The adaptor TRAF3 restrains the lineage determination of thymic regulatory T cells by modulating signaling via the receptor for IL-2

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The number of Foxp3+ regulatory T cells (Treg cells) must be tightly controlled for efficient suppression of autoimmunity with no impairment of normal immune responses. Here we found that the adaptor TRAF3 was intrinsically required for restraining the lineage determination of thymic Treg cells. T cell–specific deficiency in TRAF3 resulted in a two- to threefold greater frequency of Treg cells, due to the more efficient transition of precursors of Treg cells into Foxp3+ Treg cells. TRAF3 dampened interleukin 2 (IL-2) signaling by facilitating recruitment of the tyrosine phosphatase TCPTP to the IL-2 receptor complex, which resulted in dephosphorylation of the signaling molecules Jak1 and Jak3 and negative regulation of signaling via Jak and the transcription factor STAT5. Our results identify a role for TRAF3 as an important negative regulator of signaling via the IL-2 receptor that affects the development of Treg cells.

Tight regulation of the Foxp3+ regulatory T cell (Treg cell) population in immunity is crucial for avoiding pathogenic autoreactivity while ensuring effective protection against infectious diseases and tumor cells1. Signaling mediated by the receptor for interleukin 2 (IL-2R) is a major mechanism for controlling the development and homeostasis of Treg cells and has been widely investigated2–4. The binding of IL-2 to IL-2R activates at least three distinct signaling pathways. The signaling molecules Jak1 and Jak3, which are associated with CD122 (IL-2Rβ) and CD132 (the common γ-chain), respectively, phosphorylate each other and also IL-2Rβ and the transcription factor STAT5 (refs. 5,6). Phosphorylated STAT5 binds to the promoter and first intron of the gene encoding the transcription factor Foxp3 and is essential for initiating Foxp3 expression7,8. IL-2 also activates signaling pathways of phosphatidylinositol-3-OH kinase (PI(3)K) and the kinase Akt and of the GTPase Ras and mitogen-activated protein kinases (MAPKs). However, in contrast to STAT5, which can be directly phosphorylated by Jak3, additional intermediate molecules, such as Shc, Syk and Lck, are required for activation of the PI(3)K-Akt and Ras-MAPK pathways7,9,10. Several negative regulatory mechanisms are involved in restraining IL-2-mediated signaling. The signaling suppressors SOCS1 and SOCS3 provide negative feedback to signaling via IL-2 by associating with Jak1 and inhibiting its kinase activity11,12. The phosphatase SHP-1 dephosphorylates Jak1 and negatively regulates IL-2R–Jak1 signaling13. The tyrosine phosphatase TCPTP can also directly interact with Jak1 and Jak3 and dephosphorylate these molecules upon stimulation with IL-2 or interferon-γ (IFN-γ)14. TCPTP’s expression is ubiquitous but is higher in cells of hematopoietic origin15. The importance of TCPTP in cytokine signaling is demonstrated in vivo by TCPTP-deficient mice, which develop a severely proinflammatory phenotype and die at 3–5 weeks of age16. Notably, Treg cells are moderately more abundant in mice with T cell–specific deficiency in TCPTP17.

The adaptor TRAF3 (‘tumor-necrosis factor (TNF) receptor–associated factor 3’) participates in signaling by many members of the TNF receptor superfamily, as well as receptors of the innate immune system and the IL-17 receptor18–20. Published studies have indicated that the roles of TRAF3 are highly dependent on the cell type and receptor21. The functions regulated by TRAF3 in T cells have been examined less extensively than have those in B cells. T cell–specific deficiency in TRAF3, although it has no detectable effect on the development of conventional T cells (Tcon cells), results in diminished effector functions and impaired signaling via the T cell antigen receptor (TCR) in peripheral CD4+ T cells and CD8+ T cells22. Deficiency in TRAF3 also results in defective development and function of invariant natural killer T cells23. Another study has indicated that Treg cell–specific expression of TRAF3 is required for the induction of follicular Treg cells24. Therefore, TRAF3 has distinct roles in different T cell subsets. In the present study, we examined the molecular mechanisms by which T cell–specific deficiency in TRAF3 in mice resulted in a highly reproducible two- to threefold greater number of Treg cells than that in their TRAF3-deficient control littermates. Our results establish TRAF3 as a critical factor in regulating signaling via IL-2R to T cells, with important consequences for the development of Treg cells.

RESULTS

Effect of cell-intrinsic TRAF3 on Treg cell development

Despite the ubiquitous expression of TRAF3, CD4+ Tcon cells and CD8+ Tcon cells seem to develop normally in mice with

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T-cell–specific deficiency in TRAF3 (with loxp-flanked alleles deleted by Cre recombinase expressed from the T cell–specific Cd4 promoter (Trafsfl/loxCd4-Cre); called ‘T-Traf3−/− mice’ here), although these T cells have markedly reduced activation responses. In contrast, Cd4+Foxp3+ Treg cells showed a highly reproducible two- to threefold greater frequency in all peripheral lymphoid tissues examined from T-Traf3−/− mice than in those from their control littersates with loxp-flanked Traf3 alleles without Cre expression (Trafsfl/lox mice)22 (Supplementary Fig. 1a). The frequency and number of Treg cells, but not that of Cd4+ Tcon cells, in the thymus was also two- to threefold greater in T-Traf3−/− mice than in their control littersates (Fig. 1a–c).

To determine if this greater number was cell intrinsic, we generated chimeras by mixing wild-type (Cd45.1+) bone marrow (BM) and T-Traf3−/− (Cd45.2+) BM at a ratio of 1:1 or 20:1 and transferring the mixture into lethally irradiated wild-type (Cd45.1+Cd45.2+) host mice. Eight weeks after reconstitution of cells of the immune system, the frequency of Treg cells was still over twofold greater for T cells derived from T-Traf3−/− BM than for those derived from wild-type BM (Fig. 1d,e), which indicated that the greater number of Treg cells in T-Traf3−/− mice was a cell-intrinsic effect. Additionally, we transduced T-Traf3−/− BM with retrovirus encoding Traf3 or empty vector and transferred the BM into sublethally irradiated mice with congenital deficiency in mature B cells and T cells (Rag1−/− mice). In the resultant chimeras, overexpression of TRAF3 resulted in a much lower frequency of Treg cells than in chimeras whose T cells were derived from T-Traf3−/− BM transduced with empty vector (Fig. 1f,g). Moreover, the frequency of Treg cells was also much greater in another mouse strain with T cell–specific deficiency in TRAF3 (Traf3fl/flLck-Cre) than in their control littersates (Supplementary Fig. 1b). These results indicated that TRAF3 was required for restraining Treg cell development in a cell-intrinsic manner.

**Traf3 deficiency and Treg cell properties**

Treg cells exhibit several features that distinguish them from other T cell subsets. We thus explored whether TRAF3 deficiency affected the expression of signature proteins and functions of Treg cells. The expression of Foxp3, the immunomodulatory receptors CD152 (CTLA-4) and GITR and the cytokine receptors CD25 (IL-2Rα) and CD122 (IL-2Rβ) in Treg cells from T-Traf3−/− mice was similar to or only slightly different from that in Treg cells from their control littersates (Fig. 2a). The stability of Foxp3 expression upon in vitro stimulation of the TCR in Treg cells from T-Traf3−/− mice was similar to that seen in Treg cells from their control littersates (Supplementary Fig. 2a). In addition, Treg cells derived from splenocytes obtained from T-Traf3−/− mice and their control littersates had similar baseline amounts of apoptosis, and these cells underwent apoptosis at the same rate when costimulated with antibody to the invariant signaling protein CD3 (anti-CD3) and antibody to the coreceptor CD28 (anti-CD28) in vitro (Fig. 2b and Supplementary Fig. 2b). To further explore whether TRAF3-deficient Treg cells displayed enhanced survival in vivo, we mixed splenic wild-type (Cd45.1+) Treg cells with T-Traf3−/− (Cd45.2+) Treg cells at a ratio of 1:1 and transferred the mixture into wild-type (Cd45.1+Cd45.2+) mice. 3 weeks later, the ratio of transferred wild-type Treg cells to T-Traf3−/− Treg cells was still 1:1 in the recipients’ spleens (Fig. 2c). This result indicated that TRAF3 deficiency did not detectably alter the longevity of Treg cells.

The most important function of Treg cells is the inhibition of immune responses. An in vitro suppressive assay showed that Treg cells from T-Traf3−/− mice and their control littersates efficiently suppressed the proliferation of CD4+ Tcon cells upon stimulation of the TCR (Fig. 2d). We also found that Treg cells from T-Traf3−/− mice and their control littersates similarly suppressed the development of inflammatory bowel disease in vivo in Rag1−/− mice.
mice (Fig. 2e,f). Thus, TRAF3 deficiency did not alter the basic biological properties of Treg cells.

**Thymic origin of the abundant Treg cells in T-Traf3−/− mice**

Treg cells either are derived from the thymus (iTreg cells) or arise in the periphery. iTreg cells are a relatively stable population with sustained homeostasis, but the Treg cells that arise in the periphery are less stable and their number varies according to environmental stimuli.25,26 We stained cells with the proliferation marker Ki67 and measured incorporation of the thymidine analog BrdU to assess cell turnover. We found that the frequency of Ki67+ and BrdU+ Treg cells was similar in T-Traf3−/− mice either are derived from the thymus (iTreg cells) or arise in the periphery. Naive T cells from control littermates alone (−) or together with Treg cells from T-Traf3−/− mice (control; T-Traf3−/− Tcon). (b) Death of splenic CD4+CD25+Treg cells (among Foxp3+ cells) obtained from T-Traf3−/− mice and their control littermates and stimulated in vitro with anti-CD3 and anti-CD28, assessed by staining with annexin V (AnnV+). (c) Flow cytometry of CD4+CD25+ Treg cells isolated from wild-type (CD45.1+) and T-Traf3−/− (CD45.2+) mice and mixed at a ratio of 1:1, followed by transfer into wild-type recipients. Numbers adjacent to outlined areas indicate percent wild-type Foxp3+ cells (left) and T-Traf3−/− Foxp3+ cells (right) before transfer (left plot), or CD45.1+ cells (left) or CD45.2+ cells (right) among CD4+ Foxp3+ cells in the spleen of a host mouse at 21 d after transfer (right plot). SSC, side scatter. (d) Suppressive function of Treg cells in vitro, assessed as incorporation of [3H]thymidine (in counts per minute (c.p.m.)) by CD4+ Tcon cells from the spleens of the control littermates of T-Traf3−/− mice, cultured with Treg cells from the spleens of T-Traf3−/− mice or their control littermates at various ratios (effector:Treg; horizontal axis). (e) Weight change of Rag1−/− host mice given transfer of naive T cells from control littermates alone (−) or together with Treg cells from T-Traf3−/− mice or their control littermates (key). (f) Histological scores of sections of colon obtained from the mice in e, stained with hematoxylin and eosin and assigned scores on the basis of the degree of architectural abnormalities and inflammatory changes. *P < 0.001 (unpaired two-tailed Student’s t-test). Data are from one experiment representative of two experiments with six mice per group (a) or with three recipient mice (c) or one experiment representative of three independent experiments (b,d; error bars, s.e.m.) or are representative of two experiments with six mice per group (e,f; mean and s.e.m.).

**NIK-independent development of Treg cells in T-Traf3−/− mice**

TRAF3 deficiency in T cells results in enhanced basal activation of the noncanonical transcription factor NF-kB2 pathway22. We thus investigated the importance of this pathway in the expanded Treg cell population in T-Traf3−/− mice by breeding these mice with mice deficient in the NF-kB-inducing kinase NIK (Map3k14−/−; called ‘NIK−/−’ here) in all cell types. NIK deficiency substantially reduced the frequency of Treg cells in both NIK−/− mice and NIK−/−T-Traf3−/− mice, and we observed no difference between these mice (Supplementary Fig. 3a). This result seemed consistent with the hypothesis that enhanced NF-kB2 accounted for the increased number of Treg cells in T-Traf3−/− mice. However, it has been shown that it is instead NIK deficiency in thymic accessory cells, in particular dendritic cells, that accounts for defective development of Treg cells and other lineages of CD4+ T cells in NIK−/− mice, whereas NIK deficiency in T cells is not involved.31,32 Thus, we either transferred NIK−/− or T-Traf3−/−Nik−/− BM alone into irradiated Rag1−/− mice or mixed Rag1−/− BM with wild-type, NIK−/−, T-Traf3−/− or T-Traf3−/− BM at a ratio of 10:1 and transferred the mixtures into irradiated Rag1−/− mice. In such chimeras, the majority of dendritic cells are derived from Rag1−/− mice, which are NIK sufficient, and thymic epithelial cells are also NIK sufficient. We found that the frequency of thymic Treg cells was much higher in recipients of the mixture of Rag1−/−BM and NIK−/−BM than in recipients of NIK−/−BM, and it was even higher in mice that received the mixture of Rag1−/−BM and NIK−/− T-Traf3−/− BM, at a frequency similar to that in mice reconstituted with T-Traf3−/− BM (Supplementary Fig. 3b). Together these results indicated that the elevated basal activation of NF-kB2 in T cells from T-Traf3−/− mice did not suffice to explain their greater number of Treg cells.

**Thymic selection in T-Traf3−/− mice**

Early TCR signaling events are markedly attenuated in mature T cells from mice that lack TRAF3 relative to that in cells from their TRAF3-sufficient control littermates.23 To explore whether thymic selection is also affected and could account for the greater abundance Treg cells in T-Traf3−/− mice, we examined positive and negative selection of thymocytes. The frequency and number of thymocyte populations were similar in T-Traf3−/− mice and their control littermates (Supplementary Fig. 4a). We found no difference in the expression of TCRβ, the negative regulator CD5 or the activation marker CD69 in CD4+CD8+ double-positive (DP) thymocytes from T-Traf3−/− mice relative to the expression in DP thymocytes from their control littermates (Fig. 4a). In addition, the frequency and number of DP cells and CD4+ single-positive (CD4SP) cells were similar in T-Traf3−/− mice on the OT-II background (with transgenic expression of an ovalbumin-specific TCR) and their control OT-II littermates.
Figure 3  T_{reg} cells in T-Traf3−/− mice are derived from the thymus. (a) Flow cytometry of Foxp3+ T_{reg} cells (top) and T_{con} cells (bottom) in the spleen of T-Traf3−/− mice and their control littermates. Numbers adjacent to outlined areas indicate percent Ki67+ cells. (b) Summary of data in a. (c) Frequency of BrdU+ cells among Foxp3+ cells in the spleen of T-Traf3−/− mice and their control littermates. (d) Methylation status (open, unmethylated; filled, methylated) of CpG islands in Foxp3 CNS2 of CD4+CD25− T_{reg} cells and CD4+CD25− T_{con} cells from T-Traf3−/− mice and their control littermates; numbers above indicate position relative to the transcription start site. (e) Expression of Nrp1 by Foxp3+ T_{reg} cells from T-Traf3−/− mice and their control littermates and T_{con} cells from T-Traf3−/− mice. Isotype, isotype-matched control antibody. (f) Flow cytometry of Foxp3+ cells in the spleen of T-Traf3−/− mice and their control littermates. Numbers adjacent to outlined areas indicate percent Helios+ cells. NS, not significant (unpaired two-tailed Student’s t-test). Data are representative of three experiments with six mice per group (a,b); mean and s.e.m. in b; two experiments with four mice per group (c); mean and s.e.m.) or three experiments (d) or are from one experiment representative of three experiments with six mice per group (e,f).

Supplementary Fig. 4b). The expression of β-chain variable region 5 (Vβ5; the TCRβ chain of OT-II mice) was also similar in these groups of mice, as was the frequency of Vβ5+ cells (Supplementary Fig. 4c).

To evaluate thymic negative selection, we stimulated DP thymocytes from T-Traf3−/− OT-II mice and their control OT-II littermates with OT-II peptide–pulsed antigen-presenting cells in vitro and stained the cells with annexin V and propidium iodide. We observed similar numbers of these cells undergoing apoptosis regardless of their TRAF3 status (Fig. 4b). Additionally, expression of Nur77, a marker of TCR signaling strength33, was not different in T_{con} cells or precursors of T_{reg} cells from T-Traf3−/− OT-II mice and those from their control OT-II littermates (Supplementary Fig. 4d), which indicated that TRAF3 deficiency did not detectably alter TCR signaling in early T cell development.

TRAF3 is involved in promoting TCR- and CD28-mediated signalization24. Given the importance of CD28 signaling in the development of T_{reg} cells, we bred T-Traf3−/− mice with CD28-deficient (Cd28−/−) mice to explore whether CD28 signaling was required for the development of the abundant T_{reg} cells in T-Traf3−/− mice. Flow cytometry showed that although CD28 deficiency resulted in a lower frequency of T_{reg} cells in both T-Traf3−/− mice and their control littermates, there were still two- to threefold more T_{reg} cells in Cd28−/− T-Traf3−/− mice than in their Cd28−/− control littermates (Fig. 4c). This indicated that altered CD28 signaling was not responsible for the larger T_{reg} cell population in T-Traf3−/− mice.

According to the two-step model of T_{reg} cell development, TCR signaling is required for the selection of precursors of T_{reg} cells, while signaling induced by common γ-chain cytokines, particularly IL-2, is essential for the upregulation of Foxp3 expression24,33. Although T_{reg} cell numbers were two- to threefold greater in T-Traf3−/− thymi, the frequency of precursors of T_{reg} cells (defined as CD4+CD8−Foxp3−CD25− cells (Fig. 4d) or CD4+CD8−Foxp3+CD25+GFP+ cells (Supplementary Fig. 4e)) was similar in T-Traf3−/− mice and their control littermates (Fig. 4d and Supplementary Fig. 4e). These results further indicated

Figure 4 Unaltered thymic selection in T-Traf3−/− mice. (a) Expression of TCR-β, CD5 and CD69 in CD4+CD8+ double-positive thymocytes (DP) and CD4SP cells from T-Traf3−/− mice and their control littermates. (b) Frequency of dead cells (positive for annexin V and propidium iodide) among DP thymocytes isolated from T-Traf3−/− OT-II mice and their control OT-II littermates, then stimulated in vitro with OVA peptide–pulsed antigen-presenting cells. (c) Flow cytometry of T_{reg} cells in the thymus, spleen and lymph nodes and precursors of T_{reg} cells in the thymus of Cd28−/− and Cd28−/− T-Traf3−/− mice. Numbers adjacent to outlined areas indicate percent Foxp3+ cells (middle and bottom row) or Foxp3+ cells (top) or Foxp3−CD25+ cells (bottom) in the CD4SP population (top row). (d) Flow cytometry of CD4SP thymocytes in T-Traf3−/− mice and their control littermates. Numbers adjacent to outlined areas indicate percent Foxp3+ T_{reg} cells (top) and Foxp3−CD25− precursors of T_{reg} cells (bottom). Data are from one experiment representative of two experiments with six mice (a,c) or ten mice (d) or one experiment representative of three independent experiments with one mouse in each (b).
that thymic selection was not affected by the absence of TRAF3 in the T cell compartment. This was also true for Cd28−/− mice (Fig. 4c). Thus, we concluded that thymic selection was not responsible for greater abundance of Treg cells in Traf3−/− mice.

Inhibition of the transition from precursor to Treg cell by TRAF3

The unaltered numbers of precursors of Treg cells, in addition to the normal homeostasis and survival of Traf3−/− Treg cells, prompted us to hypothesize that the transition from precursor of a Treg cell to Foxp3+ Treg cell would be more efficient in the absence of TRAF3. To address this hypothesis, we bred T-Traf3−/− mice to mice expressing Foxp3 tagged with green fluorescent protein (Foxp3-GFP) and sorted precursors of Treg cells from the progeny of that cross for in vitro culture in the presence of IL-2. The transition from precursor to Treg cell with the addition of IL-2 was twice as efficient in the absence of TRAF3 as in its presence (Fig. 5a). Consistent with that finding, blocking CD25 in fetal thymic organ culture resulted in disappearance of TRAF3 as in its presence (Fig. 5b). These results suggested that signaling via IL-2R was not affected by the absence of TRAF3 (ref. 34). To investigate whether the observed enhanced phosphorylation of STAT5 was responsible for more efficient conversion of Treg cells from precursors in T-Traf3−/− mice, we cultured sorted precursors of Treg cells with IL-2 alone or together with trichostatin A or apicidin, which blocked this transition regardless of TRAF3 status (Fig. 5a). The unaltered numbers of precursors of Treg cells, in addition to the normal homeostasis and survival of T-CD2 positive cells from Traf3−/− mice (Fig. 4c), indicated a crucial role for enhanced phosphorylation of STAT5 in the transition process.

Histone deacetylases are required for the induction of STAT5-dependent gene transcription, including that of Foxp3 (ref. 34). To investigate whether the observed enhanced phosphorylation of STAT5 was responsible for more efficient conversion of Treg cells from precursors in T-Traf3−/− mice, we cultured sorted precursors of Treg cells with IL-2 alone or together with trichostatin A or apicidin, which are inhibitors of histone deacetylases. These inhibitors completely blocked this transition regardless of TRAF3 status (Fig. 5a), which further indicated a crucial role for enhanced phosphorylation of STAT5 in the transition process.

The generation of Treg cells in the thymus is characteristically delayed by several days relative to that of T effector cells (35). Foxp3+ Treg cells emerged at day 1 after birth in T-Traf3−/− mice, and we observed the trend of a higher frequency of Treg cells from this time onward (Fig. 5e,f). The early appearance of Treg cells in the thymus was similar to findings obtained with a mouse with transgenic expression of STAT5 with constitutively low activity3, which indicated that enhanced IL-2 signaling was able to accelerate Treg cell development. TRAF3 deficiency thus resulted in enhanced signaling via IL-2R in precursors of Treg cells, and this led to increased efficiency in the transition from precursor to Foxp3+ Treg cell.

TRAF3-mediated restraint of IL-2R signaling in Tcon cells

IL-2 signaling is essential for the homeostasis and maintenance of Treg cells. The data presented above showed that the homeostatic proliferation and survival of Treg cells from T-Traf3−/− mice were indistinguishable from that of Treg cells from their control littermates (Fig. 2 and Supplementary Fig. 2). In contrast to the finding of enhanced...
signaling via IL-2R in precursors of Treg cells, phosphorylated STAT5 was at most slightly greater in mature Treg cells from TRAF3-deficient mice than in those from their TRAF3-sufficient control littermates (Fig. 6a). Thus, TRAF3 seemed to have different roles in signaling via IL-2R in Treg cells and their precursors. It was of interest to determine whether TRAF3 also affected signaling via IL-2R in CD4+CD25− Tcon cells. The phosphorylation of STAT5, Jak1 and Jak3 was much greater in Tcon cells from Traf3−/− mice than in those from their control littermates, but the phosphorylation of Erk and Akt was not, similar to the pattern observed in precursors of Treg cells (Fig. 6b,c). Consistent with enhanced IL-2 signaling, we detected more association of STAT5 with two of its known targets (the genes encoding CD25 (IL-2Rα) and the cytokine-inducible Src homology 2 domain–containing protein CIS) in TRAF3-deficient Tcon cells than in TRAF3-sufficient Tcon cells, by chromatin-immunoprecipitation assay (Supplementary Fig. 6a,b). We further confirmed the difference between Treg cells and Tcon cells in their IL-2 signaling through the use of sorted CD4+Foxp3-GFP+ and CD4+Foxp3-GFP− splenocytes (Supplementary Fig. 6c,d). These results indicated that TRAF3 regulated IL-2R signaling differently in Treg cells and their precursors and Tcon cells, principally by altering Jak-STAT5 signaling pathways. Consistent with those findings, expression of TRAF3 protein was much lower in Treg cells than in Tcon cells (Supplementary Fig. 6e). In confirmation of the findings obtained with mouse T cells, phosphorylation of STAT5 was also augmented upon stimulation with IL-2 in human CD4+ T cells that had undergone depletion of TRAF3 through the use of small interfering RNA (Fig. 6d). Collectively, these results showed intrinsic involvement of TRAF3 in signaling via IL-2R.

Interaction between TRAF3 and TCPTP in IL-2R signaling
IL-2 binding induces the oligomerization of IL-2R components and the recruitment of Jak1 and Jak3. This receptor complex initiates the activation of downstream signaling pathways5. The enhanced phosphorylation of Jak1 and Jak3 in TRAF3-deficient cells suggested that TRAF3 exerts regulatory effects on the IL-2R complex. Immunoprecipitation of Jak3 showed that TRAF3 interacted with Jak3 upon stimulation with IL-2 (Fig. 7a). We also detected Jak1 and Jak3 among proteins immunoprecipitated from stimulated T cells with anti-TRAF3 (Fig. 7b). In human CD4+ T cells, we also observed an interaction between TRAF3 and Jak3 (Fig. 7c). Thus, TRAF3 was recruited to the IL-2R complex upon IL-2 signaling in both mouse T cells and human T cells. It has been reported that the phosphatase TCPTP associates with Jak1 and Jak3 to negatively regulate signaling via IL-2 and IFN-γ44, which we also demonstrated to be true in human T cells (Supplementary Fig. 7a). To explore whether TRAF3 affected the binding of TCPTP to the IL-2R complex, we immunoprecipitated Jak3 from Tcon cells from Traf3−/− mice and their control littermates and found that TCPTP interacted with Jak3 in CD4+ T cells from these mice, but the association was negligible in the absence of TRAF3 (Fig. 7d). Notably, when we immunoprecipitated CD122 (IL-2Rβ), we found that the recruitment of Jak1, Jak3 and SHP-1 to IL-2R was not substantially different in T cells from Traf3−/− mice and those from their control littermates (Supplementary Fig. 7b), which indicated that TRAF3 deficiency specifically affected the recruitment of TCPTP to the IL-2R complex.

There are two splice variants of TCPTP. The nuclear form (molecular mass, 45 kilodaltons (kDa)) can access both nuclear and cytoplasmic substrates and interact with Jak1 and Jak3 to dephosphorylate them in response to stimulation with IL-2 or IFN-γ44. An alternative cytoplasmic form (molecular mass, 48 kDa), unique to human T cells, is targeted to the endoplasmic reticulum15. To explore whether TRAF3 is able to interact with TCPTP, we overexpressed hemagglutinin-tagged TRAF3 and either form of TCPTP (45 kDa and 48 kDa) or a substrate-trapping mutant of that TCPTP (with substitution of alanine for the aspartic acid at position 182) in 293 human epithelial cells. In samples immunoprecipitated with anti-hemagglutinin, only the 45-kDa form of TCPTP and its substrate-trapping mutant interacted with TRAF3, but neither the 48-kDa form nor its mutant did so (Fig. 7e), consistent with the important role of the nuclear form of TCPTP in IL-2 signaling. In further support of the proposal of an interaction between TRAF3 and TCPTP, IFN-γ-induced phosphorylation of STAT1 was also enhanced in CD4+ Tcon cells in the absence of TRAF3 (Supplementary Fig. 7e). To determine which domain of TRAF3 was required for the interaction between TRAF3 and TCPTP, we transfected 293 cells to express either wild-type TRAF3 or mutant TRAF3 lacking the RING domain, the RING and zinc-finger regions or the TRAF domain, along with the 45-kDa form of TCPTP. The results showed that depletion of both the RING and zinc-finger domains of TRAF3 was needed to abrogate the binding of TRAF3 to TCPTP (Fig. 7f). Although TRAF3 has been reported to be an E3 ubiquitin ligase in certain settings, and its RING domain is required for this activity36, overexpression of ubiquitin, TRAF3 and TCPTP in 293 cells did not result in detectable ubiquitination of TCPTP (Supplementary Fig. 7d). Together these results indicated that interactions between TRAF3 and TCPTP facilitated the recruitment of TCPTP to the IL-2R complex upon stimulation with IL-2.
**Figure 7** The interaction of TRAF3 with TCPTP in signaling via IL-2R. (a, b) Immunoblot analysis of TRAF3 and Jak3 (a) and of Jak1, Jak3 and TRAF3 (b) among proteins immunoprecipitated with anti-Jak3 (a) or anti-TRAF3 (b) from CD4+CD25− T cells isolated from the spleen of wild-type mice and stimulated for 0–10 min (above lanes) in vitro with recombinant mouse IL-2 (500 U/ml) (top two blots), and immunoblot analysis of Jak3 (a) or TRAF3 (b) in whole-cell lysates of those cells (bottom blot). (c) Immunoblot analysis of TRAF3 and Jak3 among proteins immunoprecipitated with anti-Jak3 from lysates of human CD4+ T cells treated for 0–10 min (above lanes) in vitro with recombinant human IL-2 (5 ng/ml) (top two blots), and immunoblot analysis of β-actin in whole-cell lysates of those cells (bottom blot). (d) Immunoblot analysis of TCPTP and Jak3 among proteins immunoprecipitated with anti-Jak3 from CD4+CD25− T cells isolated from the spleen of Traf3−/− mice and their control littermates and stimulated as in a, b (top two blots), and immunoblot analysis of TCPTP and TRAF3 in whole-cell lysates of those cells (bottom two blots). (e) Immunoblot analysis of TCPTP and hemagglutinin-tagged TRAF3 (HA-TRAF3) among proteins immunoprecipitated, with anti-hemagglutinin, from 293 cells cotransfected with plasmids encoding hemagglutinin-tagged TRAF3 plus nuclear wild-type TCPTP (lane 1) or its substrate-trapping mutant (lane 2), or cytoplasmic wild-type TCPTP (lane 3) or its substrate-trapping mutant (lane 4) (top two blots), and immunoblot analysis of TCPTP in whole-cell lysates of those cells (bottom blot). (f) Immunoblot analysis of hemagglutinin (HA) and TCPTP among proteins immunoprecipitated with anti-TCPTP from 293 cells cotransfected with plasmids encoding nuclear TCPTP together with hemagglutinin-tagged wild-type TRAF3 (lane 1) or mutant TRAF3 lacking the RING domain (amino acids 1–113; lane 2), the RING and zinc-finger regions (amino acids 1–258; lane 3) or the TRAF domain (amino acids 382–568; lane 4) (top two blots), and immunoblot analysis of hemagglutinin in whole-cell lysates of those cells (bottom blot). Data are representative of three (a,b,d) or two (c,e,f) independent experiments.

**DISCUSSION**

A multilayered mechanism is required for the control of Treg cell development1,4,26. Although the lineage determination of precursors of Treg cells to Foxp3+ Treg cells is limited due to easily saturable niches39, our results indicated that unstranded signaling via IL-2R led to abnormal accumulation of the Treg cell population. We found TRAF3 was an important factor that controlled the number of Treg cells by negatively regulating signaling from IL-2R to precursors of Treg cells. TRAF3 was required for the recruitment of TCPTP to the IL-2R complex, an event that downregulated IL-2 signaling. Downstream of the enhanced signaling in the absence of TRAF3, the transition from precursor to Treg cell was more efficient and resulted in two- to threefold more Treg cells in T-Traf3−/− mice.

In mice with T cell–specific deficiency in TRAF3, signaling via the TCR in peripheral T cells is impaired, yet it suffices to allow the normal development of Tconv cells8,22. Here we found no evidence of altered thymic selection in T-Traf3−/− mice, which ruled this out as a potential reason for the greater number of Treg cells in these mice. The finding of a similar frequency of precursors of Treg cells in T-Traf3−/− mice and their control littermates also supported the conclusion of unaltered thymic selection. Although the development of invariant natural killer T cells in T-Traf3−/− mice is impaired, early stages in the development of such cells that require TCR signaling are normal, and only later stages are compromised31. It thus appears that TRAF3 has more important roles in TCR signaling in mature T cells than in developmentally immature T cells. Our finding that signaling via IL-2R was regulated differently by TRAF3 in precursors of Treg cells, Tconv cells and Treg cells further strengthens the concept that TRAF3 has multifaceted roles in different cell types, including distinct developmental stages, as well as different receptors in the same cell type21.

IL-2-induced signaling differs in distinct T cell subsets. Although IL-2 activates S6 kinase in both CD8+ T cells and CD4+ T cells, much higher activation is found in the former38. In contrast to its function in CD4+ Tconv cells, IL-2 fails to activate the PI(3)K-Akt pathway in Treg cells, due to high expression of the PI(3)K antagonist PTEN39,40. Foxp3 expression in Treg cells mediates a unique gene-expression profile, which may contribute to the regulation of IL-2 signaling41. In addition, the constitutive activation of IL-2 signaling in Treg cells due to high expression of CD25 may activate a negative feedback loop38,39 to alter IL-2 signaling. Consistent with our finding that the IL-2 signaling and homeostasis of mature Treg cells showed little change in the absence of TRAF3, another study has found that Treg cells are only modestly more abundant in mice with deletion of TRAF3 from mature Treg cells (Traf3fl/flFoxp3-GFP−hCre mice)24. That report further supports our observation that TRAF3 controlled the number of Treg cells specifically at the precursor stage. The lower expression of TRAF3 in mature Treg cells shown here also suggests its minimal role in this population. As TRAF3 is involved in many signaling pathways mediated by the TNF receptor superfamily, it might also affect Treg cell features to some extent through these signaling pathways.

Signaling via IL-2R is initiated by ligand-induced oligomerization of three components of IL-2R that recruit Jak1 and Jak3 and ignite a signaling cascade. TCPTP interacts with Jak1 and Jak3 and dephosphorylates them, which restrains signaling via IL-2R42. In our study here, TRAF3 deficiency caused defective recruitment of TCPTP to Jak1 and Jak3 and selectively affected the phosphorylation of STAT5, but not of Erk and Akt, which indicates that these three signaling pathways are regulated differently. Although overlapping mechanisms also exist, STAT5 can be directly phosphorylated by Jak3, while activation of PI(3)K-Akt and Ras-Erk requires additional intermediate molecules, such as Shc, Lck and Syk6,9,10. It is thus not entirely unexpected that these pathways were less affected by loss of TRAF3. Although TRAF3 has not been previously considered to regulate signaling via IL-2R, TRAF6 can compete with Jak1 for binding to the IL-2Rβ chain and negatively regulate signaling by Jak1-Erk. However, the PI(3)K-Akt and Jak3-STAT5 pathways have not been explored in such studies42. TRAF6 and TRAF3 seem to have different roles in IL-2 signaling, as they do in a variety of other signaling pathways in cells of the immune system.

The enhanced phosphorylation of Jak1 and Jak3 observed here suggested that TRAF3 facilitates the effects of TCPTP upstream of IL-2-induced phosphorylation of STAT5. Indeed, the recruitment of TCPTP to the IL-2R complex was defective in the absence of TRAF3.
The nuclear form of TCPTP has critical negative roles in cytokine signaling, but the role of the cytoplasmic form of TCPTP is less well understood. We found that only the nuclear form of TCPTP was able to interact with TRAF3, consistent with its role in signaling via IL-2R, and both wild-type TCPTP and its substrate-trapping mutant interacted equally well with TRAF3, which suggests that TRAF3 may act as a scaffold molecule to recruit TCPTP to the IL-2R complex, rather than acting as a substrate for TCPTP. Similar to our observations, a published study has shown that the nuclear form of TCPTP associates with TRAF2 in signaling via the TNF receptor and specifically regulates TNF-induced activation of MAPKs but not of NF-κB. As nuclear TCPTP translocates to the cytoplasm upon stimulation, it is possible that it interacts with TRAF3 or TRAF2 in the cytoplasm. However, the possibility that TRAF3 and/or TRAF2 also has (have) roles in the process of TCPTP translocation cannot be excluded.

The TRAF domain of TRAF3 is thought to be involved mostly in self-association and interaction with upstream receptors. However, the amino terminus of TRAF3, but not the TRAF domain, was required for interactions between NIK and TRAF3, although the TRAF domain is indispensable for association with the costimulatory receptor CD40 or the receptor for the B cell–activation factor BAFF in B cells. In addition, the amino terminus of TRAF3 is required for interaction between NIK and TRAF3 in 293 epithelial cells. Therefore, the amino terminus of TRAF3 can also be involved in protein-protein interactions. Our findings are consistent with those reports, in that the amino terminus was required for TRAF3 to associate with TCPTP. The results of our study emphasize the finding that signaling via IL-2R in the transition from precursor to Foxp3+ Treg cell was the critical checkpoint for controlling the number of Treg cells. Our observations sheds light on the molecular regulation of signaling via IL-2R and may provide additional avenues for manipulating the number of Treg cells.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Z.Y. designed and did experiments, analyzed data and wrote the manuscript; W.W.L. and L.L.S. did experiments, provided input for data interpretation and edited the manuscript; and G.A.B conceived of the research, directed the study, interpreted data and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Traf3fl/fl mice have been described46 and were backcrossed with C57BL/6 mice for at least ten generations. Traf3fl/fl mice were bred with Cd4-Cre mice (Taconic Farms) or Lck-Cre mice (Jackson Labs). Cd45.2+ C57BL/6 mice and congenic Cd45.1+ C57BL/6 mice (Jackson Labs) were bred to generate Cd45.2+Cd45.1+ mice. Cd28− mice were from Jackson Labs. Rag1−/− mice were provided by A. Schlueter (University of Iowa). Rag1−/− mice were provided by A. Schlueter (University of Iowa). NK−/− mice were originally generated by R. Schreiber (University of Washington at St. Louis) and were provided by D. Parker (Oregon Health Science University).

Mice 6–12 weeks of age were used for all experiments except where indicated otherwise. All mice were maintained under specific pathogen–free conditions at The University of Iowa and were used in accordance with guidelines of the US National Institutes of Health under an animal protocol approved by the Animal Care and Use Committee of the University of Iowa.

Retroviral transduction and bone marrow chimera. For packaging of virus, mouse Traf3 was cloned and inserted into the retrovirus backbone pMIG. The empty vector pMIG or pMIG-Traf3 was transfected together with the helper vector pCLECO into 293T epithelial cells through the use of lipofectamine (Invitrogen). Supernatants were collected after 48 h. BM cells negative for lineage markers were purified with a kit from Miltenyi, then were stimulated overnight with a combination of cytokines (IL-6, IL-3 and stem cell factor; Peprotech) and were transduced with viral supernatant. Recipient Rag1−/− mice were prepared by sublethal irradiation with 500 rads γ-ray and were allowed to 'rest' overnight. 0.5 × 10^6 transduced BM cells were transferred into host mice by intravenous injection. The resulting chimeras were analyzed 8 weeks later47.

Recipient Cd45.1+Cd45.2+ congenic C57BL/6 mice were given 1,100 rads γ-irradiation at 16 h before transfer. BM harvested from the tibiae and femurs of wild-type (Cd45.1+) mice and T-Traf3−/− (Cd45.2+) mice was depleted of B220+ and CD3+ cells by magnetic bead separation (Miltenyi) and the BM cells were mixed at a ratio of 1:1 or 2:1. 10 × 10^6 BM cells were injected intravenously into recipient mice. Mice were killed 8 weeks later for experiments. In another set of experiments, BM cells isolated from Rag1−/− mice were mixed with BM cells from wild-type, Nik−/−, T-Traf3−/− or Nik−/− Traf3−/− mice at a ratio of 10:1. 10 × 10^6 BM cells were injected intravenously into sublethally irradiated Rag1−/− mice. Mice were killed 8 weeks later for experiments. Mice were irradiated at the Free Radical & Radiation Biology Core of the University of Iowa.

Flow cytometry. Single-cell suspensions were prepared from the thymus, spleen, lymph nodes, liver and Peyer's patches and erythrocytes were lysed. For staining for flow cytometry, nonspecific staining was blocked with a combination of cytokines (IL-6, IL-3 and stem cell factor; Peprotech) and were transduced with viral supernatant. Recipient Rag1−/− mice were prepared by sublethal irradiation with 500 rads γ-ray and were allowed to 'rest' overnight. 0.5 × 10^6 transduced BM cells were transferred into host mice by intravenous injection. The resulting chimeras were analyzed 8 weeks later47.

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In vitro Treg cell assay. Treg cells were isolated from spleens of the control littermates of T-Traf3−/− mice. They were cultured for 72 h with irradiated splenocytes (2 × 10^6) and anti-CD3 (1 μg/ml; identified above) and anti-CD28 (2 μg/ml; identified above). Samples were obtained at various time points and were stained with annexin V (BD Bioscience) and anti-Foxp3 (identified above), then were analyzed by flow cytometry. For detection of the death of Treg cells ex vivo, freshly isolated splenocytes were stained with annexin V, anti-CD4, anti-CD8 and anti-Foxp3 (all identified above), then were analyzed by flow cytometry.

Assay of BrdU incorporation in vivo. Mice were given intraperitoneal administration of 2 mg BrdU (5-bromodeoxyuridine; Sigma) 24 h before analysis. For detection of the incorporation of BrdU, mice were first stained with anti-Foxp3 (identified above), followed by intracellular staining of BrdU with anti-BrdU (eBioscience) with a BrdU flow cytometry detection kit according to the manufacturers' instructions (BD Biosciences). Samples were analyzed by flow cytometry.

In vitro Treg cell suppressive assay. CD4+Cd45RBD21−CD25+ T cells (5 × 10^4) were isolated from the spleens of the control littermates of T-Traf3−/− mice. They were cultured for 72 h with irradiated splenocytes (2 × 10^6) and anti-CD3 (1 μg/ml; identified above) and anti-CD28 (2 μg/ml; identified above) in the presence of various numbers of Treg cells isolated from T-Traf3−/− mice or their control littermates. [3H]thymidine was added for the final 16 h. Cells were harvested and radioactivity was measured with a β-counter (LS6500; Beckman Coulter).

In vivo Treg cell suppressive assay. Naive CD4+Cd45RBD21−CD25+ T cells (4 × 10^4) were sorted from spleens of Traf3fl/fl mice (control littermates of T-Traf3−/− mice). They were transferred into Rag1−/− mice alone or in combination with Treg cells (4 × 10^4) from T-Traf3−/− mice or their control littermates. After reconstitution of T cells, mice were weighed weekly and monitored for signs of disease. Mice were killed 8 weeks after T cell transfer and their colons were obtained for histopathology analysis. Histological scores were assigned according to a published description48 as follows: grade 0, no changes; grade 1, minimal inflammatory infiltrates present in the lamina propria; grade 2, mild inflammation in the lamina propria, minimal to mild mucosal hyperplasia and mucin depletion; grade 3, mild to moderate inflammation in the lamina propria and moderate mucosal hyperplasia and mucin depletion; grade 4, considerable inflammatory infiltrates commonly transmural with ulceration, considerable mucosal hyperplasia and mucin depletion; grade 5, considerable transmural inflammation with ulceration, wide-spread crypt necrosis and loss of intestinal glands.

In vitro assay of the survival of Treg cells. CD4+Cd45RBD21− Treg cells were isolated from the spleen of wild-type (Cd45.1+) mice and T-Traf3−/− (Cd45.2+) mice and were mixed at a ratio of 1:1. 2 × 10^6 cells were transferred into wild-type (Cd45.1+Cd45.2+) recipient mice by intravenous injection. 21 d after transfer, splenocytes were harvested for staining and analysis by flow cytometry.

In vitro thymic negative selection. Splenocytes were isolated from the thymus of wild-type (Cd45.1+) mice and T-Traf3−/− (Cd45.2+) mice and were mixed at a ratio of 1:1. 2 × 10^6 cells were transferred into wild-type (Cd45.1+Cd45.2+) recipient mice by intravenous injection. 21 d after transfer, splenocytes were harvested for staining and analysis by flow cytometry.
Induction of Treg cells from precursor cells. Sorted precursors of Treg cells (CD4+Foxp3- GFP-CD25+) were seeded into 96-well plates in the presence of medium alone or medium containing recombinant mouse IL-2 (50 U/ml). In another set of experiments, cells were treated with 100 nM Trichostatin A (Sigma), 800 nM Apicidin (Sigma) or dimethyl sulfoxide (0.2%) and recombinant mouse IL-2 (ref. 2). 24 h later, cells were analyzed by flow cytometry.

DNA-methylation analysis. CD4+CD25+ Treg cells and CD4+CD25− Tcon cells were sorted from spleen. Only male mice were used. Genomic DNA was isolated and methylation was analyzed by bisulfite conversion of genomic DNA with an EZ DNA Methylation-Gold Kit according to the manufacturer’s instructions (Zymo Research). PCR was done as described27. PCR products were cloned to a TOPO TA Cloning kit (Invitrogen).

Depletion of TRAF3 and TCPTP with small interfering RNA. Human lymphocytes were obtained from the DeGowin Blood Center at the University of Iowa. Healthy donors 18–55 years of age provided written consent for their blood to be used in research projects, in compliance with the University of Iowa’s Institutional Review Board. Human lymphocytes were removed from whole blood with leukocyte reduction cones. CD4+ T cells were purified with Miltenyi beads and were stimulated for 24 h with 5 μg/ml antibody to human CD3 (OKT3; ebioscience) and antibody to human CD28 (CD28.2; ebioscience). Traf3 or Tcptp Trilencer-27 small interfering RNA was transfected into cells with siTran 1.0 according to the manufacturer’s instructions (Origene). 20 U/ml IL-2 was added during transfection. Cells were transfected again after 24 h. After another 24 h of incubation, cells were washed and then were allowed to ‘rest’ twice in medium without IL-2, with a interval of 6 h between. They were stimulated for various times with recombinant human IL-2 (Peprotech) and analyzed by immunoblot for the detection of phosphorylated STAT5.

Fetal thymic organ culture. For fetal thymic organ culture, thymuses were collected from fetuses at embryonic days 14–15, then were bisected and cultured in Transwell plates. Pairwise comparisons of thymic lobes with 20 μg/ml antibody to the two-tailed unpaired Student’s t-test. For comparisons of multiple groups, two-way analysis of variance was used. Statistical significance was set at a P value of <0.05. All values were calculated with Prism software (GraphPad). Sample size was not specifically predetermined, but the number of mice used was consistent with prior experience with similar experiments.

Immunoprecipitation and immunoblot analysis. Antibodies used for immunoprecipitation and immunoblot analysis included the following: anti-Jak1 (6G4), antibody to phosphorylated jak1 (3331), anti-Jak3 (D1H3), antibody to phosphorylated STAT5 (D47E7), antibody to phosphorylated Erk (D13.14.4E), antibody to phosphorylated Akt (D25E6), anti-SHP-1 (C14H6), anti-HA (C29F4) anti-STAT5 (5H7; all from Cell Signaling Technology); anti-CD122 (C-2), anti-TRAFl3 (M20) and anti-TRAFl3 (H122; all from Santa Cruz Biotech); anti-TCPTP (252294; R&D System); anti-TCPTP (6F3; Medimabs); anti-β-actin (EP1123Y; EMA Millipore); antibody to phosphorylated tyrosine (4G10; EMA Millipore); and anti-Flag (M2; Sigma). For experiments, CD4+CD25− T cells or CD4+CD25+ Treg cells were treated for various times with IL-2. Whole-cell lysates were separated by SDS-PAGE and were transferred to PVDF membranes for immunoblot analysis. Alternatively, whole-cell lysates were first precleared with magnetic beads, then the relevant antibody (all identified above) was added, followed by incubation for 4 h with rotation. Magnetic beads were added, followed by incubation overnight with rotation. Immunoprecipitates were used for immunoblot analysis. For the measurement of direct interactions between TRAF3 and TCPTP, plasmid encoding hemagglutinin-tagged wild-type TRAF3 (pMIG-HA-Traf3) or mutant TRAF3 with deletion of the RING domain (pMIG-HA-Traf3Δ–113), the RING and zinc-finger regions (pMIG-HA-Traf3Δ–258) or the TRAF domain (pMIG-HA-Traf3Δ 382–568) was transfected together with a TCPTP-encoding plasmid into 293 cells. 48 h later, 293 cells were harvested and lysed. Anti-hemagglutinin (HA–7; Sigma) was used for immunoprecipitation of TRAF3. For the TCPTP-ubiquitination assay, plasmids encoding hemagglutinin-tagged ubiquitin, Flag-tagged TRAF3 and TCPTP were transfected into 293 cells. 48 h later, proteins were denatured by boiling of cell lysates for 1 min in 1% SDS. cell lysates diluted 1:10 were immunoprecipitated with anti-TCPTP (identified above). Samples were analyzed by immunoblot, and anti-hemagglutinin (identified above) was used for detection of ubiquitination36. Plasmids encoding the 45-kDa and 48-kDa formd of TCPTP and the D182A substrate-trapping mutant of each were provided by N. Tonks (Cold Spring Harbor Laboratory)49. ImageJ software (National Institutes of Health) was used for densitometry analysis of immunobots.

Statistical analysis. Statistical differences between two means were evaluated with the two-tailed unpaired Student’s t-test. For comparisons of multiple groups, two-way analysis of variance was used. Statistical significance was set at a P value of <0.05. All values were calculated with Prism software (GraphPad). Sample size was not specifically predetermined, but the number of mice used was consistent with prior experience with similar experiments.

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