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\(^1\). 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\(^6,7\). 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\(^8\)\(^\text{–}^{11}\).

One potential explanation for this 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 mice\(^13\). 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 lymphopenia\(^14\). During that period, deletional central tolerance might be relatively relaxed because of an immature thymic medullary compartment in which most negative selection begins\(^15\). 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\(_\text{i}\) 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
Figure 1 Characterization of inducible Treg 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 Foxp3DTR+ 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 Foxp3DTR+ and Foxp3+ mice given two injections of diphtheria toxin (50 μg/kg) on consecutive days. Each data point (± 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 Treg cells was achieved after 7 d of treatment with 50 μg diphtheria toxin per kg body weight (50 μg/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 Foxp3DTR+ mice or diphtheria toxin–treated Foxp3+ control mice (Fig. 1c). The extent of Treg 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 Treg cell recovery after acute Treg 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 Treg cell numbers in Foxp3+ littermates, but near-complete elimination of Treg cells was achieved in the thymus, lymph nodes and spleen 2 d after the initial injection (Fig. 1c). On day 4, thymic Treg cells rebounded to 47% of their numbers before treatment, whereas peripheral Treg 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+ Treg 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 Treg cells, further studies are needed to address that important issue.

**Autoimmunity after neonatal Treg cell ablation**

Next we compared the autoimmune pathology developing in mice subjected to Foxp3+ Treg cell elimination at birth with that in mice with germine deletion of Foxp3. We injected Foxp3DTR+ litterers with 50 μg/kg of diphtheria toxin beginning 12 h after birth and continuing every other day thereafter. By 24 d after birth, approximately 40% of Foxp3DTR+ mice had a moribund phenotype, including failure to thrive, lack of mobility, ventral skin lesions, hunched posture and joint contractures; 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 Foxp3DTR 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 Foxp3DTR mice was reminiscent of that of Foxp3+ mice.11 One notable difference in diphtheria toxin–treated Foxp3DTR versus Foxp3+ mice was the presence of severe tail scaling only in Foxp3+ mice (data not shown).

Both diphtheria toxin–treated Foxp3DTR 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 Foxp3DTR 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′ untranscribed region of Foxp3 to produce Foxp3DTR+ (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 Foxp3DTR+ female mice would be expected to express GFP because of random X-chromosome inactivation. Accordingly, approximately 50% of the Foxp3+ peripheral Treg cells in Foxp3DTR+ 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 Treg cells expressing the knock-in allele. Furthermore, unmanipulated Foxp3DTR+ 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 Treg cell elimination, we treated Foxp3DTR mice and wild-type (Foxp3+) littermates with daily intraperitoneal injections of 50, 5.0 or 0.5 μg 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 cells.20 Diphtheria toxin did not affect the frequency of Treg cells in control Foxp3+ littermates at any dose tested, but it resulted in a dose-dependent decrease in Treg cell numbers in the lymph nodes, peripheral blood, thymuses and spleens of Foxp3DTR mice
T cell activation of Foxp3+ CD4+ T cells was similar to that of CD4+ T cells derived from age-matched Foxp3-deficient mice (Fig. 3b). Activated Foxp3+ CD4+ T cells from diphtheria toxin–treated Foxp3+DT mice and Foxp3-deficient mice also expressed Ki-67, a marker indicative of proliferation (Fig. 3). Foxp3+ CD4+ T cells from diphtheria toxin–treated Foxp3+DT 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 Treg 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 Treg 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−Treg Foxp3+ T cell subset must be controlled by Treg cells to prevent catastrophic lymphoproliferative disease.

**Treg cells and self-tolerance in adult mice**

To address the aforementioned possibility of a requirement for Treg cells in setting up and enforcing Foxp3-independent tolerance mechanisms exclusively during neonatal development, we examined the consequences of chronic ablation of Treg cells in 3-month-old mice. Like neonates, diphtheria toxin–treated Foxp3+DT 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, Treg 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+DT mice became moribund, whereas all mice succumbed to terminal disease by 3 weeks of Treg 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 Treg cell elimination.

Next we determined the kinetics of CD4+ T cell activation after Treg cell ablation. At 2 d after the initial diphtheria toxin treatment of Foxp3+DT 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−Treg CD4+ T cells were kept in check by Treg 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+DT mice subjected to Treg ablation from birth (Fig. 3).
Figure 4 Consequences of chronic T<sub>reg</sub> cell elimination in adult mice. (a) Lymphadenopathy and splenomegaly of 3- to 5-month-old Foxp<sup>3</sup>TR mice (n = 8) and Foxp<sup>3</sup> mice (n = 7) injected with diphtheria toxin every other day. (b) Survival of Foxp<sup>3</sup>TR and Foxp<sup>3</sup> 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<sub>reg</sub> cell ablation

Next we assessed the status of other immune cell types after T<sub>reg</sub> cell ablation. At 7 d after diphtheria toxin administration, we noted increases in the numbers of CD19<sup>+</sup>B220<sup>+</sup> B cells, F4/80<sup>+</sup>CD11c<sup>+</sup> macrophages, GR-1<sup>+</sup>CD11b<sup>+</sup> granulocytes and natural killer cells in the spleen and lymph nodes of Foxp<sup>3</sup>TR mice (Table 1). We also noted an increase in the frequency and an increase of nearly tenfold in the absolute numbers of CD11c<sup>+</sup> DCs expressing the major histocompatibility class II molecule I-A<sup>d</sup> in the lymph nodes of adult mice (Fig. 6a and Table 1). The percentages and absolute numbers of splenic CD11c<sup>+</sup>-I-A<sup>d</sup> cells increased 2.5- and 5-fold, respectively (Fig. 6a and Table 1). In addition, 3-week-old Foxp<sup>3</sup>TR mice treated with diphtheria toxin from birth had a fivefold higher frequency of and higher absolute numbers of CD11c<sup>+</sup>-I-A<sup>d</sup> lymph node cells than their Foxp<sup>3</sup> littermates (Fig. 6b and data not shown). We detected a similar degree of DC population expansion in age-matched Foxp<sup>3</sup> mice, suggesting that DC dysregulation is a general phenomenon resulting from the absence of T<sub>reg</sub> cells (Fig. 6b). Among CD11c<sup>+</sup> cells, the proportion of myeloid CD11b<sup>+</sup> DCs increased, whereas the proportion of DCs positive for the plasmacytoid DC marker PDCA-1 decreased after T<sub>reg</sub> 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<sup>+</sup> not PDCA-1<sup>+</sup> DCs (Fig. 6d). These results collectively suggested that T<sub>reg</sub> cells normally restrain CD11b<sup>+</sup> 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<sub>reg</sub> cell elimination diminished the apoptosis of CD11c<sup>+</sup>CD11b<sup>+</sup> DCs, we treated splenic and lymph node cells from Foxp<sup>3</sup>TR 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<sup>+</sup>CD11b<sup>+</sup> DCs from diphtheria toxin–treated Foxp<sup>3</sup>TR mice showed slightly more apoptosis than DCs from diphtheria toxin–treated Foxp<sup>3</sup> control mice (data not shown). Furthermore, CD11c<sup>+</sup>CD11b<sup>+</sup> DCs from Foxp<sup>3</sup>TR mice did not express the proliferation marker Ki-67, regardless of whether T<sub>reg</sub> cells were ablated (data not shown).

Because the lack of Ki-67 staining suggested that DCs were mostly non-proliferative after T<sub>reg</sub> cell elimination, we were able to measure the kinetics of the generation of CD11c<sup>+</sup>CD11b<sup>+</sup> DCs through continuous labeling.

Figure 5 Kinetics of T cell activation in response to acute elimination of T<sub>reg</sub> 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 Foxp<sup>3</sup>TR and Foxp<sup>3</sup> mice injected with diphtheria at 0 h and 24 h, assessed 2 d after the initial injection. Red numbers indicate percent CD4<sup>+</sup>Foxp<sup>3</sup> 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<sup>+</sup>Foxp<sup>3</sup> T cells with a CD25<sup>+</sup>, CD62L<sup>lo</sup> or CTLA-4<sup>+</sup> phenotype in Foxp<sup>3</sup>TR and Foxp<sup>3</sup> mice injected daily with diphtheria toxin as described in a,b. Data are representative of two to three experiments.
with 5-bromodeoxyuridine (BrdU). In agreement with published reports, in control mice, approximately 50% of CD11b+ DCs incorporated BrdU after 3 d of labeling (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 Foxp3ΔTIR 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 Treg cells.

In tissue culture studies, Treg cells have been shown to inhibit the maturation and to alter the cytokine production of DCs23-26. Alternatively, Treg 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 Treg 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+ Treg cells and all T cells express a single TCR specific for the lymphocytic choriomeningitis virus glycoprotein 33 epitope, the absence of Treg 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 Treg cells have provided a genetic foundation for the phenomenon of Treg cell-mediated dominant tolerance1-7. Those results suggest that Treg cells are vital in preventing autoimmunity. However, Treg cells express self-reactive TCRs, and TCR–self ligand interactions in a
certain range of increased affinity facilitate T<sub>reg</sub> cell development in the thymus.<sup>12-27,30</sup> Furthermore, many T cells with activated but not naive phenotypes in Foxp3<sup>+</sup> mice use TCRs expressed by T<sub>reg</sub> cells in wild-type mice.<sup>13</sup> Those results raise the possibility that commitment of self-reactive thymocytes escaping negative selection to the T<sub>reg</sub> cell lineage `neutralizes' most potentially pathogenic self-reactive T cells. That scenario indicates that in wild-type mice, T<sub>reg</sub> cells control relatively few Foxp3<sup>+</sup> T cells that are reactive to self or environmental antigens and that elimination of T<sub>reg</sub> cells would result in slowly progressing autoimmunity accompanied by mild lesions, in contrast to the severe and rapid autoimmunity in Foxp3<sup>−/−</sup> mice. According to that idea, autoimmunity induced after ablation of Foxp3<sup>+</sup> T<sub>reg</sub> cells in wild-type mice would be similar to that in thymanectomized mice at day 3 or in lymphopenic recipients of Foxp3<sup>+</sup> CD4<sup>+</sup> T cells. However, our analysis of T<sub>reg</sub> cell ablation in Foxp3<sup>−/−</sup> mice showed that the continuous presence of T<sub>reg</sub> cells in `normal' mice in physiological conditions was needed to prevent the devastating lymphoproliferative disease resembling that of Foxp3<sup>−/−</sup> mice. Notably, the disease induced after the elimination of T<sub>reg</sub> cells in adult mice was even more aggressive than that in neonates subjected to T<sub>reg</sub> 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<sub>reg</sub> 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 adulthood.<sup>14</sup> Furthermore, our findings emphasize the inability of reactive tolerance mechanisms established in unmanipulated adult mice (such as central and peripheral anergy) to restrain catastrophic autoimmunity beginning after T<sub>reg</sub> cells are eliminated. In addition to Foxp3<sup>−/−</sup> T<sub>reg</sub> cells, several T cell subsets, including interleukin 10–producing suppressive Foxp3<sup>+</sup> T<sub>reg</sub> cells, are report to efficiently inhibit T cell responses to foreign and self-antigens<sup>31-32</sup>. It has also been proposed that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells mediate suppression by generating at least some of those suppressive Foxp3<sup>+</sup> T<sub>reg</sub> cells<sup>33,34</sup>. A corollary to that model is that ablation of T<sub>reg</sub> 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<sup>+</sup> T<sub>reg</sub> cells to restrain autoimmunity on their own. Early studies indicated that the thymus is central to T<sub>reg</sub> cell differentiation<sup>34,35</sup>. Subsequently, it has been proposed that nonregulatory Foxp3<sup>+</sup> T cells specific to self or environmental antigens can also upregulate Foxp3<sup>+</sup> and acquire regulatory properties in the periphery.<sup>36-38</sup> However, our observation of very fast regeneration of thymic Foxp3<sup>−/−</sup> subset after transient ablation of both the thymic and peripheral T<sub>reg</sub> compartments, followed, with a considerable delay, by the peripheral Foxp3<sup>+</sup> T<sub>reg</sub> subset, indicates that the thymus serves as a chief source of T<sub>reg</sub> cell development even when the peripheral T<sub>reg</sub> cell `niche' is emptied after acute T<sub>reg</sub> cell ablation. An extremely fast pace of deterioration of mice subjected to T<sub>reg</sub> cell ablation indicates that a very high proportion of normal CD4<sup>+</sup> T cell repertoire is controlled by T<sub>reg</sub> 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<sub>reg</sub> cell ablation regimen. In addition to T cell activation, a notable consequence of T<sub>reg</sub> cell ablation was the increase in DC numbers, demonstrating that T<sub>reg</sub> cells control DC cell numbers in vivo. Reports have suggested that T<sub>reg</sub> 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<sub>reg</sub> cell ablation<sup>39</sup>. Instead, in vitro BrdU labeling experiments showed that the rate of CD11c<sup>+</sup>CD11b<sup>+</sup> DC generation was substantially accelerated after ablution of T<sub>reg</sub> 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<sub>reg</sub> cells control T cell activation by suppression of DC activation<sup>23-26</sup>. In addition, imaging studies have suggested that T<sub>reg</sub> cells diminish the ability of DCs to form stable contacts with self-reactive T cells and thereby diminish their activation<sup>41,42</sup>. Our data have not definitively indicated that T cells or DCs or both of those cell subsets are primary targets of T<sub>reg</sub> cells in vivo. Nevertheless, we propose that the DC maturation is probably the result of very early activation of CD4<sup>+</sup> T cells reactive to self and environmental antigens induced after the ablution of T<sub>reg</sub> 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<sub>reg</sub> cells. In agreement with those results, diphtheria toxin–induced ablation of T<sub>reg</sub> 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<sub>reg</sub> cell elimination triggered the production of cytokines such as GM-CSF, which is known to promote DC differentiation, by CD4<sup>+</sup> T cells. Although the mechanism of T<sub>reg</sub> cell-mediated suppression of DC activation remains unclear and its elucidation requires further investigation, we propose that Foxp3<sup>+</sup> T<sub>reg</sub> cells may indirectly influence DC dynamics by suppressing self-reactive CD4<sup>+</sup> T cells.

**METHODS**

Mice. Mice with germline deletion of Foxp3 on a C57BL/6 background (Foxp3<sup>−/−</sup> mice) have been described.<sup>1</sup> C57BL/6 mice (Foxp3<sup>+</sup>) 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<sup>−/−</sup> mice was generated by subcloning of a 7.9-kilobase XbaI fragment of Foxp3 containing exons 6–11 from a 30.8-kilobase cosmid containing the complete Foxp3 gene<sup>1</sup> into a pBluescript vector containing a PGK-DTA negative-selection cassette. A SalI restriction site was engineered in place of the Bax 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 SalI 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. BsPHI-digested genomic DNA of positive clones were then screened by Southern blot. Embryonic stem cell clones bearing the correctly targeted loci 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 DNase (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-165), 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