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The adaptor molecule TRAF3 restrains the lineage determination of thymic regulatory T cells by modulating interleukin-2 receptor signaling

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Abstract

The number of Foxp3$^+$ regulatory T (T_{reg}) cells must be tightly controlled to efficiently suppress autoimmunity while not impairing normal immune responses. Here we show that the adapter molecule TRAF3 is intrinsically required for restraining lineage determination of thymic T_{reg} cells. T cell-specific TRAF3 deficiency resulted in a 2-3 fold increase of T_{reg} cell frequency, due to more efficient transition from T precursors to Foxp3$^+$ T_{reg} cells. TRAF3 dampened interleukin-2 (IL-2) signaling by facilitating recruitment of T cell protein tyrosine phosphatase (TCPTP) to the IL-2 receptor complex, resulting in dephosphorylation of the signaling molecules Jak1 and Jak3 and negative regulation of Jak-STAT5 signaling. Our results identify a role for TRAF3 as an important negative regulator of IL-2 receptor signaling that impacts T_{reg} cell development.

Tight regulation of the Foxp3$^+$ regulatory T (T_{reg}) cell population in immunity is crucial to avoid pathogenic autoreactivity while providing effective protection against infectious diseases and tumor cells[1]. Interleukin-2 receptor (IL-2R) mediated signaling is a major mechanism controlling T_{reg} cell development and homeostasis, and has been widely investigated[2-4]. IL-2 binding to the IL-2R activates at least three distinct signaling pathways. Activation of Janus kinase (Jak) 1 and 3 associating with IL-2Rβ (CD122) and common γ chain (CD132) respectively, leads to phosphorylation of IL-2Rβ and the transcription factor STAT5[5,6]. Phosphorylated STAT5 binds to the promoter and first intron of the *Foxp3* gene

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AUTHOR CONTRIBUTIONS

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

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and is essential for initiating Foxp3 expression[7,8]. IL-2 also activates PI3K-Akt and Ras-MAPK signaling pathways. But 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 these pathways[7,9,10]. Several negative regulatory mechanisms are involved in restraining IL-2-mediated signaling. Suppressor of cytokine signaling 1 (SOCS1) and 3 play negative feedback roles in IL-2 signaling by associating with Jak1 and inhibiting its kinase activity[11,12]. The SH2 domain-containing protein phosphatase 1 (SHP-1) dephosphorylates Jak1 and negatively regulates IL-2R-Jak1 signaling[13]. T cell protein tyrosine phosphatase (TCPTP) can also directly interact with Jak1 and Jak3 and dephosphorylate these molecules upon IL-2 or interferon-γ (IFN-γ) stimulation[14]. As a tyrosine-specific phosphatase, TCPTP expression is ubiquitous, but it is expressed in higher amounts in cells of hematopoietic origin[15]. The important role of TCPTP in cytokine signaling is demonstrated *in vivo* by TCPTP-deficient mice, which show a severe pro-inflammatory phenotype and die at 3-5 weeks of age[16]. Notably, T_{reg} cells are moderately increased in T cell specific TCPTP deficient mice[17].

TNF receptor associated factor 3 (TRAF3) is an adaptor molecule that participates in signaling by many members of the TNF receptor superfamily (TNFRSF), as well as innate immune receptors and the IL-17 receptor[18-20]. Previous studies indicate that the roles of TRAF3 are highly cell type- and receptor-dependent[21]. The functions regulated by TRAF3 in T cells have been less intensively examined than those in B cells. We reported that T cell-specific deficiency in TRAF3, while having no detectable impact on development of conventional T cells, causes decreased T cell effector functions and impaired T cell receptor (TCR) signaling in peripheral $CD4^+$ and $CD8^+$ T cells[22]. Deficiency of TRAF3 also results in both defective development and function of invariant Natural Killer T (iNKT) cells[23]. Another study indicates that T_{reg} cell-specific TRAF3 expression is required for follicular T_{reg} cell (T_{FR}) induction[24]. Therefore, TRAF3 plays distinct roles in different T cell subsets.

In the current study, we examined the molecular mechanisms by which T cell-specific TRAF3 deficiency in mice results in a highly reproducible 2-3 fold increase of the T_{reg} cell numbers. Our results establish TRAF3 as a critical factor in regulating IL-2R signaling to T cells, with important consequences for T_{reg} cell development.

RESULTS

Cell-intrinsic TRAF3 impact on T_{reg} cell development

Despite the ubiquitous expression of TRAF3, conventional $CD4^+$ and $CD8^+$ T cells appeared to develop normally in T cells deficient in TRAF3 (*Cd4*^{Cre}*Traf3*$^{flox/flox}$, hereafter termed T-*Traf3*$^{-/-}$) mice, although these cells have markedly reduced activation responses[22]. In contrast, the frequency of $CD4^+Foxp3^+$ T_{reg} cells showed a highly reproducible 2-3-fold increase in frequency in all peripheral lymphoid tissues examined[22] (**Supplementary Fig. 1a**). The percentage and number of T_{reg} cells, but not conventional $CD4^+$ T (Tcon) cells in the thymus was also increased 2-3-fold in T-*Traf3*$^{-/-}$ mice compared to littermate controls (LMC) (**Fig. 1a, b, c**). To determine whether this increased number was cell intrinsic, bone marrow (BM) chimeric mice were generated by transferring mixed wild-type (WT) ($CD45.1^+$) and T-*Traf3*$^{-/-}$ ($CD45.2^+$) BM at 1:1 or 20:1 ratios into lethally irradiated WT

mice (CD45.1$^+$ CD45.2$^+$). Eight weeks after immune cell reconstitution, the percentage of T_{reg} cells still showed a >2-fold increase in T cells derived from T-*Traf3*$^{-/-}$ BM compared to those derived from WT BM (**Fig. 1d, e**), indicating that the increased T_{reg} cell number in T-*Traf3*$^{-/-}$ mice is a cell-intrinsic effect. Additionally, T-*Traf3*$^{-/-}$ BM was transduced with control or TRAF3-expressing retroviruses, and used to produce BM chimeric mice. In these mice, TRAF3 over-expression drastically reduced the percentage of T_{reg} cells compared to mice whose T cells were derived from T-*Traf3*$^{-/-}$ BM transduced with empty vector (**Fig. 1f, g**). Moreover, in another T cell-specific TRAF3 deficient mouse strain, (*Lck*^{Cre}*Traf3*$^{flox/flox}$) mice, the percentage of T_{reg} cells was also significantly increased (**Supplementary Fig. 1b**). These results indicate that TRAF3 is required for restraining T_{reg} cell development in a cell-intrinsic manner.

TRAF3 deficiency and T_{reg} cell properties

T_{reg} cells exhibit several features that distinguish them from other T cell subsets. We thus explored whether TRAF3 deficiency affects the expression of signature proteins and functions of T_{reg} cells. Expression of Foxp3, CTLA4, CD25, CD122, and GITR proteins were comparable or showed only slight differences in T_{reg} cells from LMC vs. T-*Traf3*$^{-/-}$ mice (**Fig. 2a**). The stability of Foxp3 expression upon *in vitro* TCR stimulation was similar to that seen in LMC T_{reg} cells (**Supplementary Fig. 2a**). In addition, LMC and *Traf3*$^{-/-}$ T_{reg} cells from splenocytes have similar baseline amounts of apoptosis, and these cells underwent apoptosis at the same rate when stimulated with anti-CD3 and anti-CD28 Abs *in vitro* (**Fig. 2b and Supplementary Fig. 2b**). To further explore whether TRAF3-deficient T_{reg} cells display enhanced survival *in vivo*, splenic WT T_{reg} cells (CD45.1$^+$) were mixed with *Traf3*$^{-/-}$ T_{reg} cells (CD45.2$^+$) at a 1:1 ratio and transferred into WT (CD45.1$^+$CD45.2$^+$) mice. 3 weeks later, the ratio of transferred WT and *Traf3*$^{-/-}$ T_{reg} cells was still 1:1 in the recipients' spleens (**Fig. 2c**). This result indicates that TRAF3 deficiency does not detectably alter T_{reg} cell longevity.

The most important function of T_{reg} cells is inhibition of immune responses. An *in vitro* suppressive assay showed that both LMC and *Traf3*$^{-/-}$ T_{reg} cells efficiently suppressed the proliferation of conventional CD4$^+$ T cells upon TCR stimulation (**Fig. 2d**). We also found that both LMC and TRAF3-deficient T_{reg} cells similarly suppressed the development of inflammatory bowel disease in a mouse model *in vivo* in *Rag1*$^{-/-}$ mice (**Fig. 2e, f**). Thus, TRAF3 deficiency does not alter the basic biological properties of T_{reg} cells.

Thymic origin of increased T_{reg} cells

T_{reg} cells are derived either from the thymus (tT_{reg}) or arise in the periphery (pT_{reg}). tT_{reg} cells are a relatively stable population with sustained homeostasis, but pT_{reg} cells are less stable and their number varies according to environmental stimuli[25,26]. Ki67 staining and BrDU incorporation to measure cell turnover showed that the frequency of Ki67$^+$ and BrDU$^+$ T_{reg} cells was comparable in LMC and T- *Traf3*$^{-/-}$ mice (**Fig. 3a-c**), indicating that TRAF3 deficiency does not alter T_{reg} cell homeostatic proliferation. Foxp3 expression in tT_{reg} cells is relatively stable, due to demethylation of specific conserved DNA regions[27]. T_{reg} cells in LMC and T-*Traf3*$^{-/-}$ mice displayed similar demethylation status at conserved noncoding sequence 2 (CNS2) whereas as expected CNS2 in CD4$^+$CD25$^-$ T cells was fully

methylated (**Fig. 3d**). Neuropilin-1 (Nrp1) and Helios have both been suggested as possible markers of tT cells[28-30]. Splenic T_{reg} cells in LMC and T-*Traf3*$^{-/-}$ mice expressed indistinguishable levels of Nrp1 and Helios (**Fig. 3e, f**). These results indicate that the increased T_{reg} cell population in T-*Traf3*$^{-/-}$ mice is stable and thymus-derived.

NIK independent T_{reg} cell development in T-*Traf3*$^{-/-}$ mice

TRAF3 deficiency in T cells results in enhanced basal activation of the non-canonical NF-κB2 pathway[22]. We thus investigated the importance of this pathway in the expanded T_{reg} cell population in T-*Traf3*$^{-/-}$ mice, by breeding these mice with NF-κB inducing kinase deficient (*Nik*$^{-/-}$, also known as *Map3k14*$^{-/-}$) mice, which lack NIK in all cell types. NIK deficiency substantially reduced T_{reg} cell frequency in both *Nik*$^{-/-}$ and T-*Traf3*$^{-/-}$ *Nik*$^{-/-}$ mice, and the difference in T_{reg} cell percentage between these mice disappeared (**Supplementary Fig. 3a**). This result seemed consistent with the hypothesis that enhanced NF-κB2 activation accounts for the increased T_{reg} cell number in T-*Traf3*$^{-/-}$ mice. However, it has been shown that it is instead NIK deficiency in thymic accessory cells, in particular dendritic cells (DCs), that accounts for defective development of T_{reg} cells and other $CD4^+$ T cell lineages in *Nik*$^{-/-}$ mice, whereas T cell NIK deficiency is not involved[31,32]. Thus, we mixed *Rag1*$^{-/-}$ BM with *Nik*$^{-/-}$, T-*Traf3*$^{-/-}$ or *Nik*$^{-/-}$ T-*Traf3*$^{-/-}$ BM at a 10:1 ratio and transferred this mixed BM into irradiated *Rag1*$^{-/-}$ mice. In this model, the majority of DCs are derived from *Rag1*$^{-/-}$ mice, which are NIK sufficient, and thymic epithelial cells are also NIK sufficient. We found that the percentage of thymic T_{reg} cells was significantly increased in recipients of *Rag1*$^{-/-}$:*Nik*$^{-/-}$ BM compared to recipients of *Nik*$^{-/-}$ BM mice, and was further increased in mice receiving *Rag1*$^{-/-}$:*Nik*$^{-/-}$ T-*Traf3*$^{-/-}$ BM, to levels comparable to those seen in mice reconstituted with T-*Traf3*$^{-/-}$ BM (**Supplementary Fig. 3b**). Taken together, these results indicate that the elevated basal T cell NF-κB2 activation in T-*Traf3*$^{-/-}$ mice does not suffice to explain their increased T_{reg} cell number.

Thymic selection in T-*Traf3*$^{-/-}$mice

Early TCR signaling events in mature T cells lacking TRAF3 are markedly reduced[22]. To explore whether thymic selection is also impacted, and could account for the increased T_{reg} cells in T-*Traf3*$^{-/-}$ mice, positive and negative selection of thymocytes was examined. Frequencies and numbers of thymocyte populations were comparable between LMC and T-*Traf3*$^{-/-}$ mice (**Supplementary Fig. 4a**). No differences in expression of TCR-β, CD5 and CD69 were seen when comparing double positive (DP) thymocytes from LMC and T-*Traf3*$^{-/-}$ mice (**Fig. 4a**). In addition, the frequency and numbers of DP and CD4 single positive (CD4SP) cells were similar between LMC OTII and T-*Traf3*$^{-/-}$ OTII mice (**Supplementary Fig. 4b**). The percentage and expression of Vβ5 (TCR β chain of OTII mice) were also indistinguishable (**Supplementary Fig. 4c**).

To evaluate thymic negative selection, LMC OTII and T-*Traf3*$^{-/-}$ OTII DP thymocytes were stimulated with OTII peptide-pulsed APC *in vitro* and stained for Annexin V and propidium iodide (PI). Similar numbers of these cells undergoing apoptosis were observed, irrespective of TRAF3 status (**Fig. 4b**). Additionally, expression of Nur77, a marker of TCR signaling strength[33], was not different in T_{reg} cells or T_{reg} precursors from LMC vs. T-*Traf3*$^{-/-}$ mice

(**Supplementary Fig. 4d**), indicating that TRAF3 deficiency does not detectably alter TCR signaling in early T cell development.

Our previous report implicates TRAF3 as promoting TCR and CD28-mediated signaling[22]. Given the important role of CD28 signaling in tT_{reg} cell development, T-*Traf3*$^{-/-}$ mice were bred with CD28 deficient (*Cd28*$^{-/-}$) mice, to explore whether CD28 signaling is required for the development of increased T_{reg} cells in T-*Traf3*$^{-/-}$ mice. Flow cytometric analysis showed that although CD28 deficiency decreased the T_{reg} cell percentage in both LMC and T-*Traf3*$^{-/-}$ mice, there were still 2-3-fold more T_{reg} cells in *Cd28*$^{-/-}$T-*Traf3*$^{-/-}$ mice than in *Cd28*$^{-/-}$ LMC mice (**Fig. 4c**), indicating that altered CD28 signaling is not responsible for the increased tT_{reg} cell population in T-*Traf3*$^{-/-}$ mice.

According to a two-step model of T_{reg} cell development, TCR signaling is required for T_{reg} precursor selection, while signaling induced by common γ chain cytokines, particularly IL-2, is essential for the upregulation of Foxp3[2,3]. Although T_{reg} cell numbers were increased 2-3 fold in the T-*Traf3*$^{-/-}$ thymus, the percentage of T_{reg} precursors was comparable in LMC and T-*Traf3*$^{-/-}$ mice (defined as $CD4^+CD8^-Foxp3^-CD25^+$ in **Fig. 4d**, or $CD4^+CD8^-Foxp3^-CD25^+GITR^+$ in **Supplementary Fig. 4e**). These results further indicate that thymic selection is not affected by the absence of TRAF3 in the T cell compartment. This was also true in *Cd28*$^{-/-}$ mice (**Fig. 4c, upper panels**). Thus we conclude that alterations in thymic selection are not responsible for increased T_{reg} cells in T-*Traf3*$^{-/-}$ mice.

TRAF3 inhibition of Treg precursor to Treg cell transition

Unaltered numbers of T_{reg} precursors, in addition to the normal homeostasis and survival of *Traf3*$^{-/-}$ T_{reg} cells, prompted us to hypothesize that the transition from T_{reg} precursor to $Foxp3^+$ T_{reg} cell is more efficient in the absence of TRAF3. To address this hypothesis, T-*Traf3*$^{-/-}$ mice were bred to Foxp3-GFP mice and T_{reg} precursors were sorted for *in vitro* culture in the presence of IL-2. Results show that transition from T_{reg} precursor to T_{reg} cell with the addition of IL-2 was twice as efficient in the absence of TRAF3 (**Fig. 5a**). Consistent with this finding, blocking CD25 in fetal thymus organ culture (FTOC) resulted in disappearance of the advantage of over-development of T_{reg} cells in T-*Traf3*$^{-/-}$ thymus (**Fig. 5b, c**). These results suggested that IL-2R signaling was enhanced in *Traf3*$^{-/-}$ T_{reg} precursors. Indeed, IL-2-activated phosphorylation of STAT5 in *Traf3*$^{-/-}$ T_{reg} precursors was markedly elevated in comparison to LMC T_{reg} precursors (**Fig. 5d**), with no discernible change in the MAPK p- Erk or p-Akt (**Supplementary Fig. 5a, b**). Additionally, the expression of CD25, CD122 and CD132 in T_{reg} cell precursors was not impacted by the absence of TRAF3 (**Supplementary Fig. 5c**). Thus, TRAF3 specifically regulates IL-2-induced activation of STAT5 in T_{reg} precursors.

Histone deacetylases (HDACs) are required for induction of STAT5-dependent gene transcription, including that of Foxp3[34]. To investigate whether the observed enhanced phosphorylation of STAT5 is responsible for more efficient T_{reg} cell conversion from precursors in T-*Traf3*$^{-/-}$ mice, sorted T_{reg} precursors were cultured with IL-2 and HDAC inhibitors Trichostatin A (TSA) or apicidin. These inhibitors completely blocked the

transition in both LMC and *Traf3*$^{-/-}$ T_{reg} precursors (**Fig. 5a**), further indicating the crucial role of enhanced p-STAT5 in the transition process.

Generation of T_{reg} cells in the thymus is characteristically delayed by several days compared to T effector cells[35]. Foxp3^{+} T_{reg} cells emerged at day 1 after birth in T-*Traf3*$^{-/-}$ mice and the trend of increased percentages of T_{reg} cells was observed from this time onward (**Fig. 5e, f**). The early appearance of T_{reg} cells in the thymus is similar to findings in a mouse expressing transgenic STAT5 that is constitutively active at a low level[2], indicating that enhanced IL-2 signaling can accelerate T_{reg} cell development. TRAF3 deficiency thus results in enhanced IL-2R signaling in T_{reg} precursors, and this leads to increased efficiency of the transition from precursor to Foxp3^{+} T_{reg} cells.

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

IL-2 signaling is essential for the homeostasis and maintenance of T_{reg} cells. Data presented here show that homeostatic proliferation and survival of *Traf3*$^{-/-}$ T_{reg} cells were indistinguishable from LMC T_{reg} cells (**Fig. 2 and Supplementary Fig. 2**). In contrast to findings of enhanced IL-2R signaling in T_{reg} precursors, p-STAT5 in TRAF3-deficient mature T_{reg} cells was at most slightly increased compared to LMC T_{reg} cells (**Fig. 6a**). Thus, TRAF3 appears to play different roles in IL-2R signaling in T_{reg} precursors vs. T_{reg} cells. It was thus of interest to determine whether TRAF3 also affects IL-2R signaling in conventional CD4^{+}CD25^{-} T cells. Phosphorylation of STAT5, Jak1 and Jak3 was robustly elevated in *Traf3*$^{-/-}$ Tcon cells, but phosphorylation of Erk and Akt was not, similar to the pattern observed in T_{reg} precursors (**Fig. 6b, c**). Consistent with enhanced IL-2 signaling, increased STAT5 association with two of its known target genes, *IL-2Rα* chain and Cytokine inducible SH2-containing protein (*Cis*) was detected in TRAF3 deficient T cells by chromatin-immunoprecipitation (ChIP) assay (**Supplementary Fig. 6a, b**). The difference in IL-2 signaling in T_{reg} cells and Tcon cells was further validated with sorted CD4^{+}Foxp3GFP^{+} and CD4^{+}Foxp3GFP^{-} splenocytes (**Supplementary Fig. 6c, d**). These results indicate that TRAF3 differentially regulates IL-2R signaling in T_{reg} cells, T_{reg} precursors and Tcon cells, principally by altering Jak-STAT5 signaling pathways. Consistent with this finding, protein expression of TRAF3 in T_{reg} cells are much lower than in Tcon cells (**Supplementary Fig. 6e**). Validating findings in mouse T cells, STAT5 phosphorylation was also augmented upon IL-2 stimulation in normal human CD4^{+} T cells, when TRAF3 was depleted with siRNA (**Fig. 6d**). Collectively, these results show TRAF3 is intrinsically involved in IL-2R signaling.

Interaction between TRAF3 and TCPTP in IL-2R signaling

IL-2 binding induces oligomerization of IL-2R components and recruitment of Jak1 and Jak3. This receptor complex initiates activation of downstream signaling pathways[5]. The elevated phosphorylation of Jak1 and Jak3 in TRAF3-deficient T cells suggests that TRAF3 exerts regulatory effects upon the IL-2R complex. Immunoprecipitation of Jak3 showed that TRAF3 interacts with Jak3 upon IL-2 stimulation (**Fig. 7a**). Jak1 and Jak3 were also detected in TRAF3 immunoprecipitates from stimulated T cells (**Fig. 7b**). In human CD4^{+} T cells, we also observed interaction between TRAF3 and Jak3 (**Fig. 7c**). Thus, TRAF3 was recruited to the IL-2R complex upon IL-2 signaling in both mouse and human T cells. It has

been reported that the phosphatase TCPTP associates with Jak1 and Jak3 to negatively regulate IL-2 and IFN-γ signaling[14], which was also demonstrated to be true in human T cells (**Supplementary Fig. 7a**). To explore whether TRAF3 affects the binding of TCPTP to the IL-2R complex, Jak3 was immunoprecipitated from both LMC and *Traf3*$^{-/-}$ Tcon cells. TCPTP clearly interacted with Jak3 in LMC CD4^{+} T cells, but the association was negligible in the absence of TRAF3 (**Fig. 7d**). Notably, when CD122 was immunoprecipitated, recruitment of Jak1, Jak3 and SHP-1 to the IL-2R was not significantly changed in *Traf3*$^{-/-}$ T cells compared to LMC T cells (**Supplementary Fig. 7b**), indicating that TRAF3 deficiency specifically affects recruitment of TCPTP to the IL-2R complex. There are two splice variants of TCPTP. The nuclear form (45kD) can access both nuclear and cytoplasmic substrates, and interact with Jak1 and Jak3 to dephosphorylate them in response to IL-2 or IFN-γ stimulation[14]. An alternative cytoplasmic form (48kD), unique to human T cells, is targeted to the endoplasmic reticulum[15]. To explore whether TRAF3 can interact with TCPTP, HA-TRAF3, both forms of TCPTP (45kD and 48kD) and their substrate-trapping mutants (D182A) were overexpressed in 293 epithelial cells. In HA immunoprecipitates, only the 45kD form of TCPTP and its substrate-trapping mutant interacted with TRAF3, but not the 48kD form and its mutant (**Fig. 7e**), consistent with the important role of the nuclear form of TCPTP in IL-2 signaling. Further supporting the interaction between TRAF3 and TCPTP, IFN-γ-induced STAT1 phosphorylation was also enhanced in conventional CD4^{+} T cells in the absence of TRAF3 (**Supplementary Fig. 7c**). To test which domain of TRAF3 is required for the interaction between TRAF3 and TCPTP, either WT TRAF3, or mutant TRAF3 was co-transfected with the 45kD form of TCPTP into 293 cells. Results show that deletion of both the RING and Zinc finger domains was needed to abrogate TRAF3 binding 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 activity[36], overexpression of ubiquitin, TRAF3 and TCPTP in 293 cells did not result in detectable ubiquitination of TCPTP (**Supplementary Fig. 7d**). Taken together, these results clearly indicate that interactions between TRAF3 and TCPTP facilitate TCPTP recruitment to the IL-2R complex upon IL-2 stimulation.

Discussion

A multilayered mechanism is required for controlling T_{reg} cell development[1,4,26]. Although the lineage determination from T_{reg} precursors to Foxp3^{+} T_{reg} cells is limited due to easily saturable niches[37], our results indicate that unrestrained IL-2R signaling can lead to an abnormal accumulation of the tT_{reg} cell population. We demonstrate TRAF3 to be an important factor controlling tT_{reg} cell numbers by negatively regulating IL-2R signals to T_{reg} precursors. TRAF3 was required for TCPTP recruitment to the IL-2R complex, an event that downregulates IL-2 signaling. Downstream of this enhanced signaling in the absence of TRAF3, the transition from T_{reg} precursors to T_{reg} cells was more efficient, resulting in 2-3 fold more tT_{reg} cells in T-*Traf3*$^{-/-}$ mice.

In T cell-specific TRAF3-deficient mice, TCR signaling in peripheral T cells is impaired, yet it suffices to allow normal development of Tcon cells[22]. Here, we found no evidence of altered thymic selection in T-*Traf3*$^{-/-}$ mice, ruling out this potential reason for increased T_{reg} cell number in these mice. The comparable percentage of T_{reg} precursors in LMC and

T-*Traf3*$^{-/-}$ mice also supports the conclusion of unaltered thymic selection. Although iNKT cell development in T-*Traf3*$^{-/-}$ mice is impaired, early stages of iNKT cell development which require TCR signaling are normal, and only later stages are compromised[23]. It thus appears that TRAF3 plays more important roles in TCR signaling in mature T cells than in developmentally immature T cells. Our current findings that IL-2R signaling in T_{reg} precursors, conventional T cells and T_{reg} cells was differentially regulated by TRAF3, further strengthen the concept that TRAF3 plays multifaceted roles in serving different cell types, including distinct developmental stages, as well as distinct receptors within the same cell type[21].

IL-2-induced signaling differs in distinct T cell subsets. Although IL-2 activates S6 kinase in both $CD8^+$ and $CD4^+$ T cells, much higher activation was found in the former[38]. Compared to $CD4^+$ conventional T cells, IL-2 fails to activate the PI3K-Akt pathway in T_{reg} cells, due to high expression of phosphatase and tensin homolog protein (PTEN)[39,40]. Foxp3 expression in T_{reg} cells mediates a unique gene expression profile, which may contribute to regulation of IL-2 signaling[41]. In addition, the constitutive activation of IL-2 signaling in T_{reg} cells due to high expression of CD25 may activate a negative feedback loop[38,39], to alter IL-2 signaling. Consistent with our results that IL-2 signaling and homeostasis of mature T_{reg} cells show little change in the absence of TRAF3, another study found that T_{reg} cell number is only modestly increased in *Traf3*$^{flox/flox}$*Foxp3*$^{GFP\text{-}hCre}$ mice in which TRAF3 is depleted from mature T cells[24]. This report further supports our observation that TRAF3 controls T_{reg} cell number specifically at the precursor stage. Decreased expression of TRAF3 in mature T_{reg} cells shown here also suggest its minimal role in this population. As TRAF3 is involved in many TNFRSF-mediated signaling pathways, it might also impact T_{reg} cell features to some extent through these signaling pathways.

IL-2R signaling is initiated by ligand-induced oligomerization of three IL-2R components which recruit Jak1 and Jak3 and ignite a signaling cascade. TCPTP interacts with Jak1 and Jak3 and dephosphorylates them, restraining IL-2R signaling[14]. In the current study, TRAF3 deficiency caused defective recruitment of TCPTP to Jak1 and Jak3 and selectively affected phosphorylation of STAT5, but not Erk and Akt, indicating that these three signaling pathways are differentially regulated. Although overlapping mechanisms also exist, STAT5 can be directly phosphorylated by Jak3, while activation of PI3K-Akt and Ras-Erk requires additional intermediate molecules, e.g. Shc, Lck and Syk[6,9,10]. It is thus not entirely surprising that these pathways are less affected by loss of TRAF3. Although TRAF3 was not previously considered as regulating IL-2R signaling, TRAF6 can compete with Jak1 for binding to the IL-2Rβ chain and negatively regulate Jak1-Erk signaling. However, PI3KAkt and Jak3-STAT5 pathways were not explored[42]. TRAF6 and TRAF3 appear to play different roles in IL-2 signaling, as they do in a variety of other signaling pathways in immune cells.

TCPTP can directly dephosphorylate p-STAT5 in the nucleus upon prolactin stimulation, independent of receptor recruitment[43]. However, the elevated p-Jak1 and p-Jak3 observed here suggest that TRAF3 facilitates TCPTP effects upstream of IL-2-induced STAT5 phosphorylation. Indeed, the recruitment of TCPTP to the IL-2R complex is defective in the absence of TRAF3. The nuclear form of TCPTP plays critical negative roles in cytokine

signaling, but the role of the cytoplasmic form of TCPTP is less understood. We found that only the nuclear form of TCPTP can interact with TRAF3, consistent with its role in IL-2R signaling, and both WT TCPTP and its substrate-trapping mutant can equally interact with TRAF3, suggesting that TRAF3 may act as a scaffold molecule to recruit TCPTP to the IL-2R complex, rather than as a TCPTP substrate. Similar to our observations, the nuclear form of TCPTP associates with TRAF2 in TNFR signaling, and specifically regulates TNF-induced MAPK but not NF-κB activation[44]. As nuclear TCPTP translocates to the cytoplasm upon stimulation, it is quite possible that it interacts with TRAF3 or TRAF2 in the cytoplasm. However, the possibility that TRAF3 and/or TRAF2 also play roles in the process of TCPTP translocation cannot be excluded.

The TRAF domain of TRAF3 is thought to be mostly involved in self-association and interaction with upstream receptors. However, the N-terminus of TRAF3, but not the TRAF domain is required for interactions between NIK and TRAF3, although the TRAF domain is indispensable for association with CD40 or BAFFR in B cells[45]. In addition, the N-terminus of TRAF3 is required for interaction between NIK and TRAF3 in 293 epithelial cells[46]. Therefore, the N-terminus of TRAF3 can also be involved in protein-protein interactions. Our findings are consistent with these reports, in that the N-terminus is required for TRAF3 to associate with TCPTP.

Results of the present study emphasize that IL-2R signaling in the transition stage from T_{reg} cell precursors to Foxp3$^+$ T_{reg} cells is the critical checkpoint for controlling T_{reg} cell numbers. Our observations shed light on the molecular regulation of IL-2R signaling and may provide novel avenues for manipulation of T_{reg} cell numbers.

ONLINE METHODS

Mice

Traf3$^{flox/flox}$ mice were previously described[47] and backcrossed with C57BL/6 mice for at least 10 generations. *Traf3*$^{flox/flox}$ mice were bred with *Cd4*^{Cre} mice (Taconic Farms, Hudson, NY) and *Lck*^{Cre} mice (Jackson Labs, Bar Harbor, ME). CD45.2$^+$ C57BL/6 and congenic CD45.1$^+$ C57BL/6 mice (Jackson Labs) were bred to generate CD45.2 and CD45.1 double positive mice. *Cd28*$^{-/-}$ mice were purchased from Jackson Labs. *Rag1*$^{-/-}$ mice were provided by Dr. Fayyaz Sutterwala (University of Iowa, Iowa City, IA). Foxp3GFP mice were originally generated by Dr. Alexander Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY) and provided by Dr. Thomas J. Waldschmidt (University of Iowa). OTII transgenic mice were provided by Dr. Annette Schlueter (University of Iowa). *Nik*$^{-/-}$ mice were originally generated by Dr. Robert Schreiber (University of Washington at St. Louis, St. Louis, MS) and provided by Dr. David Parker (Oregon Health Science University, Portland, OR). Mice of 6-12 weeks of age were used for all experiments except when indicated. All mice were maintained under specific pathogen-free conditions at The University of Iowa and were used in accordance with National Institutes of Health guidelines under an animal protocol approved by the Animal Care and Use Committee of the University of Iowa.

Retrovirus transduction and bone marrow chimeras

For virus packaging, the mouse *Traf3* gene was cloned and inserted into the retrovirus backbone pMIG. pMIG or pMIG-*Traf3* and helper vector pCLECO were co-transfected into 293T epithelial cells using lipofectamine (Invitrogen, Grand Island, NY). Supernatant was harvested after 48hr. Lineage negative BM cells were purified using a Miltenyi kit (Cambridge, MA), and were stimulated overnight with a cytokine combination (IL-6, IL-3, SCF) (Peprotech, Rocky Hill, NJ), and transduced with viral supernatant. Recipients *Rag1*$^{-/-}$ mice were prepared by sublethal irradiation with 500 rads γ-ray and rested overnight. 0.5×10^6 transduced BM cells were transferred by i.v. injection. The resulting chimeras were analyzed 8 wks later[48].

Recipient CD45.1$^+$CD45.2$^+$ congenic C57BL/6 mice were given 1100 rads γ-irradiation 16 hr before transfer. BM cells harvested from the tibiae and femurs of WT (CD45.1$^+$) and T-*Traf3*$^{-/-}$ (CD45.2$^+$) mice were depleted of B220$^+$ and CD3$^+$ cells by Miltenyi magnetic bead separation and mixed at a 1:1 or 20:1 ratio. 10×10^6 BM cells were injected intravenously into recipient mice. Mice were euthanized 8 wks later for experiments.

In another set of experiments, BM cells isolated from *Rag1*$^{-/-}$ mice were mixed with BM cells from WT, *Nik*$^{-/-}$, T-*Traf3*$^{-/-}$, *Nik*$^{-/-}$ *Traf3*$^{-/-}$ mice at a 10:1 ratio. 10×10^6 BM cells were injected intravenously into sublethally irradiated *Rag1*$^{-/-}$ mice. Mice were euthanized 8 wks later for experiments. Mouse irradiation was conducted at the Free Radical & Radiation Biology Core, University of Iowa.

Flow cytometry

Single-cell suspensions were prepared from thymus, spleen, lymph nodes, liver and Peyer's Patch, and erythrocytes were lysed. For flow cytometry staining, cells were blocked with anti-mouse CD16/CD32 mAb and stained with fluorescently labeled antibodies against CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), Foxp3 (FJK-16s), B220 (RA3-6B2), CD122 (5H4), CD132(TUGh4), GITR (DTA-1), CTLA4 (UC10-4B9), CD25 (eBio7D4), Ki67 (SolA15), Helios (22F6), Neuropilin (761705), Nur77 (12.14), CD24 (M1/69), CD5 (53-7.3), CD69 (H1.2F3), TCR-β (H57-597), CD45.2 (104) and CD45.1 (A20). For intracellular phosphoprotein staining, cells were fixed immediately after treatment, then permeabilized and stained with fluorescently labeled Abs against surface markers and anti-p-STAT5 (D47E7) or p-Erk (D13.14.4E) or p-Akt (D25E6) Ab, followed by anti-rabbit–APC secondary Ab. All antibodies were purchased from eBioscience (San Diego, CA), BD Biosciences (San Jose, CA), R&D System (Minneapolis, MN) or Cell Signaling Technology (Danvers, MA). For T_{reg} precursor cell sorting, CD8$^+$ T cells were depleted with Miltenyi beads and the remaining cells were stained for CD4, CD8 and CD25. CD4 single positive Foxp3GFP$^-$CD25$^+$ cells were sorted as T_{reg} precursor cells. Flow cytometric analysis and cell sorting were performed using a BD FACS LSRII or Aria at The University of Iowa Flow Cytometry Facility. Results were analyzed with FlowJo software (TreeStar).

Foxp3 expression stability assay

CD4$^+$CD25$^+$ T_{reg} cells were purified from spleen with Miltenyi beads, and cultured in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT),

10 mM HEPES (Sigma, St. Louis, MO), 1 mM sodium pyruvate (Sigma), 50 μM 2-ME (Gibco), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). Cells were stimulated with 1ug/ml anti-CD3 Ab and 2ug/ml anti-CD28 Ab (eBioscience) and/or 50U rmIL-2 (Peprotech) for 72 hrs. Frequency and expression level of Foxp3 were measured by flow cytometry.

T_{reg} cell death assay

To measure *in vitro* cell death, enriched splenic $CD4^+CD25^+$ T_{reg} cells were stimulated with 1μg/ml anti-CD3 and 2 μg/ml anti-CD28 Abs. Samples were taken at various time points and stained for Annexin V (BD Bioscience) and Foxp3 and analyzed by flow cytometry. To detect *ex vivo* T_{reg} cell death, freshly isolated splenocytes were stained for Annexin V, CD4, CD8 and Foxp3 and analyzed by flow cytometry.

Bromodeoxyuridine (BrDU)*in vivo* incorporation assay

Mice were administered 2 mg BrdU (Sigma) i.p. 24 hr prior to analysis. To detect BrdU incorporation, cells were first stained for Foxp3 followed by intracellular BrdU staining with anti-BrdU Ab (clone PRB-1; eBioscience) using a BrdU flow cytometry detection kit (BD Biosciences) according to the manufacturers' instructions. Samples were analyzed using flow cytometry.

In vitro T_{reg} cell suppressive assay

$CD4^+CD25^-$ $CD62L^{hi}$ T cells (5×10^4) were isolated from LMC spleen. They were cultured for 72 hrs with irradiated splenocytes (2×10^5) and anti-CD3 Ab (2μg/ml) in the presence or absence of various numbers of T cells. ^{3}H-thymidine was added for the last 16 hrs. Cells were harvested and CPM were measured on a β-counter (LS6500, Beckman Coulter, CA, USA).

In vivo T_{reg} cell suppressive assay

Naïve $CD4^+CD45RB^{hi}CD25^-$ T cells (4×10^5) were sorted from LMC spleen. They were transferred into *Rag1*$^{-/-}$ mice alone or in combination with LMC or T-*Traf3*$^{-/-}$ T cells (4×10^5). After T cell reconstitution, mice were weighed weekly and monitored for signs of disease. Mice were euthanized at 8 weeks after T cell transfer and their colons were used for histopathology analysis. Histological score was performed according to previous description[49]. Briefly, grade 0, no changes were observed; 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, marked inflammatory infiltrates commonly transmural with ulceration, marked mucosal hyperplasia and mucin depletion, and multifocal crypt necrosis; grade 5, marked transmural inflammation with ulceration, wide-spread crypt necrosis and loss of intestinal glands.

In vivo T_{reg} cell survival assay

$CD4^+CD25^+$ T_{reg} cells were isolated from spleen in WT ($CD45.1^+$) and T-*Traf3*$^{-/-}$ mice ($CD45.2^+$), and mixed at a 1:1 ratio. 2×10^6 cells were transferred into WT recipient mice ($CD45.1^+CD45.2^+$) by intravenous injection. 21 days after transfer, splenocytes were harvested for staining and flow cytometry analysis.

In vitro thymic negative selection

T cell- depleted splenocytes were irradiated and pulsed with 10 μg/ml OVA peptide 323-339 (ISQAVHAAHAEINEAGR) (AnaSpec, San Jose, CA). 2×10^5 LMC and T-*Traf3*$^{-/-}$ OTII thymocytes were stimulated with 1×10^6 OTII peptide-pulsed antigen presenting cells for different time point. They were stained for CD4, CD8, Annexin V and propidium iodide (PI) and analyzed by flow cytometry.

T_{reg} cell induction from precursors

Sorted T_{reg} precursor cells ($CD4^+Foxp3GFP^-CD25^+$) were seeded into 96-well plates in the presence of medium alone or medium containing rmIL-2 (50U/ml). In another set of experiments, cells were treated with 100 nM Trichostatin A (Sigma, St. Louis, MO), 800 nM Apicidin (Sigma, St. Louis, MO), or DMSO (0.2%) and rmIL-2[2]. 24 hrs later, cells were analyzed by flow cytometry.

DNA methylation analysis

$CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ conventional T cells were sorted from spleen. Only male mice were used. Genomic DNA was isolated and methylation analysis was performed by bisulfite conversion of genomic DNA using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA) following the manufacturer instructions. PCR were performed as previously described[27]. The PCR product was cloned using the TOPO TA Cloning kit (Invitrogen).

TRAF3 and TCPTP depletion by siRNA

Human lymphocytes were obtained from the DeGowin Blood Center at the University of Iowa. Blood from healthy donors aged 18-55 years gave 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 using leukocyte reduction cones. $CD4^+$ T cells were purified with Milteyni beads and stimulated with 5μg/ml anti-human CD3 and CD28 Abs (eBioscience) for 24 hrs. *Traf3* or *Tcptp* Trilencer-27 siRNA was transfected into cells with siTran 1.0 (Origene, Rockville, MD), according to the manufacturer's instructions. 20U/ml IL-2 was added during transfection. Cells were transfected again after 24 hrs. After another 24 hr incubation, cells were washed and rested in regular medium without IL-2 twice with a 6 hr interval. They were stimulated with rhIL-2 (Peprotech) for indicated times and Western blots were performed for p-STAT5 detection.

Fetal thymic organ culture

For fetal thymic organ culture, E14-E15 fetal thymuses were harvested, bisected and cultured in trans-well plates. Pairwise comparisons of thymic lobes with 20 μg/ml of isotype

control Ab or CD25 blocking Ab (3C7) (ebioscience) were set up. Medium with antibodies was changed on day 5. Single cell suspensions were stained with fluorescently-labeled anti-CD4, anti-CD8, anti-CD25, anti-Foxp3 Abs on day 10 and analyzed by flow cytometry.

Chromatin Immunoprecipitation assay

1×10^7 $CD4^+CD25^-$ cells isolated from spleen were stimulated with 500U/ml rmIL-2 for 1 hr. Cells were crosslinked and nuclei were subjected to chromatin shearing according to the manufacturer's instructions (truCHIP Chromatin Shearing Reagent Kit, Covaris, Woburn, MA). STAT5 was immunoprecipitated overnight at 4 °C with anti-STAT5 Ab (Cell Signaling Technology). The precipitated DNA was quantified by real-time PCR to determine the relative abundance of STAT5 associated target DNA. Specific primers for analysis of the chromatin binding to STAT5 are for IL-2Rα chain: 5'-GCATGATATGATGTGCAGTTTCTTC-3' and 5'-TCAGGACTGGTGGTTGGTTG-3'; and for Cis: 5'-GTCCAAAGCACTAGACGCCTG-3' and 5'-TTCCCGGAAGCCTCATCTT-3'.

Immunoprecipitation (IP) and Western blots

$CD4^+CD25^-$ T cells or $CD4^+CD25^+$ T_{reg} cells were treated with IL-2 for different time points. Whole cell lysates were separated by SDS-PAGE and transferred to PVDF membranes for Western blots. Alternatively, whole cell lysates were first pre-cleared with magnetic beads, then the relevant Ab was added and incubated for 4 hrs with rotation. Magnetic beads were added and incubated with rotation overnight. IP products were used for Western blots. To measure direct interactions between TRAF3 and TCPTP, the pMIG-HA-*Traf3*, or pMIG-HA-*Traf3* mutant (Δ1-113) (RING domain deleted), or pMIG-HA-*Traf3* mutant (Δ 1-258) (both RING and Zinc Finger regions deleted), or pMIG-HA-*Traf3* mutant (Δ 382-568) (TRAF domain deleted) plasmid was co-transfected with a TCPTP-encoding plasmid into 293 cells. 48 hrs later, 293 cells were harvested and lysed. Anti-HA Ab (HA-7, Sigma) was used for TRAF3 IP. For TCPTP ubiquitination assay, HA-tagged ubiquitin, FLAG-tagged TRAF3 and TCPTP were transfected into 293 cells. 48 hrs later, cell lysates were denatured by boiling in 1% SDS for 1 min. 1:10 diluted cell lysates were immunoprecipitated with anti-TCPTP Ab. Western blotting was performed and anti-HA antibody was used for ubiquitination detection[36]. Plasmids carrying 45KD and 48KD forms of TCPTP and their 182D/A substrate-trapping mutants were kindly provided by Dr. Nicholas Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY)[50]. Abs used for IP and Western blots include: Anti-Jak1 (6G4), p-Jak1, Jak3 (D1H3), CD122 (C-2), SHP-1 (C14H6), TCPTP (252294), TCPTP (6F3), STAT5 (3H7), p-STAT5 (D47E7), p-Erk (D13.14.4E), p-Akt (D25E6), β-actin (EP1123Y), p-Y (4G10), FLAG (M2), TRAF3 (M20) and TRAF3 (H122) Abs. They were purchased from Cell Signaling Technology, Santa Cruz Biotech, R&D System, Sigma, EMA Millipore (Billerica, MA), or Medimabs (Montreal, Quebec, Canada). Densitometry analysis was done using ImageJ software (National Institutes of Health) for Western blots.

Statistical analysis

Results are presented as mean values ± SEM. Statistical differences between two means were evaluated using the two-tailed unpaired Student's t-test. For comparisons of multiple groups, two-way ANOVA 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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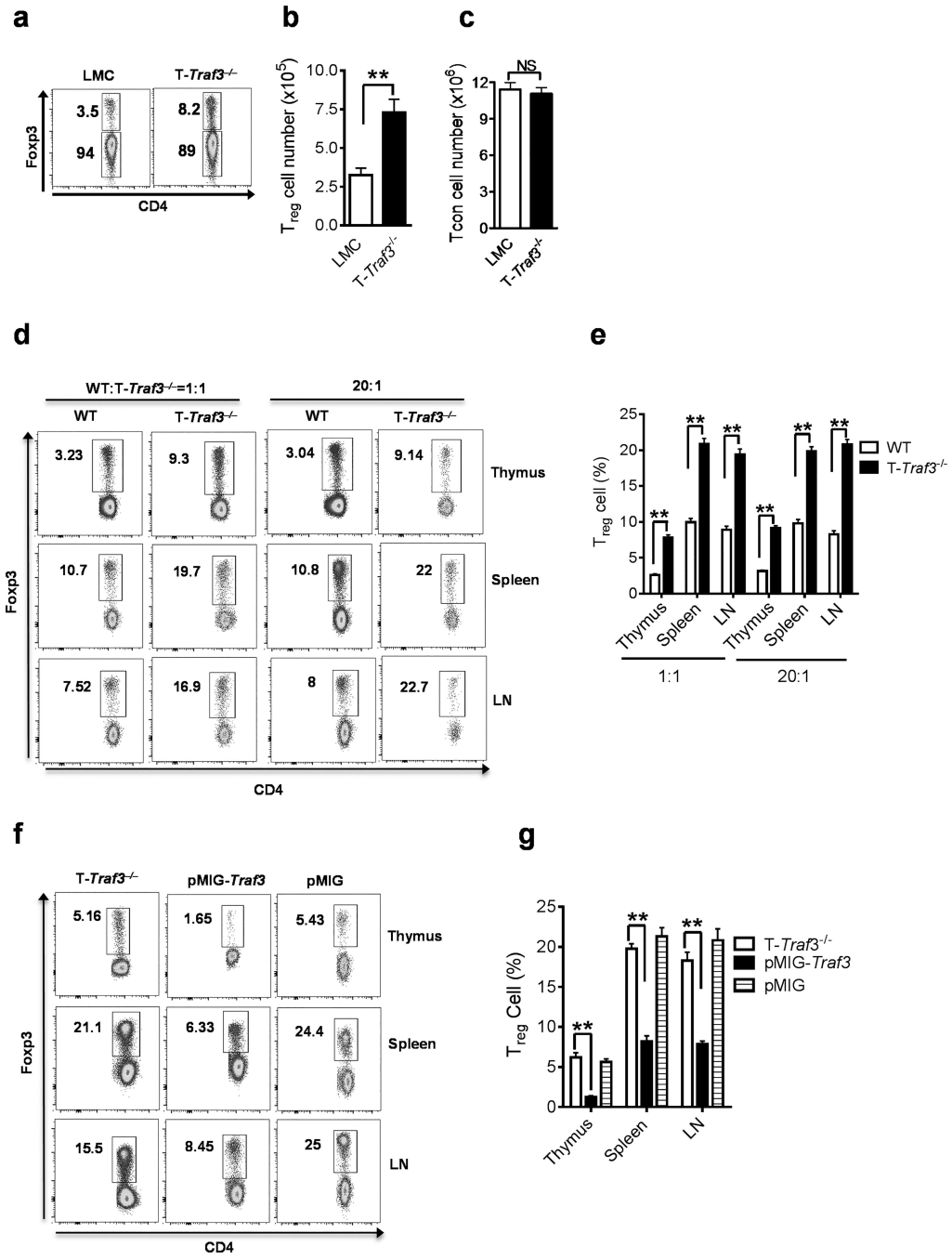

Figure 1. TRAF3 restrains T_{reg} cell development in a cell intrinsic manner

(**a**) Frequency of representative mice and (**b, c**) number of T_{reg} cells and Tcon cells among thymic CD4 single positive cells in LMC (littermate controls) or T-*Traf3*$^{-/-}$ (mean ± SEM, n= 10 mice).. (**d**) Frequency of Foxp3^{+} cells in CD4 single positive cells in indicated tissues. WT and T-*Traf3*$^{-/-}$ immune cells in BM chimeric mice were distinguished by congenic markers CD45.1^{+} and CD45.2^{+} respectively (representative of 6 mice). (**e**) Summary of data in (d) (mean ± SEM, n= 6 mice). LN: inguinal lymph nodes.. (**f**) Percentage of Foxp3^{+} cells of CD4 single positive cells in indicated tissues from BM chimeric mice (representative of 4

mice). BM cells isolated from T-*Traf3*$^{-/-}$ mice (GFP^{-}) were transduced with viral vectors encoding GFP (pMIG) or TRAF3 and GFP (pMIG-*Traf3*) (GFP^{+}). (**g**) Summary of data in (f) (mean ± SEM, n= 4 mice). **p<0.001. NS, not significant (unpaired two-tailed Student's *t*-test).

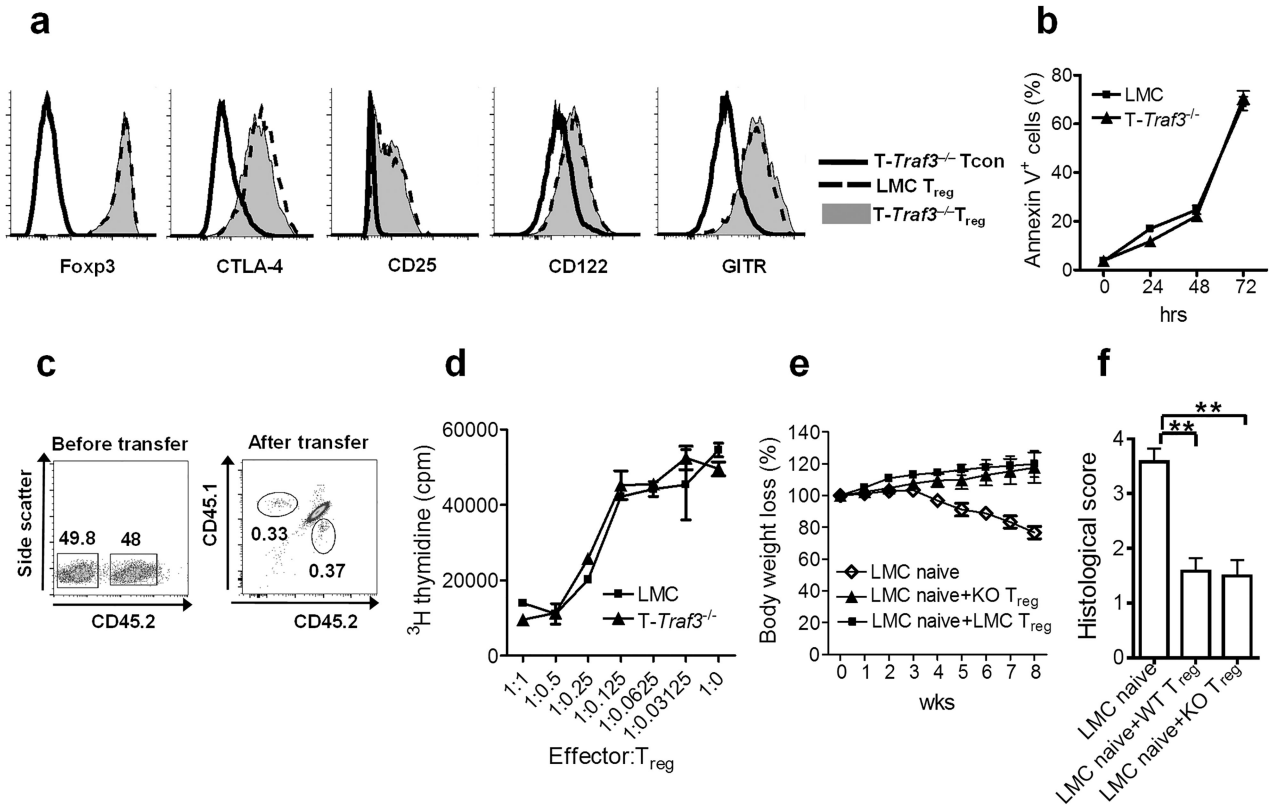

Figure 2. TRAF3 deficiency does not alter T_{reg} cell properties

(**a**) Expression of Foxp3, CTLA-4, CD25, CD122 and GITR in $CD4^+Foxp3^+$ cells in spleen. $CD4^+Foxp3^-$ T conventional cells (Tcon) were used as control. Data are representative of one of six mice for each group. (**b**)Frequency of T-*Traf3*$^{-/-}$ and LMC death measured by Annexin V^+ staining amongst $Foxp3^+$ cells. Splenic $CD4^+CD25^+$ T_{reg} cells were stimulated *in vitro* with anti-CD3 and CD28 Abs. Data represent one of three individual experiments. (**c**) $CD4^+CD25^+$ T_{reg} cells isolated from WT ($CD45.1^+$) and T-*Traf3*$^{-/-}$ mice ($CD45.2^+$) were mixed at a 1:1 ratio and transferred into WT recipients ($CD45.1^+CD45.2^+$). Left panel shows ratio of WT and T-*Traf3*$^{-/-}$ $Foxp3^+$ cells before transfer. Right panel shows percentage of $CD45.1^+$ or $CD45.2^+$ cells in $CD4^+$ $Foxp3^+$ cells in spleen at 21 days after transfer. Data represent one of three recipient mice. (**d**) Suppressive function of T_{reg} cells was measured *in vitro* as described in Methods. Data represent one of three individual experiments. (**e**) Weight loss of *Rag1*$^{-/-}$ mice transferred with naive LMC T cells or a mixture of LMC or T-T*raf3*$^{-/-}$ (KO) T_{reg} cells, as indicated. (mean ± SEM, n= 6 mice/ group). (**f**) Hematoxylin and eosin stained colon sections from mice in (**e**) were scored as in Methods. **p<0.001. (mean ± SEM, n= 6 mice/group) (unpaired two-tailed Student's *t*-test).

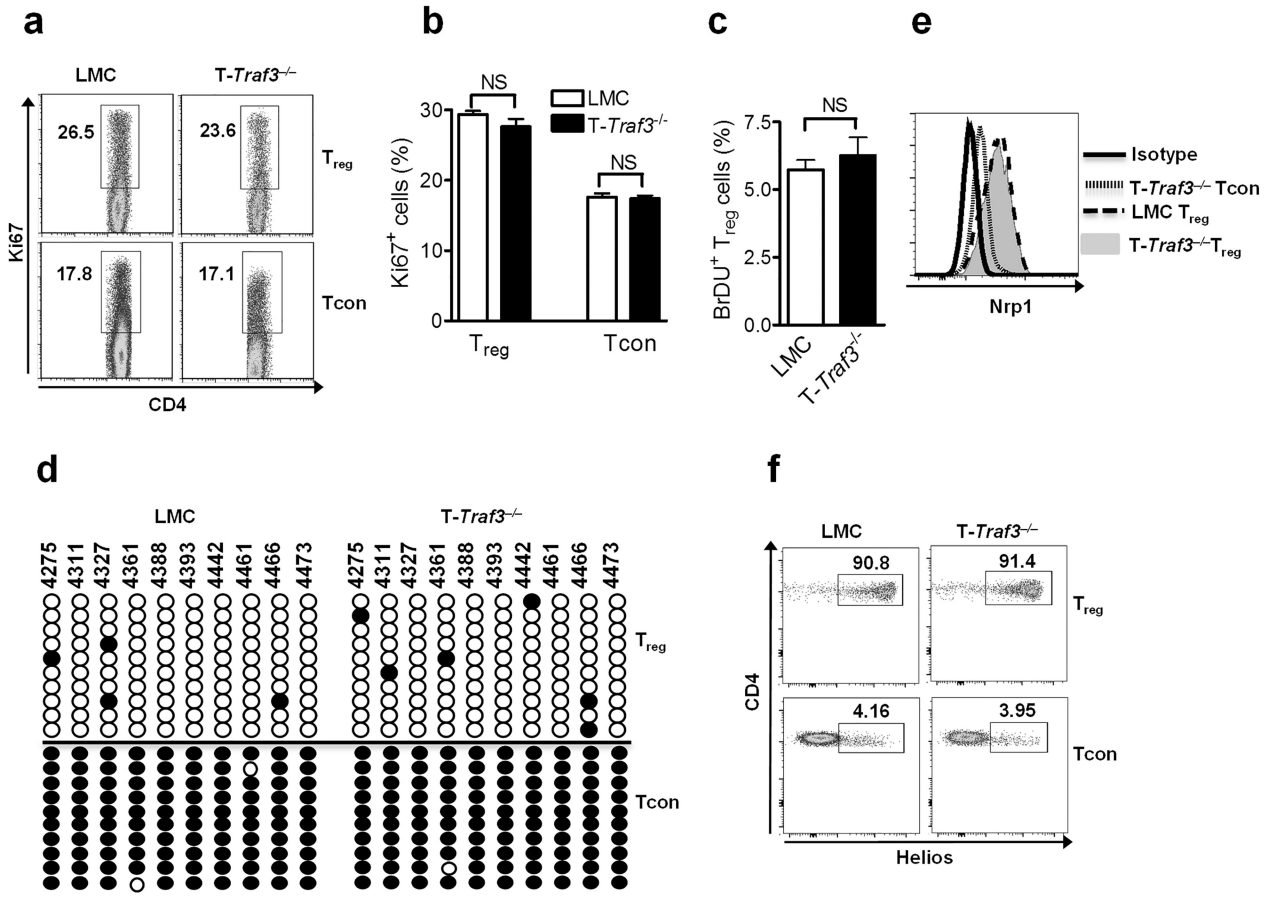

Figure 3. T_{reg} cells in T-T*raf3–/–* mice are derived from the thymus

(**a**) Frequency of $Ki67^+$ cells in $Foxp3^+$ cells in spleen and (**b**) data summarized from (a) (mean ± SEM, n= 6 mice/group). (**c**) Frequency of BrDU positive cells in $Foxp3^+$ cells in spleen. (mean ± SEM, n= 4 mice/group). (**d**) The methylation status of CpG islands within *Foxp3* CNS2 was determined in $CD4^+CD25^+$ T_{reg} cells and $CD4^+CD25^-$ conventional T cells. The upper number indicates position of CpGs relative to the transcription start site of the *Foxp3* gene. Open circle: unmethylated CpGs; filled circle: methylated CpGs. (**e and f**) Expression of Nrp1 (**e**) and percentage of $Helios^+$ cells (**f**) in $Foxp3^+$ cells in spleen. Data are representative of one of 6 mice. NS, not significant (unpaired two-tailed Student's *t*-test).

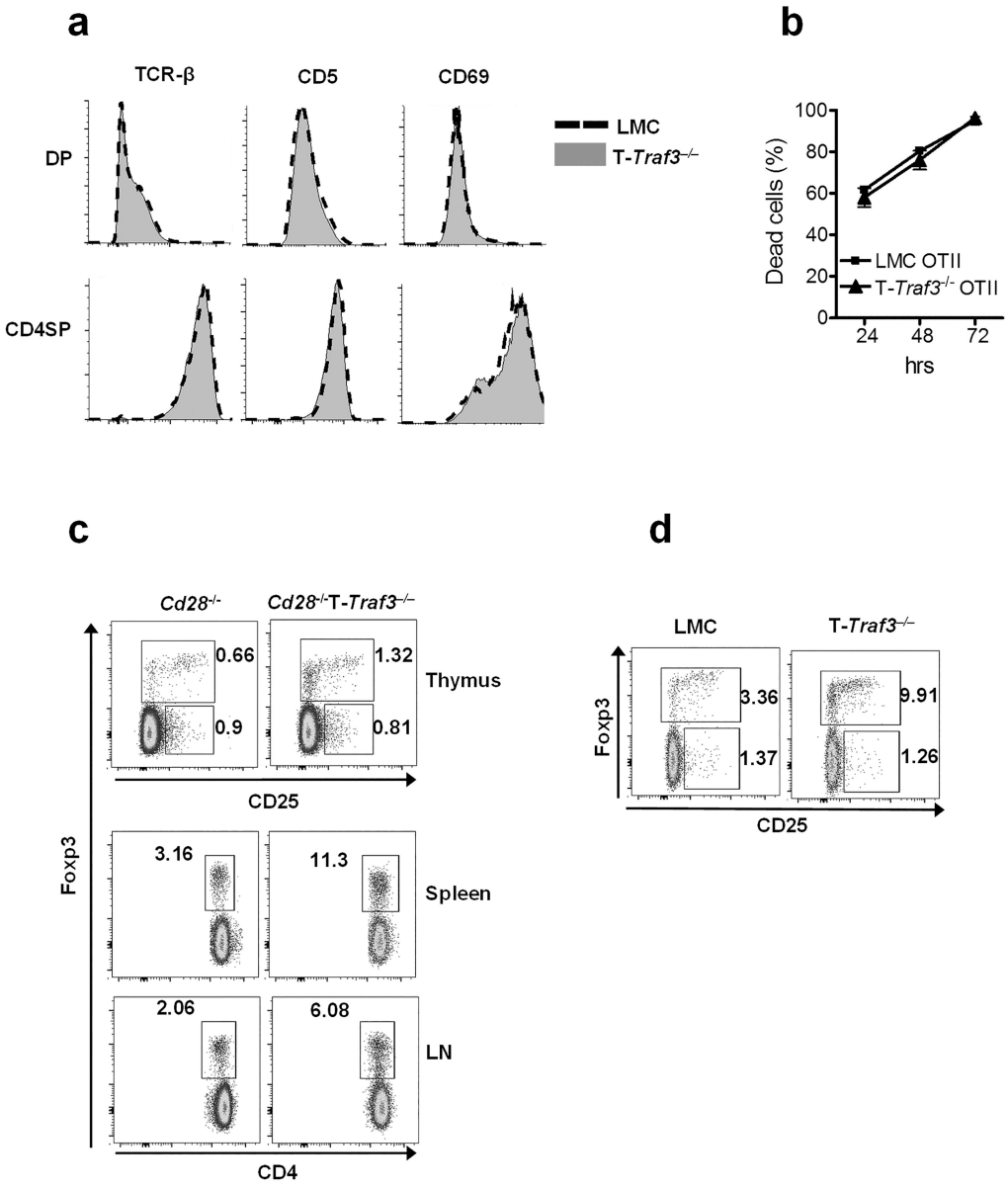

Figure 4. Unaltered thymic selection in T-*Traf3*$^{-/-}$ mice

(**a**) Expression of TCR-β, CD5 and CD69 in CD4^{+}CD8^{+} double positive (DP) thymocytes and CD4 single positive (SP) cells. (**b**) Thymocyte death measured by percentage of Annexin V and propidium iodide positive cells in DP thymocytes. Thymocytes isolated from LMC OTII and T-*Traf3*$^{-/-}$ OTII TCR transgenic mice were stimulated with OVA peptide-pulsed APC. Data represent one of three individual experiments, each with one mouse. (**c**) Flow cytometric analysis of T_{reg} cells and precursor cells in *Cd*28$^{-/-}$ and *Cd*28$^{-/-}$T-*Traf3*$^{-/-}$ mice. Numbers represent percentage of Foxp3^{+} cells, or Foxp3^{-}CD25^{+} cells in the CD4SP population. (**d**) Percentage of Foxp3^{+} T_{reg} cells and Foxp3^{-}CD25^{+} T_{reg} precursors in CD4SP thymocytes in LMC and T-*Traf3*$^{-/-}$ mice. Data are representative of one of six mice (**a** and **c**) or ten mice (**d**).

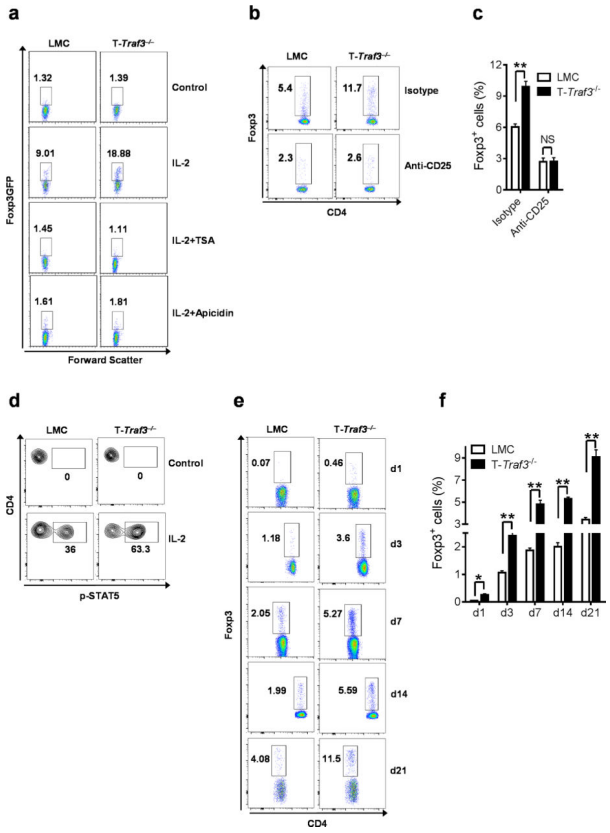

Figure 5. Enhanced IL-2-dependent transition from precursor to T_{reg} cell in the absence of TRAF3

(**a**) Thymic $CD4^+Foxp3GFP^-$ $CD25^+$ T_{reg} precursors were sorted and cultured with 50 U/ml rmIL-2 with/without TSA or Apicidin for 24hr. Frequency of $Foxp3GFP^+$ cells was analyzed by flow cytometry. (**b**) Percentage of $Foxp3^+$ cells in CD4SP thymocytes from FTOC, cultured for 10 d with anti-CD25 blocking Ab or isotype control Ab, is shown. Summarized data are shown in (**c**). (6 fetuses for each group). (**d**) Sorted T_{reg} precursors were incubated with 50U rmIL-2 for 20 min. Phosphorylation of STAT5 was stained and analyzed by flow cytometry. (**e**) Percentage of $Foxp3^+$ T cells in CD4 SP thymocytes in newborn d1 to d21-old mice. (**f**) Summarization of data shown in (**e**). (mean ± SEM, n= 6 mice/group). Data represent one of three individual experiments with one mouse in each (**a** and **d**). *p<0.05, **p<0.001. NS, not significant (unpaired two-tailed Student's *t*-test).

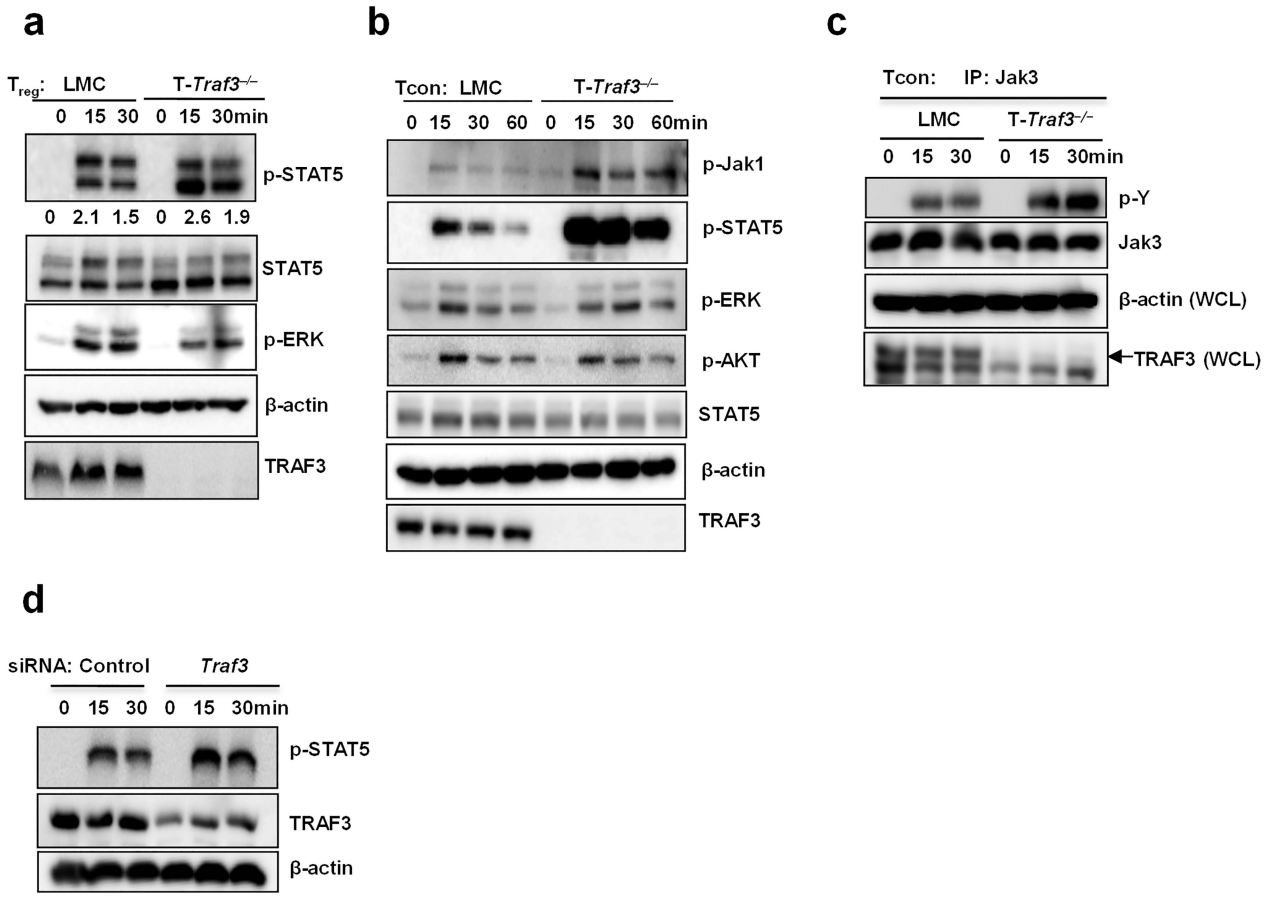

Figure 6. TRAF3 restrains IL-2 signaling in conventional CD4$^+$ CD25$^-$ T cells

(**a**) CD4$^+$CD25$^+$ T$_{reg}$ cells were stimulated with 50U/ml rmIL-2. Immunoblots of cell lysates probed for p-STAT5, STAT5, p-Erk, TRAF3 and β-actin are shown. Numbers under p-STAT5 blot show results of densitometric analysis (p-STAT5 vs. total STAT5). (**b, c**) CD4$^+$CD25$^-$ T cells were stimulated with 500U/ml rmIL-2. Immunoblots of cell lysates probed for p-Jak1, p-STAT5, p-Erk, p-Akt, STAT5, TRAF3 and β-actin (**b**). Jak3 immunoprecipitates (IPs) were blotted with anti-p-tyrosine (p-Y) and Jak3 Abs, and whole cell lysates (WCL) were blotted with anti-TRAF3 and β-actin Abs (**c**). (**d**) Activation of IL-2R signaling in human CD4$^+$ T cells under the indicated conditions. Human CD4$^+$ T cells were transfected with *Traf3* siRNA or control scrambled siRNA and treated with 5ng/ml rhIL- 2. Immunoblots of cell lysates probed for p-STAT5, TRAF3, STAT5 and β-actin are shown. Data represent three (**a, c**), five (**b**) and two (**d**) individual experiments.

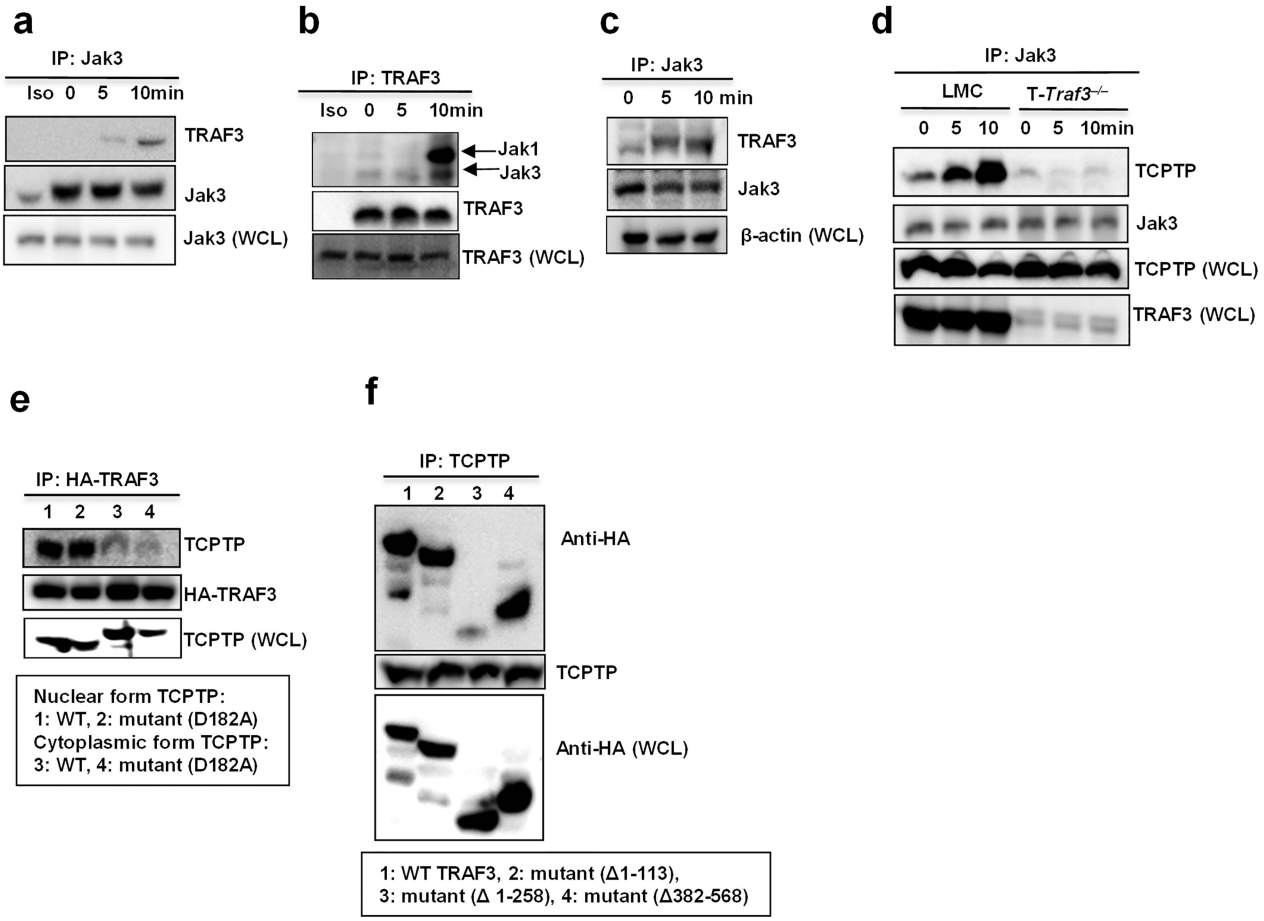

Figure 7. TRAF3 interaction with TCPTP in IL-2R signaling

(**a,b,d**) $CD4^+CD25^-$ T cells isolated from spleen were stimulated with 500 U/ml rmIL-2. Jak3 IPs were blotted for TRAF3 (**a**). TRAF3 IPs were blotted for Jak1 and 3 (**b**). Jak3 IPs were blotted for TCPTP (**d**). (**c**) Human $CD4^+$ T cells treated with 5 ng/ml rhIL-2 were lysed and immunoprecipitated for Jak3. IPs were blotted for TRAF3. (**e**) 293 cells were co-transfected with plasmids encoding HA-TRAF3 and the nuclear or cytoplasmic form of TCPTP WT or its substrate-trapping mutant. IPs for HA-TRAF3 were probed for TCPTP. (**f**) 293 cells were co-transfected with plasmids encoding nuclear form of TCPTP WT and HA-TRAF3 WT and its mutants. IPs for TCPTP were probed for HA. Data represent three individual experiments in (**a, b and d**) and two individual experiments in (**c, e and f**).