

Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice

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Mice lacking the transcription factor *Foxp3* (*Foxp3*⁻) lack regulatory T (T_{reg}) cells and develop fatal autoimmune pathology. In *Foxp3*⁻ mice, many activated effector T cells express self-reactive T cell receptors that are expressed in T_{reg} cells in wild-type mice. Thus, in wild-type mice, most self-reactive thymocytes escaping negative selection are diverted into the T_{reg} lineage, and whether T_{reg} cells are critical in self-tolerance in wild-type mice remains unknown. Here, acute *in vivo* ablation of T_{reg} cells demonstrated a vital function for T_{reg} cells in neonatal and adult mice. We suggest that self-reactive T cells are continuously suppressed by T_{reg} cells and that when suppression is relieved, self-reactive T cells become activated and facilitate accelerated maturation of dendritic cells.

The transcription factor *Foxp3* is specifically expressed in regulatory T cells (T_{reg} cells) and is required for their development^{1–3}. Loss-of-function mutations in the gene encoding *Foxp3* in mice and humans result in a lack of T_{reg} cells and in fatal autoimmune pathology beginning at a very early age and affecting multiple organs^{4–7}. In contrast, depletion of T_{reg} cells by neonatal thymectomy, by adoptive transfer of naive T cell samples depleted of T_{reg} cells into lymphopenic hosts or by treatment of mice with antibodies specific for CD25, which is highly expressed on most T_{reg} cells, results in a much milder and more slowly progressing disease^{8–11}.

One potential explanation for that discrepancy is provided by the observation that protective *Foxp3*⁺ T_{reg} cells, which constitute 10–15% of peripheral CD4⁺ T cells, express self-reactive T cell receptors (TCRs)¹². In *Foxp3*-deficient mice, many activated T cells express TCRs normally found in T_{reg} cells in wild-type mice¹³. Those data suggest that many cells expressing T_{reg} cell-associated TCRs are not deleted in the absence of *Foxp3* and instead enter the pool of self-reactive T cells, which contribute to disease progression.

An extreme version of that line of reasoning is that most self-reactive T cells that escape negative selection are diverted into the T_{reg} cell lineage and that diversion itself is mainly responsible for preventing T cell-mediated autoimmunity. An additional, non-mutually exclusive possibility is that T_{reg} cells are needed to establish tolerance exclusively during early postnatal development, when self-reactive recent thymic emigrants might undergo 'preferential' homeostatic proliferation because of moderate lymphopenia¹⁴. During that period, deletional central tolerance might be relatively relaxed because of an immature thymic medullary compartment in which most negative selection begins¹⁵. In addition, T cells of the early wave in neonatal mice display TCRs with increased cross-reactivity and, therefore, self-reactive potential, because of a developmental delay in expression of the terminal deoxynucleotidyl transferase enzyme^{16,17}. Finally, the presence

of T_{reg} cells early in life may facilitate the differentiation of T cells having suppressive properties but lacking *Foxp3* expression^{18,19}. Thus, the relative importance of T_{reg} cell-mediated suppression versus *Foxp3*-independent suppressive mechanisms, including infectious tolerance and immunosuppression mediated by induced regulatory T cells (T_r1 cells) and T helper type 3 cells, remains unknown. Similarly, whether *Foxp3*⁺ T_{reg} cells are differentially required for protection against autoimmunity in neonatal versus adult mice is also not clear.

To address those principal outstanding issues, we generated 'knock-in' mice containing the coding sequence of a toxin receptor inserted into the 3' untranslated region of *Foxp3*. Using those mice, we acutely ablated the entire T_{reg} cell population by injecting toxin for varying periods of time and analyzed the functional consequences of T_{reg} cell ablation in neonatal and adult mice. In newborn and adult mice, purging of T_{reg} cells resulted in an autoimmune disease similar in severity to that of *Foxp3*-deficient mice. Our results emphasize the importance of T_{reg} cell-mediated suppression throughout the lifespan of mice and the inadequacy of other forms of peripheral tolerance established in adult mice. Furthermore, we identified other immune cells that were activated after T_{reg} cell ablation. Our data suggest that T cells, probably expressing self-reactive TCRs, are targets of continuous T_{reg} cell-mediated suppression. After T_{reg} cell ablation, those T cells became activated, produced secreted and membrane-bound cytokines and facilitated dendritic cell (DC) maturation. Those events probably led to the recruitment of additional self-reactive T cell clones and thereby established a 'vicious cycle' of autoimmune hyper-reactivity.

RESULTS

Inducible T_{reg} cell ablation in mice

To generate mice allowing specific elimination of T_{reg} cells *in vivo*, we designed a targeting construct in which we inserted cDNA encoding the

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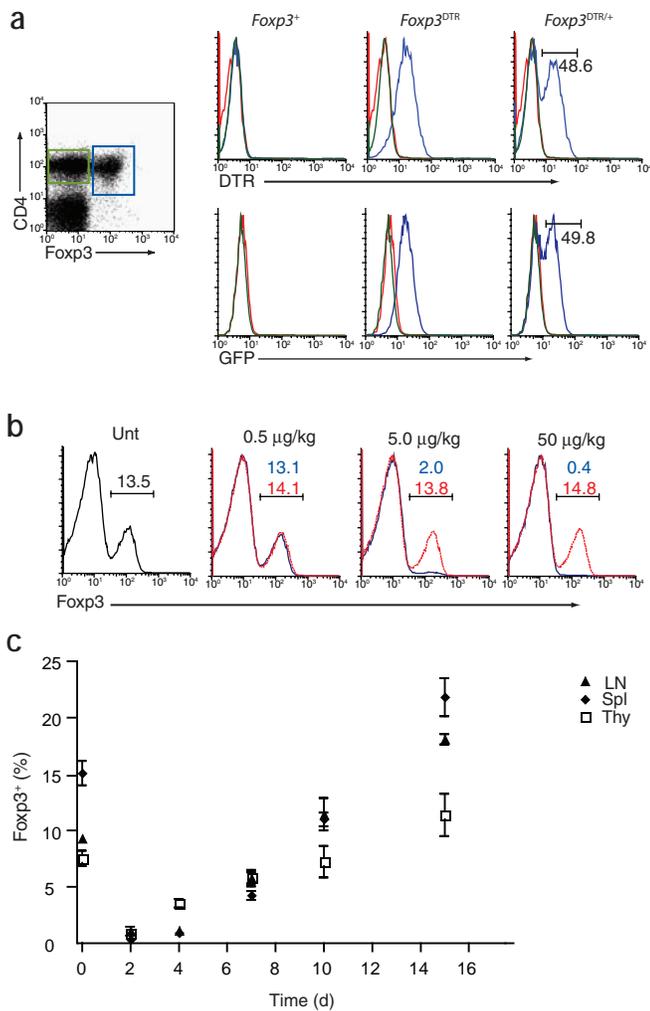


Figure 1 Characterization of inducible T_{reg} cell ablation. (a) Flow cytometry of DTR expression (top row) and GFP fluorescence (bottom row) by $CD4^{+}Foxp3^{+}$ (blue) and $CD4^{+}Foxp3^{-}$ (green) lymph node cells (mouse identification, above histograms). Colors in histograms correspond to gated populations in the dot plot (left); red lines, isotype control (DTR plots) or $CD4^{+}Foxp3^{+}$ cells from $Foxp3^{+}$ mice (GFP plots). (b) Flow cytometry of Foxp3 expression in $CD4^{+}$ lymph node cells from untreated $Foxp3^{+}$ mice (Unt; far left) or from $Foxp3^{DTR}$ mice (blue lines) and $Foxp3^{+}$ mice (red lines) treated with diphtheria toxin (right three; dose, above histogram). Numbers above bracketed lines indicate percent $Foxp3^{+}$ cells among $CD4^{+}$ cells. (c) Flow cytometry of the recovery of $CD4^{+}Foxp3^{+}$ cells from the lymph nodes (LN), spleens (Spl) and thymuses (Thy) of 8- to 10-week-old $Foxp3^{DTR}$ and $Foxp3^{+}$ mice given two injections of diphtheria toxin (50 μ g/kg) on consecutive days. Each data point (\pm s.e.m.) represents three to four mice. Data are representative of two to five experiments.

(Fig. 1b and data not shown). Complete elimination of T_{reg} cells was achieved after 7 d of treatment with 50 μ g diphtheria toxin per kg body weight (50 μ g/kg), the highest dose tested. After 2 d of diphtheria toxin injections, 0.4% of $CD4^{+}$ T cells expressed Foxp3⁺; that indicated over 97% depletion within 48 h compared with Foxp3⁺ T cells in untreated $Foxp3^{DTR}$ mice or diphtheria toxin–treated $Foxp3^{+}$ control mice (Fig. 1c). The extent of T_{reg} cell elimination remained constant throughout the time course and was not further augmented at later time points (Fig. 1c and data not shown).

To determine the rate of T_{reg} cell recovery after acute T_{reg} cell depletion, we treated mice with diphtheria toxin at 0 h and 24 h and monitored the subsequent appearance of Foxp3⁺ cells in the thymus, lymph nodes and spleen. Administration of diphtheria toxin did not affect T_{reg} cell numbers in $Foxp3^{+}$ littermates, but near-complete elimination of T_{reg} cells was achieved in the thymus, lymph nodes and spleen 2 d after the initial injection (Fig. 1c). On day 4, thymic T_{reg} cells rebounded to 47% of their numbers before treatment, whereas peripheral T_{reg} cells were present at 8% and 6% of pretreatment numbers in lymph node and spleen, respectively. The Foxp3⁺ T cell subset in the thymus was fully recovered by day 10, and pretreatment numbers of Foxp3⁺ T_{reg} cells in the lymph nodes and spleen were reached between days 10 and 15 (Fig. 1c). Although those tissue-specific recovery kinetics can be considered as evidence supporting the idea that the thymus is central to the generation of T_{reg} cells, further studies are needed to address that important issue.

Autoimmunity after neonatal T_{reg} cell ablation

Next we compared the autoimmune pathology developing in mice subjected to Foxp3⁺ T_{reg} cell elimination at birth with that in mice with germline deletion of Foxp3. We injected $Foxp3^{DTR}$ litters with 50 μ g/kg of diphtheria toxin beginning 12 h after birth and continuing every other day thereafter. By 24 d after birth, approximately 40% of $Foxp3^{DTR}$ mice had a moribund phenotype, including failure to thrive, lack of mobility, ventral skin lesions, hunched posture and conjunctivitis; the remaining mice became moribund within 27 d of birth (Fig. 2a and data not shown). In addition, untreated $Foxp3^{-}$ mice and diphtheria toxin–treated $Foxp3^{DTR}$ mice developed similar lymphoproliferative syndromes, as shown by a similar degree of lymphadenopathy, which was not detected in control mice (Fig. 2b). The severe and rapid pathology of diphtheria toxin–treated $Foxp3^{DTR}$ mice was reminiscent of that of $Foxp3^{-}$ mice^{1,21}. One notable difference in diphtheria toxin–treated $Foxp3^{DTR}$ versus $Foxp3^{-}$ mice was the presence of severe tail scaling only in $Foxp3^{-}$ mice (data not shown). Both diphtheria toxin–treated $Foxp3^{DTR}$ mice and untreated $Foxp3^{-}$ mice showed severe tissue pathology manifested by massive lymphocytic and mononuclear infiltrates in liver sinusoids, lung interstitium and epidermis of the skin and other organs (Fig. 2c–e and data not shown). In contrast, we found no tissue pathology in diphtheria toxin–treated $Foxp3^{+}$ littermates of $Foxp3^{DTR}$ mice or in untreated $Foxp3^{+}$ littermates of $Foxp3^{-}$ mice.

human diphtheria toxin receptor (DTR) fused to sequences encoding green fluorescent protein (GFP) and equipped with an internal ribosome entry site (IRES) into the 3' untranslated region of *Foxp3* to produce '*Foxp3*^{DTR}' (Supplementary Fig. 1 online). We detected GFP fluorescence and DTR expression exclusively in Foxp3⁺ T cells, not in Foxp3⁻ CD4⁺ T cells (Fig. 1a). If the DTR–GFP 'knock-in' allele did not perturb the normal expression pattern of *Foxp3*, which is an X chromosome–linked gene, half of the Foxp3⁺ cells in *Foxp3*^{DTR/+} female mice would be expected to express GFP because of random X-chromosome inactivation. Accordingly, approximately 50% of the Foxp3⁺ peripheral T_{reg} cells in *Foxp3*^{DTR/+} female mice expressed GFP. These data indicated that insertion of the IRES–DTR–GFP cassette in the *Foxp3* locus did not alter the competitive fitness of T_{reg} cells expressing the knock-in allele. Furthermore, unmanipulated *Foxp3*^{DTR} hemizygous male or homozygous female mice showed no overt signs of autoimmunity or immune dysfunction up to 5 months of age, the maximum period monitored (data not shown).

To identify the dose of diphtheria toxin that induced T_{reg} cell elimination, we treated *Foxp3*^{DTR} mice and wild-type (*Foxp3*⁺) littermates with daily intraperitoneal injections of 50, 5.0 or 0.5 μ g diphtheria toxin per kg body weight for 7 consecutive days. That treatment regimen was based on an study showing that a 7-day injection regimen is required for efficient elimination of CD4⁺ T cells²⁰. Diphtheria toxin did not affect the frequency of T_{reg} cells in control *Foxp3*⁺ littermates at any dose tested, but it resulted in a dose-dependent decrease in T_{reg} cell numbers in the lymph nodes, peripheral blood, thymuses and spleens of *Foxp3*^{DTR} mice

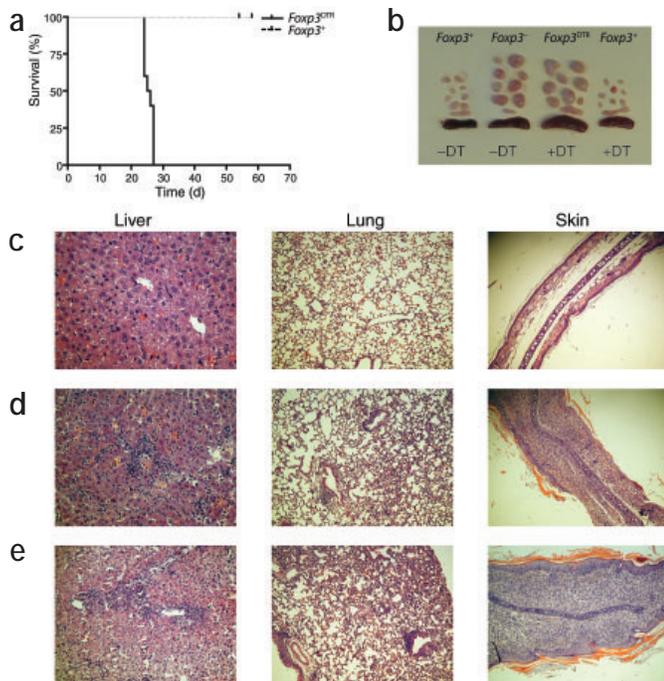


Figure 2 Consequences of chronic T_{reg} cell elimination in neonates. (a) Survival of progeny of $Foxp3^{DTR}$ and $Foxp3^{DTR/+}$ mice injected with diphtheria toxin from birth every other day. Mice were monitored for signs of terminal autoimmune disease and were killed once moribund. (b) Splenomegaly and lymphadenopathy in 3-week-old $Foxp3^{DTR}$ ($n = 10$) and $Foxp3^{+}$ ($n = 6$) littermates treated with diphtheria toxin (+DT) as described in a, and in untreated (-DT) $Foxp3^{-}$ ($n = 5$) and $Foxp3^{+}$ ($n = 4$) littermates. (c–e) Hematoxylin and eosin staining of sections from livers, lungs and skin of 3-week-old $Foxp3^{+}$ mice (c) or $Foxp3^{DTR}$ mice (d) treated with diphtheria toxin as described in a, and of age-matched, untreated $Foxp3^{-}$ mice (e). Original magnification, $\times 25$. Representative data are from two independent experiments.

Because T_{reg} cells suppress the proliferation and activation of $Foxp3^{-}$ nonregulatory T cells, we next determined whether $CD4^{+}$ T cells were hyperactivated in $Foxp3^{DTR}$ mice that were chronically exposed to diphtheria toxin from birth. We compared the activation status of $Foxp3^{-}$ $CD4^{+}$ T cells from diphtheria toxin-treated $Foxp3^{DTR}$ mice with that of cells from diphtheria toxin-treated $Foxp3^{+}$ littermates. $Foxp3^{-}$ $CD4^{+}$ T cells from diphtheria toxin-treated $Foxp3^{DTR}$ mice had higher expression of T cell activation markers, including CD25, CTLA-4 and ICOS, and lower expression of CD62L (indicative of an activated status) than did cells from diphtheria toxin-treated $Foxp3^{+}$ littermates (Fig. 3a). The extent of

T cell activation of $Foxp3^{-}$ $CD4^{+}$ T cells was similar to that of $CD4^{+}$ T cells derived from age-matched $Foxp3^{-}$ mice (Fig. 3b). Activated $Foxp3^{-}$ $CD4^{+}$ T cells from diphtheria toxin-treated $Foxp3^{DTR}$ mice and $Foxp3^{-}$ mice also expressed Ki-67, a marker indicative of proliferation (Fig. 3). $Foxp3^{-}$ $CD4^{+}$ T cells from diphtheria toxin-treated $Foxp3^{+}$ and untreated $Foxp3^{+}$ control mice had low expression of those activation markers, ruling out the possibility of nonspecific T cell activation due to potential contamination of diphtheria toxin preparations with bacterial products. These results collectively showed that elimination of T_{reg} cells in neonates induced an autoimmune syndrome similar to that of $Foxp3^{-}$ mice, with the exception of the difference in the severity of gross skin lesions. These data indicated that self-reactive T cells that failed to commit to the T_{reg} cell lineage were not the only cause of the fatal autoimmune lesions in $Foxp3^{-}$ mice; in wild-type mice, self-reactive T cells present in the 'non- T_{reg} ' $Foxp3^{-}$ T cell subset must be controlled by T_{reg} cells to prevent catastrophic lymphoproliferative disease.

T_{reg} cells and self-tolerance in adult mice

To address the aforementioned possibility of a requirement for T_{reg} cells in setting up and enforcing $Foxp3$ -independent tolerance mechanisms exclusively during neonatal development, we examined the consequences of chronic ablation of T_{reg} cells in 3-month-old mice. Like neonates, diphtheria toxin-treated $Foxp3^{DTR}$ adult mice developed pathology manifested by severe lymphadenopathy and splenomegaly, wasting disease characterized by weight loss, failure to thrive and reduced mobility, and severe conjunctivitis (Fig. 4 and data not shown). Notably, T_{reg} cell elimination in adult nonlymphopenic mice resulted in an even more rapid development of terminal autoimmune disease than in neonates. As early as 10 d after the first injection of diphtheria toxin, some $Foxp3^{DTR}$ mice became moribund, whereas all mice succumbed to terminal disease by 3 weeks of T_{reg} cell ablation (Fig. 4b). Therefore, $Foxp3$ -independent recessive and dominant tolerance mechanisms established in adult mice are not sufficient to protect mice from fatal autoimmunity after T_{reg} cell elimination.

Next we determined the kinetics of $CD4^{+}$ T cell activation after T_{reg} cell ablation. At 2 d after the initial diphtheria toxin treatment of $Foxp3^{DTR}$ mice, we detected an increase of about 2% in the proportion of $CD4^{+}$ cells expressing the T cell activation markers CTLA-4 and CD25 compared with that of control $Foxp3^{+}$ mice (Fig. 5a,b). This result suggested that in normal mice, many non- T_{reg} $CD4^{+}$ T cells were kept in check by T_{reg} cells. The relative and absolute numbers of $CD4^{+}$ T cells with an activated phenotype, including higher expression of CTLA-4 or CD25 and lower expression of CD62L, gradually reached a plateau by 6 d after diphtheria toxin treatment (Fig. 5c,d). At that time, the extent of $CD4^{+}$ T cell activation was similar to that in 3-week-old $Foxp3^{DTR}$ mice subjected to T_{reg} ablation from birth (Fig. 3).

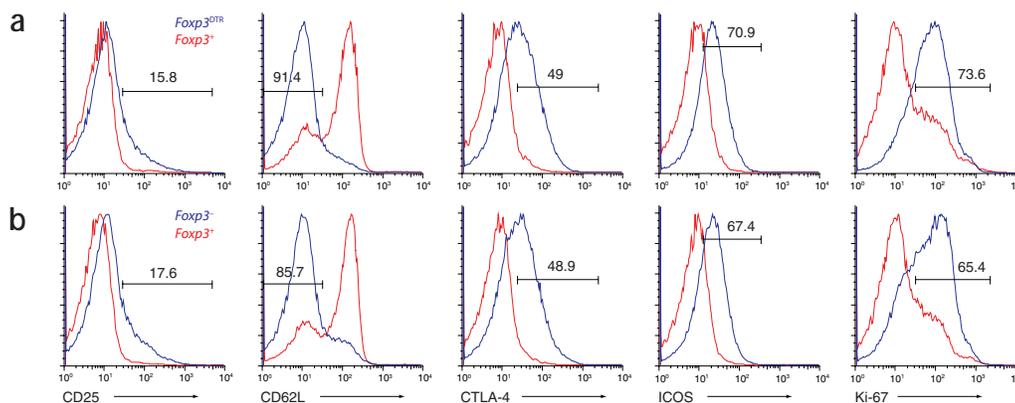


Figure 3 T cell activation in $Foxp3^{-}$ and $Foxp3^{DTR}$ mice depleted of T_{reg} cells from birth.

(a) Expression of activation markers on splenic $CD4^{+}Foxp3^{-}$ T cells from 3-week-old $Foxp3^{DTR}$ mice (blue lines) and $Foxp3^{+}$ mice (red lines) injected with diphtheria toxin from birth every other day. (b) Expression of activation markers on splenic $Foxp3^{-}$ $CD4^{+}$ T cells from age-matched $Foxp3^{+}$ mice (red lines) and $Foxp3^{-}$ mice (blue lines). Numbers above bracketed lines indicate percent cells positive for marker (below histogram). Data are representative of two independent experiments.

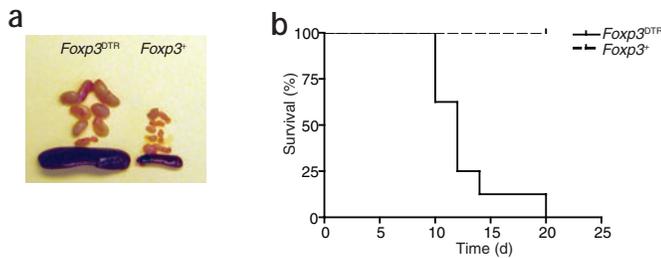


Figure 4 Consequences of chronic T_{reg} cell elimination in adult mice. (a) Lymphadenopathy and splenomegaly of 3- to 5-month-old $Foxp3^{DTR}$ mice ($n = 8$) and $Foxp3^+$ mice ($n = 7$) injected with diphtheria toxin every other day. (b) Survival of $Foxp3^{DTR}$ and $Foxp3^+$ mice treated with diphtheria toxin as described in a. Mice were examined for clinical signs of disease and were killed once moribund. Representative data are from two independent experiments.

Activation of DCs after T_{reg} cell ablation

Next we assessed the status of other immune cell types after T_{reg} cell ablation. At 7 d after diphtheria toxin administration, we noted increases in the numbers of $CD19^+B220^+$ B cells, $F4/80^+CD11c^-$ macrophages, $GR-1^+CD11b^+$ granulocytes and natural killer cells in the spleen and lymph nodes of $Foxp3^{DTR}$ mice (**Table 1**). We also noted an increase in the frequency and an increase of nearly tenfold in the absolute numbers of $CD11c^{hi}$ DCs expressing the major histocompatibility class II molecule $I-A^b$ in the lymph nodes of adult mice (**Fig. 6a** and **Table 1**). The percentages and absolute numbers of splenic $CD11c^{hi}I-A^b+$ cells increased 2.5- and 5-fold, respectively (**Fig. 6a** and **Table 1**). In addition, 3-week-old $Foxp3^{DTR}$ mice treated with diphtheria toxin from birth had a fivefold

higher frequency of and higher absolute numbers of $CD11c^{hi}I-A^b+$ lymph node cells than their $Foxp3^+$ littermates (**Fig. 6b** and data not shown). We detected a similar degree of DC population expansion in age-matched $Foxp3^+$ mice, suggesting that DC dysregulation is a general phenomenon resulting from the absence of T_{reg} cells (**Fig. 6b**). Among $CD11c^+$ cells, the proportion of myeloid $CD11b^+$ DCs increased, whereas the proportion of DCs positive for the plasmacytoid DC marker PDCA-1 decreased after T_{reg} cell elimination (**Fig. 6c**). Nevertheless, both DC subsets increased in absolute numbers (data not shown). Furthermore, there was a moderate but reproducible increase in the expression of DC maturation markers, including CD80 and CD40, on $CD11b^+$ but not PDCA-1 $^+$ DCs (**Fig. 6d**). These results collectively suggested that T_{reg} cells normally restrain $CD11b^+$ DC maturation.

Next we sought to determine whether the increases in DC numbers were caused by enhanced DC survival or proliferation. To test whether T_{reg} cell elimination diminished the apoptosis of $CD11c^+CD11b^+$ DCs, we treated splenic and lymph node cells from $Foxp3^{DTR}$ mice with diphtheria toxin for 7 consecutive days, stained them with fluorescence-tagged annexin V or z-VAD-fmk and analyzed them by flow cytometry. Instead of showing less apoptosis, $CD11c^+CD11b^+$ DCs from diphtheria toxin-treated $Foxp3^{DTR}$ mice showed slightly more apoptosis than DCs from diphtheria toxin-treated $Foxp3^+$ control mice (data not shown). Furthermore, $CD11c^+CD11b^+$ DCs from $Foxp3^{DTR}$ mice did not express the proliferation marker Ki-67, regardless of whether T_{reg} cells were ablated (data not shown).

Because the lack of Ki-67 staining suggested that DCs were mostly non-proliferative after T_{reg} cell elimination, we were able to measure the kinetics of the generation of $CD11c^+CD11b^+$ DCs through continuous labeling

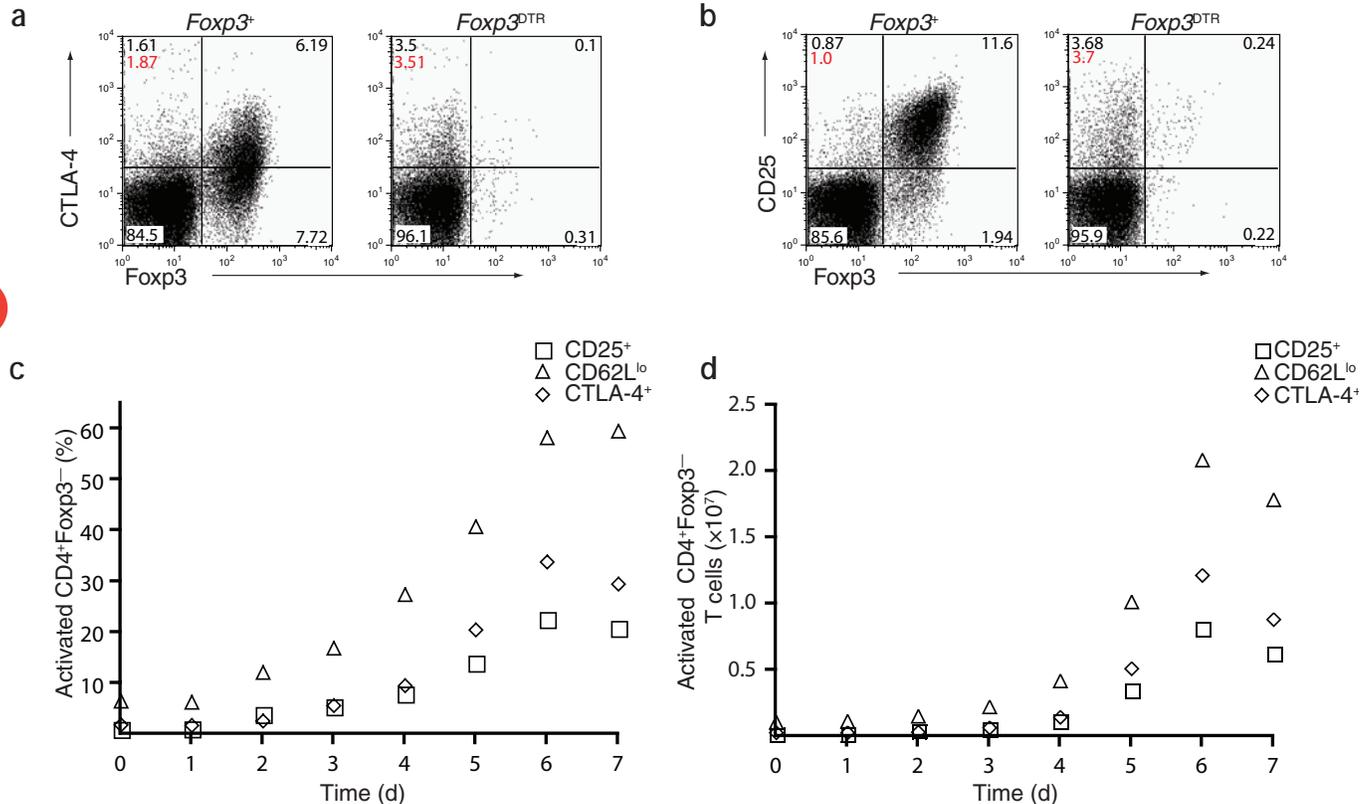


Figure 5 Kinetics of T cell activation in response to acute elimination of T_{reg} cells from adult mice. (a,b) Flow cytometry of intracellular CTLA-4 (a) and surface CD25 (b) expression on lymph node cells isolated from 4- to 5-week-old $Foxp3^{DTR}$ and $Foxp3^+$ mice injected with diphtheria toxin at 0 h and 24 h, assessed 2 d after the initial injection. Red numbers indicate percent $CD4^+Foxp3^-$ T cells expressing CTLA-4 (a) or CD25 (b); black numbers indicate percent of total cells in each quadrant. (c,d) Percentages (c) and absolute numbers (d) of $CD4^+Foxp3^-$ T cells with a $CD25^+$, $CD62L^{lo}$ or CTLA-4 $^+$ phenotype in $Foxp3^{DTR}$ and $Foxp3^+$ mice injected daily with diphtheria toxin as described in a,b. Data are representative of two to three experiments.

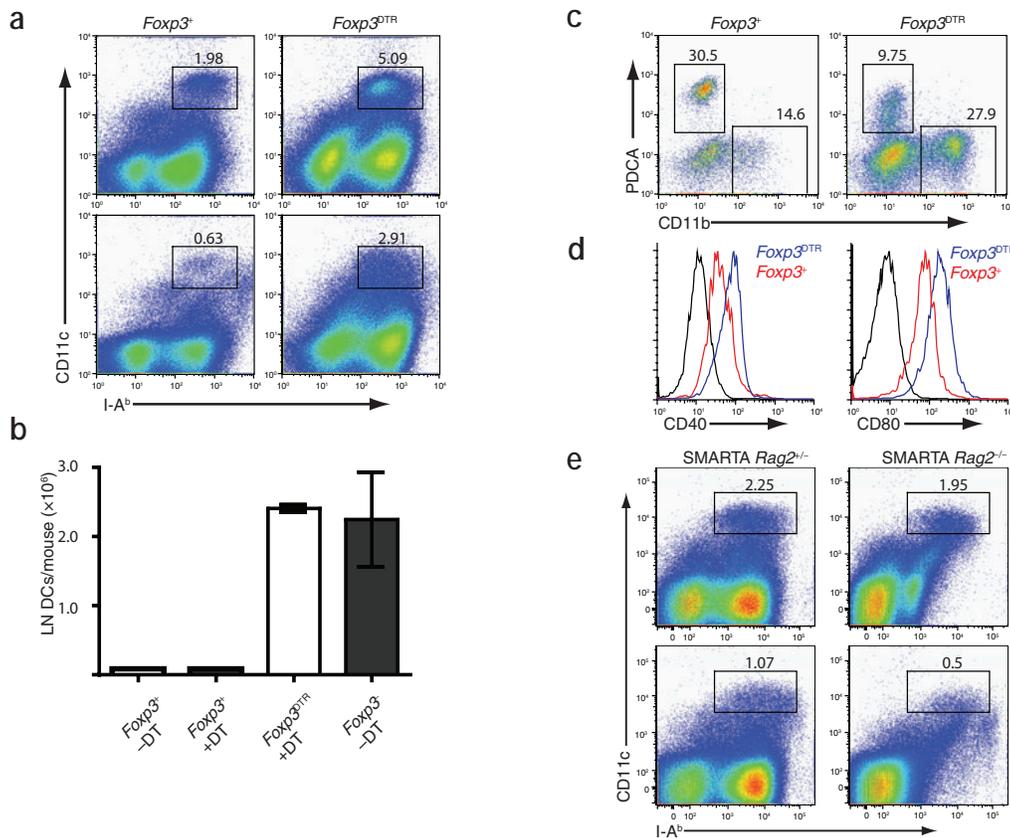


Figure 6 Increase in DC numbers after T_{reg} cell elimination. (a–c) Analysis of splenocytes and lymph node cells from $Foxp3^{DTR}$ and $Foxp3^+$ mice 4–8 weeks of age injected daily with diphtheria toxin. (a) Flow cytometry of splenocytes (top) and lymph node cells (bottom) isolated and stained on day 7 with antibodies specific for CD11c and I- A^b . Numbers above boxed areas indicate percent cells in outlined gate. (b) Absolute numbers of CD11c^{hi}I- A^b ⁺ cells in lymph nodes of 3-week-old $Foxp3^{DTR}$ and $Foxp3^+$ mice treated with diphtheria toxin from birth (+DT), and in age-matched, untreated (–DT) $Foxp3^+$ and $Foxp3^+$ littermates. (c) Expression of CD11b and PDCA-1 on CD11c⁺CD3[–] lymph node cells. (d) Expression of CD40 and CD80 on CD11c^{hi}I- A^b ⁺CD11b⁺ lymph node cells from $Foxp3^{DTR}$ mice (blue lines) and $Foxp3^+$ mice (red lines). Black lines, isotype control. (e) CD11c and I- A^b expression on splenocytes (top) and lymph node cells (bottom) from SMARTA $Rag2^{+/-}$ and SMARTA $Rag2^{-/-}$ mice (two to three mice per group). Numbers above boxed areas indicate percent cells in outlined gate. Representative data are from two independent experiments.

with 5-bromodeoxyuridine (BrdU). In agreement with published reports, in control mice, approximately 50% of CD11b⁺ DCs incorporated BrdU after 3 d of labeling²² (**Supplementary Fig. 2** online). Between the days 2 and 3 of diphtheria toxin treatment, we detected a 20% increase in the percentage of BrdU⁺ CD11b⁺ DCs in $Foxp3^{DTR}$ mice. The frequency of BrdU⁺ CD11b⁺ DCs progressively increased throughout the time course,

suggesting more efficient generation of CD11b⁺ DCs from precursors after ablation of T_{reg} cells.

In tissue culture studies, T_{reg} cells have been shown to inhibit the maturation and to alter the cytokine production of DCs^{23–26}. Alternatively, T_{reg} cells may indirectly affect DC maturation by suppressing self-reactive CD4⁺ T cells that themselves induce the maturation of DCs. Consistent with the

latter possibility, activated T cells generated after T_{reg} cell elimination expressed GM-CSF, interleukin 13, RANKL and CD40L, cell surface molecules that promote DC development and maturation (**Supplementary Fig. 3** online). In addition, we detected no DC activation in SMARTA TCR-transgenic mice on the $Rag2^{-/-}$ (recombination-activating gene 2–deficient) genetic background (**Fig. 6e**). As these mice lack $Foxp3^+$ T_{reg} cells and all T cells express a single TCR specific for the lymphocytic choriomeningitis virus glycoprotein 33 epitope, the absence of T_{reg} cells itself was not sufficient to trigger DC activation.

DISCUSSION

The identification of mutations in the gene encoding *Foxp3* as the cause of aggressive autoimmunity in human patients with IPEX syndrome (immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) and in the mutant mouse strain *scurfy* and the subsequent discovery of the essential function of *Foxp3* in the development of T_{reg} cells have provided a genetic foundation for the phenomenon of T_{reg} cell–mediated dominant tolerance^{1–7}. Those results suggest that T_{reg} cells are vital in preventing autoimmunity. However, T_{reg} cells express self-reactive TCRs, and TCR–self ligand interactions in a

Table 1 Analysis of expanded cell subsets in response to T_{reg} cell elimination

	Lymph node			Spleen		
	<i>Foxp3</i> ⁺	<i>Foxp3</i> ^{DTR}	'Fold change'	<i>Foxp3</i> ⁺	<i>Foxp3</i> ^{DTR}	'Fold change'
CD19 ⁺ B220 ⁺	4.43 × 10 ⁷ ± 1.66 × 10 ⁷	1.39 × 10 ⁸ ± 3.72 × 10 ⁷	3.13	8.52 × 10 ⁷ ± 1.49 × 10 ⁷	1.52 × 10 ⁸ ± 4.89 × 10 ⁷	1.78
TCRβ ⁺ CD4 ⁺	3.76 × 10 ⁷ ± 1.28 × 10 ⁷	7.97 × 10 ⁷ ± 2.07 × 10 ⁷	2.12	2.45 × 10 ⁷ ± 3.77 × 10 ⁶	5.65 × 10 ⁷ ± 1.33 × 10 ⁷	2.31
TCRβ ⁺ CD8 ⁺	2.12 × 10 ⁷ ± 7.51 × 10 ⁶	6.45 × 10 ⁷ ± 2.15 × 10 ⁷	3.05	1.40 × 10 ⁷ ± 2.36 × 10 ⁶	4.71 × 10 ⁷ ± 2.44 × 10 ⁷	3.37
CD11c ^{hi} I- A^b ⁺	6.36 × 10 ⁵ ± 1.58 × 10 ⁵	6.24 × 10 ⁶ ± 2.76 × 10 ⁶	9.81	2.57 × 10 ⁶ ± 9.19 × 10 ⁵	1.27 × 10 ⁷ ± 3.89 × 10 ⁶	4.93
GR-1 ^{hi} CD11b ⁺	1.46 × 10 ⁵ ± 8.82 × 10 ⁴	2.69 × 10 ⁶ ± 1.78 × 10 ⁶	18.44	5.49 × 10 ⁶ ± 2.19 × 10 ⁶	2.89 × 10 ⁷ ± 1.18 × 10 ⁷	5.26
F4/80 ⁺ CD11c [–]	7.22 × 10 ⁵ ± 2.25 × 10 ⁵ (n = 4)	7.12 × 10 ⁶ ± 5.86 × 10 ⁵ (n = 3)	9.86	1.44 × 10 ⁷ ± 1.47 × 10 ⁶ (n = 4)	6.23 × 10 ⁷ ± 1.72 × 10 ⁷ (n = 3)	4.34
NK1.1 ⁺	1.07 × 10 ⁶ ± 3.12 × 10 ⁵	7.83 × 10 ⁶ ± 2.72 × 10 ⁶	7.3	4.81 × 10 ⁶ ± 1.46 × 10 ⁶	1.98 × 10 ⁷ ± 7.96 × 10 ⁶	4.13

Lymph node cells and splenocytes were isolated from $Foxp3^+$ and $Foxp3^{DTR}$ mice after 7 d of T_{reg} cell elimination by digestion with collagenase D. Mean ± s.d. values are based on $Foxp3^+$ mice (n = 6) and $Foxp3^{DTR}$ mice (n = 5) unless otherwise noted.

certain range of increased affinity facilitate T_{reg} cell development in the thymus^{12,27–30}. Furthermore, many T cells with activated but not naive phenotypes in *Foxp3*⁻ mice use TCRs expressed by T_{reg} cells in wild-type mice¹³. Those results raise the possibility that commitment of self-reactive thymocytes escaping negative selection to the T_{reg} cell lineage ‘neutralizes’ most potentially pathogenic self-reactive T cells. That scenario indicates that in wild-type mice, T_{reg} cells control relatively few *Foxp3*⁻ T cells that are reactive to self or environmental antigens and that elimination of T_{reg} cells would result in slowly progressing autoimmunity accompanied by mild lesions, in contrast to the severe and rapid autoimmunity in *Foxp3*⁻ mice.

According to that idea, autoimmunity induced after ablation of *Foxp3*⁺ T_{reg} cells in wild-type mice would be similar to that in thymectomized mice at day 3 or in lymphopenic recipients of *Foxp3*⁻ CD4⁺ T cells. However, our analysis of T_{reg} cell ablation in *Foxp3*^{DTR} mice showed that the continuous presence of T_{reg} cells in ‘normal’ mice in physiological conditions was needed to prevent the devastating lymphoproliferative disease resembling that of *Foxp3*⁻ mice.

Notably, the disease induced after the elimination of T_{reg} cells in adult mice was even more aggressive than that in neonates subjected to T_{reg} cell ablation. We propose that this is at least partially due to the fact that overall, the T cell compartment, including self-reactive T cells, is much larger in adult mice than in neonates. Those results exclude the possibility that T_{reg} cells, by limiting the activation and population expansion of self-reactive T cells facilitated by transient lymphopenia inherent to neonates, are critical early in life but not during adulthood¹⁴. Furthermore, our findings emphasize the inability of recessive tolerance mechanisms established in unmanipulated adult mice (such as central and peripheral anergy) to restrain catastrophic autoimmunity beginning after T_{reg} cells are eliminated. In addition to *Foxp3*⁺ T_{reg} cells, several T cell subsets, including interleukin 10–producing suppressive *Foxp3*⁻ T cells, are reported to efficiently inhibit T cell responses to foreign and self antigens^{31,32}. It has also been proposed that CD4⁺CD25⁺ T_{reg} cells mediate suppression by generating at least some of those suppressive *Foxp3*⁻ T cells³³. A corollary to that model is that ablation of T_{reg} cells in adults should result in a less aggressive and severe disease than that in neonates. However, the opposite trend observed indicates the inability of *Foxp3*⁻ T_{reg} cells to restrain autoimmunity on their own.

Early studies indicated that the thymus is central to T_{reg} cell differentiation^{34,35}. Subsequently, it has been proposed that ‘nonregulatory’ *Foxp3*⁻ T cells specific to self or environmental antigens can also upregulate *Foxp3* and acquire regulatory properties in the periphery^{36–39}. However, our observation of very fast regeneration of thymic *Foxp3*⁺ subset after transient ablation of both the thymic and peripheral T_{reg} compartments, followed, with a considerable delay, by the peripheral *Foxp3*⁺ T_{reg} subset, indicates that the thymus serves as a chief source of T_{reg} cell development even when the peripheral T_{reg} cell ‘niche’ is emptied after acute T_{reg} cell ablation.

An extremely fast pace of deterioration of mice subjected to T_{reg} cell ablation indicates that a very high proportion of normal CD4⁺ T cell repertoire is controlled by T_{reg} cells. That idea is supported by the finding of a doubling of the proportion of peripheral T cells expressing activation markers after less than 48 h of administration of a T_{reg} cell ablation regimen. In addition to T cell activation, a notable consequence of T_{reg} cell ablation was the increase in DC numbers, demonstrating that T_{reg} cells control DC cell numbers *in vivo*. Reports have suggested that T_{reg} cells kill immature myeloid DC in a granzyme-dependent way *in vitro*; however, we failed to find evidence to support that idea, as neither DC apoptosis nor survival was altered after T_{reg} cell ablation⁴⁰. Instead, *in vivo* BrdU labeling experiments showed that the rate of CD11c⁺CD11b⁺ DC generation was substantially accelerated after ablation of T_{reg} cells.

We also noted an increase in the expression of activation markers on DCs. That result is in apparent agreement with *in vitro* studies proposing

that T_{reg} cells control T cell activation by suppression of DC activation^{23–26}. In addition, imaging studies have suggested that T_{reg} cells diminish the ability of DCs to form stable contacts with self-reactive T cells and thereby diminish their activation^{41,42}. Our data have not definitively indicated that T cells or DCs or both of those cell subsets are primary targets of T_{reg} cells *in vivo*. Nevertheless, we propose that the DC maturation is probably the result of very early activation of CD4⁺ T cells reactive to self and environmental antigens induced after the ablation of T_{reg} cells. The idea of dependence of DC activation and population expansion in the presence of self-reactive T cells is consistent with our observation that DCs failed to expand their populations and did not have an activated phenotype in TCR-transgenic *Rag2*-deficient mice lacking T_{reg} cells. In agreement with those results, diphtheria toxin-induced ablation of T_{reg} cells combined with antibody-mediated depletion of total CD4 T cell subset prevented the expansion of DC numbers (J.M.K. and A.Y.R., unpublished observations). Furthermore, T_{reg} cell elimination triggered the production of cytokines such as GM-CSF, which is known to promote DC differentiation, by CD4⁺ T cells. Although the mechanism of T_{reg} cell-mediated suppression of DC activation remains unclear and its elucidation requires further investigation, we propose that *Foxp3*⁺ T_{reg} cells may indirectly influence DC dynamics by suppressing self-reactive CD4⁺ T cells.

METHODS

Mice. Mice with germline deletion of *Foxp3* on a C57BL/6 background (*Foxp3*⁻ mice) have been described¹. C57BL/6 mice (*Foxp3*⁺) were purchased from Jackson Laboratories. All mice were bred and maintained in specific pathogen-free conditions in the animal facility at University of Washington in accordance with the institutional guidelines. The targeting construct for *Foxp3*^{DTR} mice was generated by subcloning of a 7.9-kilobase *Xba*I fragment of *Foxp3* containing exons 6–11 from a 30.8-kilobase cosmid containing the complete *Foxp3* gene¹ into a pBluescript vector containing a PGK-DTA negative-selection cassette. A *Sal*I restriction site was engineered in place of the *Bae*I site in the 3′ untranslated region of *Foxp3* upstream of the polyadenylation signal. The targeting construct was generated by first cloning of the DTR-GFP cDNA from the CD11c-DTR-GFP plasmid by PCR amplification into the IRES-MCS-BGHpA-FRT-NEO-FRT shuttle vector. The IRES-DTR-GFP fragment was then cloned into the *Sal*I site inserted into *Foxp3*. The linearized targeting construct was introduced by electroporation into R1 embryonic stem cells and neomycin-resistant clones were screened by PCR across the 3′ arm for evidence of homologous recombination. *Bsp*HI-digested genomic DNA of positive clones were then screened by Southern blot. Embryonic stem cell clones bearing the correctly targeted locus were injected into C57BL/6 blastocysts, and chimeric male offspring were mated to ‘FLP-deleter’ mice for excision of the PGK-Neo cassette. Transmission of the targeted allele was confirmed by PCR.

Cell isolation. Single-cell suspensions from lymph nodes (superficial cervical, mandibular, axillary, lateral axillary, superficial inguinal and mesenteric), spleen and thymus were generated by mechanical disruption. For DC studies, tissue samples were minced in 5% (volume/volume) FCS in RPMI medium containing 2 mg/ml of collagenase D (Roche) and 20 μg/ml of DNaseI (Roche). Collagenase digestion was done for 30 min at 37 °C and single-cell suspensions were prepared by passage of cells through a filter with a pore size of 100 μm (BD Falcon).

Reagents and flow cytometry. Diphtheria toxin was purchased from Sigma and was reconstituted according to the manufacturer’s protocol. Frozen diphtheria toxin stocks were frozen and thawed once and 50 μg/kg of diphtheria toxin was injected intraperitoneally unless otherwise noted. Phycoerythrin-conjugated antibody to CD25 (anti-CD25; PC61), allophycocyanin-anti-*Foxp3* (FJK-16S), allophycocyanin-anti-CD11c (N418), phycoerythrin-anti-CD80 (16-10A1), phycoerythrin-anti-CD40L (MR1), anti-RANKL (IK22/5), allophycocyanin-anti-CD19 (MB19-1) and allophycocyanin-anti-F4/80 (BM8) were purchased from eBioscience; phycoerythrin-, FITC- or biotin-anti-CD11b (M1/70), phycoerythrin- or

biotin-anti-CD11c (HL3), peridinin chlorophyll protein-anti-CD4 (L3T4), phycoerythrin-anti-CD8 α (53-6.7), phycoerythrin- or FITC-anti-I-A^b (AF6-120), FITC-anti-CD40 (HM 40.3), phycoerythrin- or FITC-anti-TCR β (H57-597), phycoerythrin-anti-CTLA4 (UC10-4F10-11), phycoerythrin-anti-ICOS (7E.17G9), phycoerythrin-anti-CD62L (MEL14), peridinin chlorophyll protein-CD3 (2C11), allophycocyanin-annexin V, anti-BrdU, phycoerythrin-anti-Ki-67, phycoerythrin- or FITC-anti-CD45R (B220) and FITC-anti-Gr-1 were purchased from BD Pharmingen; biotin-anti-HB-EGF (anti-DTR; BAF259) was from R&D Systems; FITC-z-VAD-fmk was from Promega; and biotin-anti-mPDCA-1 was from Miltenyi Biotec. For annexin V, z-VAD-fmk and intracellular anti-Foxp3 staining, cells were stained according to the manufacturer's instructions. Cells were analyzed on a FACSCanto (BD Biosciences) with FloJo software (Tree Star).

Cytokine analysis. CD4⁺ cells were purified with anti-CD4 beads and an autoMACs (Miltenyi Biotec). Sorted CD4⁺ cells (1×10^6) were stimulated for 14 h with 50 ng/ml of phorbol 12-myristate 13-acetate and 200 ng/ml of ionomycin and supernatants were analyzed with a cytokine array (Allied Biotech). Interleukin 13 was measured by enzyme-linked immunosorbent assay (R&D Systems).

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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