at 36 h. With Tregs, IFN- γ secretion was induced between 6–12 h but was not enhanced 18–36 h into the culture (Fig. 1E), even at high concentrations of the polarizing cytokine recombinant IL-12 (rIL-12) (Fig. S1B). Tregs could passively limit positive signals for Th1 differentiation (18) or actively secrete suppressive cytokines. Th1 differentiation depends, in addition to IL-12, on IL-2 and IFN- γ signaling (19, 20). However, neither cytokine appeared limiting in Treg cocultures, because cytokine receptor signaling in target T cells was intact (Fig. S1A), and exogenous provision of IL-2 or IFN- γ could not rescue IFN- γ production (Fig. S1B). Tregs also may regulate immunity through consumption of key cytokines; however, Tregs derived from IFN- γ receptor (IFN- γ R^{−/−}) and IL-12 receptor (IL-12R^{−/−})-deficient mice maintained inhibitory function in Th1 cultures (Fig. 1F). Loss of IFN- γ was also not associated with increased cell death of target T-cells by 7-amino-actinomycin D (7AAD) or expression of antiapoptotic B-cell lymphoma 2 (Bcl-2) (Fig. 1G). Therefore, we conclude that Tregs do not suppress IFN- γ secretion by interrupting Th1 differentiation by limiting key cytokines.

We next tested the requirement for immunosuppressive cytokines (21–25) in suppressing IFN- γ production. Target T cells isolated from dominant-negative TGF- β receptor type II (TGF- β RII^{−/−}) mice (which are unable to respond to TGF- β) remained sensitive to Treg inhibition of IFN- γ (Fig. S2A), suggesting that suppression of IFN- γ is independent of direct TGF- β signaling in target T cells. Similarly, suppression of IFN- γ remained intact in the absence of IL-35, using Epstein-Barr virus-induced gene 3 (Ebi3^{−/−}) or p35^{−/−} Tregs (Fig. S2B) and IL-10 (Fig. S2C and D). Therefore, suppressive cytokines are not essential for acute regulation of IFN- γ by Tregs during Th1 priming. Consistent with IFN- γ suppression being independent of cytokine consumption or a Treg-secreted cytokine, transwell cultures in which Tregs were separated physically from the target T cells failed to support IFN- γ inhibition (Fig. 1H). Thus, Tregs require close proximity with the

targets (and/or antigen-presenting cells, APCs) to mediate IFN- γ suppression during Th1 priming.

Acute Regulation of IFN- γ but No Inhibition of Lineage Commitment.

Thus far we have observed Treg inhibition of IFN- γ production during the early stages of Th1 differentiation. To determine if Tregs disrupt T-helper cell differentiation itself, target T cells were isolated after primary Th1 culture with Tregs and were recultured for 6 d without Tregs and without polarizing cytokines. Th1-primed target T cells expanded and produced IFN- γ but not IL-4 (Fig. 2A and B) (or IL-17) regardless of primary culture with Tregs. To test functional flexibility, Th1-primed target T cells cultured with Tregs were recultured in Th2-polarizing conditions in the absence of Tregs. Compared with Th2 cells, Th1-primed target T-cell populations primed with or without Tregs made significantly less IL-4 (Fig. 2C), demonstrating a functional commitment to the Th1 lineage. Our data suggest Tregs acutely inhibit IFN- γ but do not inhibit commitment to the Th1 differentiation program. An extension of this idea would predict that Tregs also should be capable of acute regulation of IFN- γ production from established Th1 effectors. Indeed, once differentiated, Th1 cells remain susceptible to acute regulation of IFN- γ by Tregs (Fig. 2D), as previously proposed (26).

Regulation of IFN- γ Transcripts Independent of Tbet. To determine how Tregs modulate Th1 function, we performed a targeted transcriptional array for genes associated with CD4⁺ T-cell differentiation. Target T-cell mRNA was isolated from Th1-primed cultures in the presence or absence of Tregs. The presence of Tregs led to the selective transcriptional down-regulation of IFN- γ at 36 h (Fig. 3A) with kinetics matching that seen for IFN- γ protein (Fig. 1E and Fig. S3A). However, many genes, including other Th1 effector molecules such as TNF- α and the Th1 lineage-specific transcription factor Tbet, were not modulated by Tregs at 36 h (Fig. 3A). Consistent with effective Th1 differentiation, GATA-

Fig. 2. Acute regulation of IFN- γ by Tregs but no inhibition of lineage commitment. Naïve DO11.10⁺CD25⁺Thy1.2⁺CD4⁺ T cells (targets) and DO11.10⁺Thy1.1⁺CD25⁺CD4⁺ cells (Tregs) were cultured with APC/OVAp under Th1-priming conditions (primary culture). On day 6, target T cells were sorted and restimulated, without Tregs, with APC/OVAp and rIL-2 (**A** and **B**) or with rIL-2 and rIL-4 (**C**) (secondary culture) for 6 d. Then cells were washed and restimulated for 6 h with plate-bound anti-TCR β mAb (tertiary culture). Frequency of cytokine producers was assessed by intracellular cytokine staining. (**A**) Representative dot plot of cytokine production from target T cells in tertiary culture. (**B**) Percentage and fold expansion of IFN- γ producers in tertiary culture. (**C**) Percentage of IL-4 producers in tertiary culture after primary culture in Th1-priming conditions with or without Tregs and in secondary culture in Th2-priming conditions without Tregs. Labels in **A** to **C** refer to conditions in primary culture. (**D**) Thy1.1⁺CD4⁺ Th1 effector cells were stimulated with APC and anti-CD3 mAb with or without Tregs for 18–24 h and were assessed for IFN- γ using the cytokine secretion assay. Data are from at least three individual experiments. ** $P < 0.005$, *** $P < 0.0005$, ns, not significant.

binding protein 3 (GATA3) was not induced in the presence or absence of Tregs (Fig. S3B).

To assess potential posttranscriptional control of Tbet expression, we measured Tbet protein levels by FACS. Similar levels of Tbet expression were found in target T cells cultured alone or with Tregs throughout the first 36 h (Fig. 3B and Fig. S3C), despite ongoing Treg suppression of IFN- γ (Fig. 3B). Tbet appears to be expressed in two independent waves with a late (post-48 h) IL-12/signal transducer and activator of transcription 4 (STAT4)-dependent stage being critical for Th1 lineage commitment and high-level IFN- γ production (27). To determine if Tregs interfere with this late Th1 lineage decision, we examined STAT4 signaling and sustained Tbet expression. STAT4 signaling was largely unaffected by Tregs in the late phase (48 h and beyond) (Fig. 3C), consistent with prolonged Tbet expression (Fig. 3D). As a measure of Tbet function, we assessed expression of the Tbet gene target chemokine (C-X-C motif) receptor 3 (CXCR3) and found no difference between target T cells cultured alone and with Tregs (Fig. 3E). Thus, Tregs reduce transcript levels of IFN- γ without blocking Tbet expression. Intact Tbet likely accounts for our observed imprinting of Th1 function in target T cells primed with Tregs (Fig. 2).

Control of Effector Function Independent of Differentiation in Vivo.

To test Treg modulation of Th1 differentiation formally in vivo, we established a model to identify the initial acquisition

of CD4 effector function directly. Cytokines were measured directly ex vivo by intracellular cytokine staining in the presence of Brefeldin A, eliminating the need to reactivate cells in vitro, similar to the methods of McLachlan et al. (26). Using DO11.10⁺ T-cell receptor (TCR) transgenic mice expressing a Foxp3-GFP reporter, we isolated naïve DO11.10⁺ target T cells (CD4⁺CD62L^{hi}CD44^{lo}GFP⁺Thy1.2⁺) and DO11.10⁺ Tregs (CD4⁺GFP⁺Thy1.2⁺). Carboxyfluorescein succinimidyl ester (CFSE)-labeled naïve T cells were transferred with or without Tregs to immunocompetent Thy1.1⁺ WT BALB/c mice immunized with ovalbumin peptide in incomplete Freund's adjuvant (OVAp/IFA). On days 3 and 5 after immunization, transferred target T cells (Thy1.2⁺ Foxp3[−]) were assessed for proliferation, expansion, and cytokines.

As in previous reports, Tregs did not prevent target T-cell entry into division (Fig. 4A) (14) but did compromise expansion (total number of target T cells: $2.1 \times 10^6 \pm 0.2$ target T cells alone vs. $0.5 \times 10^6 \pm 0.6$ target T cells with Tregs) (see Fig. 4B for IFN- γ numbers) (13). In addition to a reduced number of primed effector cells, the remaining activated pool was compromised in its ability to make IFN- γ . Direct ex vivo IFN- γ production was measured early in the draining lymph node (on days 3 and 5) and in tissue at the site of immunization (ear dermis) (on day 5; no transferred cells were detected day 3). Target T cells produced IFN- γ at both sites, but this production was suppressed by twofold in the presence of Tregs in both the lymph node (Fig. 4B) and

Fig. 3. Regulation of IFN- γ transcripts is independent of Tbet. Naïve CD25[−]Thy1.1⁺CD4⁺ T cells (targets) were cultured in Th-1 priming conditions as in Fig. 1 alone or with CD25⁺Thy1.2⁺CD4⁺ (Tregs). (**A**) At 36 h, target T cells were enriched by removal of Thy1.2⁺ Tregs, and mRNA was purified for quantitative RT-PCR. Transcripts are normalized to unstimulated target T cells. (**B**) Naïve target T cells were stimulated as in **A** with or without Tregs for 36 h. Tbet and IFN- γ expression in target T cells was determined by intracellular staining. (**C**) Phosphorylated Stat-4 protein in the target T cells with or without Tregs. (**D**) Tbet expression in target T cells from **A** at day 6. (**E**) CXCR3 expression on target T cells from **A** at day 4, unstimulated target T cells $3.4\% \pm 0.2$ SEM. CXCR⁺. All FACS data are gated on Thy1.1⁺ target T cells; numbers show the percentage of gated. Data are from at least three individual experiments.

Fig. 5. Compartmentalized requirement for Treg-derived IL-10 in the inflamed ear dermis. CFSE-labeled naïve DO11.10⁺Thy1.2⁺CD4⁺ target T cells were transferred intravenously with or without WT DO11.10⁺Foxp3GFP⁺ or IL-10^{-/-} (IL-10 KO) DO11.10⁺Foxp3GFP⁺ Tregs into congenic Thy1.1 BALB/c mice immunized in the ear with OVA/IFA. On day 5, cells were isolated from the draining lymph node and ear in buffers containing Brefeldin A and were assessed for ex vivo IFN-γ, gated on Thy1.2⁺Foxp3⁺CD4⁺ target T cells. (A) Number and frequency of IFN-γ+ target T cells in the draining lymph node (LN). (B) Cell numbers of WT and IL-10^{-/-} DO11.10⁺Thy1.2⁺Foxp3GFP⁺ Tregs in the ear. (C) Representative dot plot of ex vivo IFN-γ in the ear, gated on Thy1.2⁺Foxp3⁺CD4⁺ target T cells. Numbers represent percentage of target T cells making IFN-γ. (D) Fold-change in IFN-γ frequency of target T cells in the ear tissue in the presence of WT or IL-10^{-/-} Tregs. Mean fold change ± SEM: with WT Tregs, 0.65 ± 0.33 (n = 16); with IL-10^{-/-} Tregs, 0.98 ± 0.23 (n = 10). Data are from at least three independent experiments.

of the target T cells (13). Our studies extend these findings to show that the acute suppression of IFN-γ by Tregs, similar to that reported in ref. 13, does not necessarily disrupt Th1 differentiation: The effector pool was fully able to mount an IFN-γ response when removed from the Tregs. Our data are consistent with the need for Tregs to be present both in the lymph node and the infected/inflamed tissue for full regulatory activity (7–10). Thus, we support a model in which Tregs regulate developing immunity centered around acute regulation of cytokines (Fig. S5), similar to the conclusions regarding CD8 function (12). Such a mechanism would attenuate the effector response but leave appropriate programming for immune function intact. This mechanism has distinct advantages for subsequent responses to pathogens but comes at the expense of generating pathogenic autoreactive T cells.

We find a tissue-specific requirement for Treg IL-10 in regulating IFN-γ by CD4 T cells in the inflamed skin. IL-10 was not required for the regulation of T-cell expansion or IFN-γ production during Th1 priming in the lymph node. Similarly, deletion of IL-10 specifically in the Treg compartment did not lead to systemic immune dysregulation but rather to a local disruption at multiple tissue sites (11). Thus, IL-10 seems to play an essential role in Treg control in inflamed tissues. This context-specific requirement for IL-10 could be caused by IL-10-sensitive tissue-specific APCs or could be driven by stage-specific sensitivity of the effector T cells (29, 30). We have been unable to find a direct role for IL-10 in regulating IFN-γ by Th1 cells; therefore we suggest that Treg-derived IL-10 in the inflamed tissue acts indirectly, likely on the APCs, to regulated IFN-γ production by Th1 cells. The data suggest that the efficiency of specific Treg mechanisms depends on whether the critical control point for a given inflammatory response lies in the attenuation of lymph node generation/expansion of effectors or in the targeting of the effectors in the inflamed tissue—in essence, in prevention or cure, respectively.

The mechanism of IFN-γ regulation during Th1 priming remains unknown. Treg consumption of cytokines did not play a role; neither did the suppressive cytokines IL-10, TGF-β, or IL-35 (Fig. S2) nor negative regulation via CTLA-4 (31). Tregs led to a selective defect in IFN-γ but not other Th1 cytokines such as TNF-α and lymphotoxin. Tregs could modulate specific control points in IFN-γ transcription or modify general activation signals for which IFN-γ is differentially sensitive. Western blot analyses of nuclear factor of activated cells (NFAT) and NF-κB failed to reveal Treg-mediated attenuation of these signals at the time of active IFN-γ inhibition, suggesting there is no global disruption in TCR signaling. IFN-γ and TNF-α production appear differentially sensitive to extracellular ATP and cAMP (32), a pathway known to mediate some Treg functions (33, 34). However, addition of a cAMP antagonist did not disrupt Treg control of IFN-γ in this Th1 culture system (87.2% suppression of IFN-γ producers with Tregs vs. 91.7% suppression with Tregs plus cAMP antagonist). Alternatively, provision of CD28 costimulation may rescue the

expression of cytokines differentially regulated by Tregs. Costimulatory signals such as CD28 can stabilize cytokine transcripts by increasing stabilizing proteins that bind to adenylate uridylate-rich sequences (35). Although we show that steady-state IFN-γ transcripts are reduced, Tregs may mediate this effect by limiting the stabilization of the cytokine message rather than by a direct effect on transcription. CD28 signaling may override such regulation (36) and be sufficient to stabilize IL-2 and TNF-α but insufficient to stabilize IFN-γ.

An emerging concept is that Treg function may be conditioned by the inflammatory milieu so that Tregs activated under Th1 or Th2 conditions are better equipped to regulate the corresponding effectors. Treg interferon regulatory factor 4 (Irf4) ablation led to dysregulated Th2 immunity (37); Tbet-deficient Tregs were less able to control Th1 inflammation (38); and STAT3-deficient Tregs failed to regulate Th17 pathology (39). In our studies, Tregs from IFN-γR^{-/-} and IL-12R^{-/-} mice retain the intrinsic capacity to regulate IFN-γ during Th1 priming despite developing in the absence of IFN-γ/IL-12 signals. Thus, inflammation-induced expression of Tbet and CXCR3 may not impart better regulatory function per se but rather may help localize Tregs to sites of Th1 inflammation (38).

In vivo, we show Tregs attenuate early IFN-γ in the lymph node and in the antigen-bearing tissue, the latter step being dependent on Treg-derived IL-10. We suggest a lack of disruption of Th1 programming but control over effector function may explain the rapid activation and cytokine production of tissue effector cells following Treg-specific ablation in an autoimmune diabetes setting (40). Indeed fast production of IFN-γ (first by natural killer cells and then by T cells), but not of other cytokines such as TNF-α, appeared to be an important trigger of diabetogenesis following Treg depletion. We also found a selective loss of IFN-γ over TNF-α production by Tregs and a specific loss of IFN-γ and TNF-α dual producers, which are reported to be closely linked to protection against the pathogen *Leishmania major* (41). Therefore, Tregs may potentially regulate immune function by targeting IFN-γ production.

The generation of functional immune effectors is a multistep process that starts in the lymph node and extends to the tissue site of infection or inflammation. With the increasing interest in using Treg-based therapies, particularly for autoimmune disease, it is important to understand at what stage Tregs disrupt immune function. Our studies show that Tregs can regulate IFN-γ synthesis acutely without disruption of Th1 differentiation. Thus, in a therapeutic setting, the adoptive transfer of Tregs could control effector function and acutely attenuate autoimmune pathology but might not lead to long-term immunotolerance.

Materials and Methods

Mice. WT BALB/c, C57BL/6, Thy1.1, DO11.10⁺, Foxp3GFP BALB/c, DO11.10⁺Foxp3GFP, dnTGF-βRII^{-/-}, and IL-10^{-/-} mice were from Jackson

Laboratories. IL-10^{-/-} DO11.10*Foxp3GFP mice were bred and maintained at the University of Rochester (Rochester, NY). IL-12Rβ1^{-/-} and IL-12Rβ2^{-/-} mice were provided by D. Fairweather (The Johns Hopkins University, Baltimore, MD) and E. M. Lord (University of Rochester, Rochester, NY), respectively, and Ebi3^{-/-} mice were obtained from R. S. Blumberg (Harvard University, Cambridge, MA).

Cell Purification. CD4⁺CD44^{low} naïve CD4⁺ T cells and CD4⁺ CD25⁺ Tregs were isolated using standard MACS columns (Miltenyi Biotech) or sorting by FACS (BD Bioscience). Further details can be found in *SI Materials and Methods*.

In Vitro Suppression Assay. Target T cells (10⁷/mL) were labeled with 1 μM CFSE (Molecular Probes) for 5 min at room temperature. Then 1 × 10⁵ CD25⁺ Thy1.1⁺ CD4⁺ T cells were cultured with 1 × 10⁵ APC, 1 μg/mL anti-CD3 mAb (2C11), and 1 μg/mL anti-CD28 mAb (37.51) with or without 1 × 10⁵ CD25⁺ Thy1.2⁺ CD4⁺ T cells in 96-well U-bottomed plates. Th1-priming conditions were 10–20 ng rIL-12, anti-IL-4 Ab 11B11, 10 units/mL of recombinant human IL-2 (rhIL-2). Cells were cultured in complete RPMI 1640 with 10% (vol/vol) heat-inactivated FCS (Hyclone).

Quantitative Real-Time RT-PCR. RT-PCR was performed using standard techniques. Further details can be found in *SI Materials and Methods*.

Flow Cytometry. All FACS analyses used standard intracellular staining techniques or the cytokine capture assay, as described (15). Further details can be found in *SI Materials and Methods*.

In Vivo Suppression Assay. For the in vivo suppression assay, 0.5–1 × 10⁶ sorted naïve CD4⁺CD62L^{high}CD44^{low} KJ-126⁺ cells were CFSE labeled (5 μM) and injected i.v. alone or with equal numbers of sorted CD4⁺Foxp3GFP⁺KJ-126⁺ Tregs into congenic Thy1.1 BALB/c mice. Mice were immunized intradermally in the ear with OVAp/IFA (1 μM OVAp per ear). Three to five days after immunization cells were isolated from the ears and draining lymph node in buffers containing Brefeldin A and were assessed ex vivo for function. Where indicated, on day 5 after immunization target T cells (Thy1.2⁺ Foxp3GFP⁻) were sorted, and 100,000 target T cells were injected alone into fresh congenic mice and rested for 5 d before challenge with OVAp/IFA. Further details can be found in *SI Materials and Methods*.

Statistical Analysis. A paired student's *t* test was performed for the in vitro data. A Mann–Whitney test was performed for the in vivo data. **P* ≤ 0.05, ***P* ≤ 0.005, and ****P* ≤ 0.0005.

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