Tumor necrosis factor receptor superfamily costimulation couples T cell receptor signal strength to thymic regulatory T cell differentiation

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Abstract

Regulatory T (T_{reg}) cells express tumor necrosis factor receptor superfamily (TNFRSF) members, but their role in thymic T_{reg} development is undefined. We demonstrate that T_{reg} progenitors highly express the TNFRSF members GITR, OX40, and TNFR2. Expression of these receptors correlates directly with T cell receptor (TCR) signal strength, and requires CD28 and the kinase TAK1. Neutralizing TNFSF ligands markedly reduced T_{reg} development. Conversely, TNFRSF agonists enhanced T_{reg} differentiation by augmenting IL-2R/STAT5 responsiveness. GITR-ligand costimulation elicited a dose-dependent enrichment of lower-affinity cells within the T_{reg} repertoire. *In vivo*, combined inhibition of GITR, OX40 and TNFR2 abrogated T_{reg} development. Thus TNFRSF expression on T_{reg} progenitors translates strong TCR signals into molecular parameters that specifically promote T_{reg} differentiation and shape the T_{reg} repertoire.

The development of T lymphocytes with randomly generated T cell receptors (TCRs) allows for diverse and productive immune responses, but also raises the risk for autoimmunity. The thymic medulla contains antigen presenting cells (APCs) that display self-peptide:major histocompatibility (MHC) complexes to immature CD4 and CD8 single positive (SP) thymocytes, and plays a critical role in limiting autoimmunity[1]. This occurs in part by deletion of a substantial fraction of autoreactive thymocytes via the process of negative selection. However, some autoreactive T cells escape deletion in the thymus, and thus alternative mechanisms to control autoreactivity have evolved including the production of thymic-derived regulatory T cells (T_{reg})[2]. The transcription factor Foxp3 plays a critical role in this process as inactivating mutations in *Foxp3* abrogate T_{reg} development and cause lethal multi-organ lymphoproliferative disease[3, 4]. More subtle perturbations in T_{reg} development and/or function, such as reduced responsiveness to interleukin-2 (IL-2), are also associated with autoimmune diseases such as Type 1 Diabetes[5-7]. T_{reg} development occurs via a multistep differentiation process that preferentially drives high affinity autoreactive T cells to express *Foxp3*. A key question is how thymocytes translate relative differences in TCR signal strength into distinct developmental programs that give rise to naïve CD4^{+} T cells or regulatory T cells, respectively.

Thymic T_{reg} development occurs via a two-step process[8, 9]. An initial TCR-CD28-driven signaling event leads to expression of CD25 and CD122 (the α and β subunits of the IL-2 receptor) and c-REL-dependent chromatin remodeling at the *Foxp3* locus[10-15]. A second, TCR-independent but cytokine-dependent step occurs when CD25^{+}Foxp3^{−} T_{reg} progenitors receive an IL-2 signal, which via a STAT5-dependent process drives *Foxp3* expression to complete the Treg differentiation process[8, 9, 16].

How high affinity TCR stimuli drive a molecular program that results in T_{reg} differentiation remains poorly defined. A hallmark of T_{reg} progenitor cells that might play a role in this process is their high expression of the TNFRSF member TNFRSF18, (called GITR hereafter). As GITR expression precedes the induction of Foxp3 in developing T_{reg} cells, we hypothesized that costimulation via GITR may support conversion of T_{reg} progenitors into mature Foxp3^{+} T_{reg} cells in the thymus.

In addition to GITR, several other TNFRSF members are expressed on T_{reg} cells[17]. We screened thymocytes by flow cytometry and found that GITR, TNFRSF4 (called OX40 herafter), and TNFRSF1B (called TNFR2 hereafter) are uniquely overexpressed on T_{reg} progenitors when compared to conventional CD4SP thymocytes. Multiple APCs including dendritic cell subsets and medullary thymic epithelial cells express the corresponding ligands GITR-L, OX40-L, and TNF. We found that TNFRSF expression by T_{reg} progenitors strongly correlates with TCR signal strength. Thus, CD4SP thymocytes encountering the highest affinity TCR signals most strongly upregulate GITR, OX40 and TNFR2. This process occurred via a TAK1-and CD28-dependent pathway as TAK1- and CD28-deficient T_{reg} progenitors fail to express GITR, OX40, and TNFR2 and do not convert into mature Foxp3^{+} T_{reg} cells. Stimulation of wild type T_{reg} progenitors with either GITR-L or OX40-L promoted their conversion into mature Foxp3^{+} T_{reg} cells at much lower doses of IL-2. In contrast, the addition of neutralizing antibodies to TNFSF members in neonatal thymic organ cultures markedly inhibited T_{reg} development. Likewise, blocking signaling

collectively through OX40, TNFR2 and GITR completely abrogated T_{reg} development *in vivo* indicating that GITR, OX40 and TNFR2 function in a cell-intrinsic manner to program T_{reg} differentiation. Finally, competition for TNFRSF costimulation skews the T_{reg} repertoire toward higher affinity TCRs, as increasing GITR-L availability dose-dependently broadens the T_{reg} repertoire by enriching for cells with reduced TCR signal strength. These findings support a model in which high-affinity TCR signals in CD4SP thymocytes are translated into a molecular program that entrains T_{reg} development via increased expression of specific TNFRSF members. Ligation of GITR, OX40 or TNFR2 on T_{reg} progenitors enhances the ability of T_{reg} progenitors to compete for limiting amounts of IL-2 and thereby helps define the developmental niche for T_{reg} cells in the thymus.

Results

T_{reg} progenitors highly express GITR, OX40 and TNFR2

A prominent feature of T_{reg} progenitors is their high-level expression of GITR, which is upregulated prior to Foxp3 during T_{reg} development (refs 8, 9 and Fig. 1a). Studies to elucidate a role for GITR in T_{reg} cells have primarily focused on the effects of costimulation on mature T_{reg} cells in peripheral lymphoid organs[18-20]. However, whether GITR costimulation plays any role during thymic T_{reg} development is unknown. Because of the large potential for redundancy within the TNFRSF, we sought to clarify which of these receptors are expressed during T_{reg} development. Staining Foxp3-GFP thymocytes with specific antibodies to evaluate expression by conventional (non-T_{reg}) CD4SP thymocytes, T_{reg} progenitors, and mature T_{reg} cells (gated as CD4$^+$CD8$^-$CD25$^-$Foxp3$^-$, CD4$^+$CD8$^-$CD25$^+$Foxp3$^-$, and CD4$^+$CD8$^-$CD25$^+$Foxp3$^+$, respectively) revealed that in addition to GITR, T_{reg} progenitors also highly express OX40 and TNFR2 (Fig. 1a). Mature Foxp3$^+$ T_{reg} cells continue to express high amounts of these TNFRSF members in the thymus (Fig. 1a) and in peripheral lymphoid organs (data not shown). T_{reg} progenitors do not globally overexpress TNFRSF members, as 4-1BB, CD30, and TNFR1 were not detectable above background in thymic T_{reg} progenitors or T_{reg} cells (Fig. 1a and data not shown). CD27, which is already expressed on DP thymocytes, is highly expressed by all CD4SP thymocytes but was not further upregulated by T_{reg} progenitors or mature Foxp3$^+$ T_{reg} cells (Fig. 1a, Supplementary Fig. 1). Thus, GITR, OX40, and TNFR2 are highly upregulated on T_{reg} progenitors during thymic T_{reg} development.

APCs in the thymic medulla express GITR-L, OX40-L and TNF

For signaling, homotrimeric TNFRSF members require ligation by specific TNFSF ligands[21]. Various APC subsets have been described to express TNFSF ligands upon activation in secondary lymphoid organs, but the constitutive expression of GITR-L, OX40-L, and TNF within the thymic APC subsets that actually mediate T_{reg} differentiation has not been defined. Medullary thymic epithelial cells (mTEC; CD45$^-$EpCAM$^+$UEA1$^+$BP-1^{lo}) constitutively express GITR-L, OX40-L, and TNF (Fig. 1b). Both GITR-L and TNF are expressed by CD8α$^+$ and SIRPα$^+$ conventional DC subsets (CD11C$^+$B220$^-$), and by plasmacytoid DCs (CD11C$^+$B220$^+$), whereas OX40-L expression is restricted to mTEC. Since TNFR2 can bind both membrane-bound and soluble TNF [22, 23], we evaluated TNF expression by surface staining and by intracellular staining after fixation and

permeabilization. We were able to detect membrane bound TNF on DCs (data not shown) albeit at much lower levels than total TNF (Fig. 1b).

We used immunofluorescence microscopy to define the pattern of TNFSF ligand expression within the thymic microenvironment. Co-staining slides with anti-Keratin 5, which marks the thymic medulla (the location of thymic T_{reg} differentiation), showed a high degree of overlap with OX40-L expression (Fig. 1c). Similar results were obtained when staining for GITR-L and TNF (data not shown). Thus, the specific ligands that bind the TNFRSF members present on developing T_{reg} cells are expressed by APCs at the site of thymic T_{reg} maturation.

GITR, OX40 and TNFR2 correlate with TCR signal strength

When we gated on T_{reg} progenitors and plotted the expression of GITR versus OX40 (Fig. 2a) or GITR versus TNFR2 (data not shown), we found that cells expressing the most GITR also express the most OX40 and TNFR2, and vice versa. This finding prompted us to ask the question if TNFRSF expression is related to TCR signal strength in T_{reg} progenitors. For these experiments, we used Nur77-GFP BAC reporter mice, which provide a reliable readout of TCR signal strength via GFP expression[24]. We found a significant positive correlation between Nur77-GFP fluorescence and GITR, OX40 and TNFR2 expression in T_{reg} progenitors (Fig. 2a,b). In contrast, expression of CD27, a TNFRSF member recently reported to drive thymic Treg development via the inhibition of apoptosis[25], did not correlate significantly with Nur77-GFP (Fig. 2b). Therefore, thymic T_{reg} progenitors receiving the strongest TCR signals during development are engendered with the highest expression of GITR, OX40, and TNFR2. Moreover, mature T_{reg} cells express substantially more OX40 and GITR than T_{reg} progenitors (Fig. 1a) suggesting that T_{reg} progenitors expressing relatively more of these TNFRSF members have a selective advantage in thymic T_{reg} differentiation.

TAK1 and CD28 govern GITR, OX40 and TNFR2 expression

To identify the pathways downstream of the TCR that entrain expression of specific TNFRSF members we used mice that lack the MAPK kinase kinase TAK1 (also known as MAP3K7) specifically in T cells ($cd4^{Cre} \times Tak1^{FL/FL}$ mice; Tak1 KO). TAK1 links TCR signaling to NF-κB activation[26] and thus is a key signaling intermediate downstream of the TCR. Previous studies have documented that TAK1-deficiency profoundly blocks thymic T_{reg} differentiation but did not examine effects on T_{reg} progenitors as these studies preceded the identification of this cell population[26, 27]. We found that T_{reg} progenitors could be detected in $Cd4^{cre} \times Tak1^{FL/FL}$ mice although their numbers are reduced (data not shown). These T_{reg} progenitors fail to express GITR, OX40, and TNFR2 on their surface and do not convert into mature Foxp3+ T_{reg} cells (Fig. 2c,d and data not shown). In contrast, CD27 expression was not affected by TAK1-deficiency. Thus, TAK1 is required for TCR-induced expression of GITR, OX40, and TNFR2 on T_{reg} progenitors.

We also examined the surface expression of TNFRSF in T_{reg} progenitors from CD28-deficient mice, as CD28 plays an important role in T_{reg} development[28-30]. $Cd28^{-/-}$ mice showed a significant reduction in GITR and OX40 expression on T_{reg} progenitors relative to

BALB/c controls; a more modest trend toward reduced TNFR2 expression was also observed (Fig. 2c,d, bottom panels). In contrast, CD27 surface expression on T_{reg} progenitors was unaffected by the absence of CD28. Similar results were obtained with $Cd28^{AYAA}$ knockin mice (data not shown), which lack the distal proline-rich motif that was previously shown to be required for T_{reg} development[13]. This was not a strain-specific effect as we found a similar reduction in the expression of GITR and OX40 on T_{reg} progenitors from $Cd28^{-/-}$ mice on the C57Bl/6 background (Supplementary Fig. 2). Thus, CD28 signals are also required for upregulation of GITR and OX40 expression (and likely TNFR2) on T_{reg} progenitors.

GITR, OX40 or TNFR2 costimulation drives T_{reg} maturation

To determine if signaling through GITR, OX40 or TNFR2 directly augments T_{reg} development by promoting maturation of T_{reg} progenitors, we utilized an assay that models step 2 of T_{reg} development *in vitro* (i.e., the IL-2-dependent conversion of T_{reg} progenitors into mature Foxp3+ T_{reg} cells). Purified T_{reg} progenitors sorted from Foxp3-GFP reporter mice were incubated in culture with low dose IL-2 with or without agonist TNFSF ligands. Conversion was assessed as the frequency of cells that acquired Foxp3-GFP expression during the course of the incubation. The addition of GITR-L–Fc, OX40-L–Fc, or TNF plus IL-2 enhanced conversion to Foxp3$^+$ T_{reg} cells by ~2-3 fold when compared to treatment with IL-2 alone (Fig. 3a,b). In contrast, three independent CD27 agonists (CD70–Fc, soluble CD70, and an agonist monoclonal antibody to CD27) did not enhance T_{reg} development (Fig. 3a,b and data not shown). T_{reg} progenitors do not express 41BB or CD30, and as expected, stimulation with 41BB-L or CD30-L did not affect T_{reg} development (data not shown).

One potential mechanism by which TNFRSF agonists promote T_{reg} development is to enhance sensitivity to IL-2. To directly test this, we examined CD25 expression on both Foxp3-GFP$^-$ T_{reg} progenitors and Foxp3-GFP$^+$ mature T_{reg} cells that had been stimulated with IL-2 ± GITR-L–Fc. CD25 expression was significantly enhanced on both T_{reg} progenitors and mature T_{reg} cells (Fig. 3c,d). To examine the relative effect of TNFRSF co-stimulation on IL-2-dependent T_{reg} differentiation we carried out a dose-response experiment. Stimulation of T_{reg} progenitors with 1 U/mL IL-2 plus GITR-L or OX40-L resulted in 50% maximal conversion to mature T_{reg} cells; in the absence of GITR-L or OX40-L co-stimulation, almost 10-fold more IL-2 was required to achieve the same effect (Fig. 3e). Agonist GITR-L–Fc, OX40-L–Fc, or TNF did not promote T_{reg} maturation in the absence of IL-2 (data not shown). Finally, we observed that GITR-L–Fc costimulation enhanced IL-2-dependent STAT5 phosphorylation in T_{reg} progenitors, suggesting that GITR-L acts directly on the IL-2R signaling pathway (Fig. 3f). These data demonstrate that GITR, OX40, or TNFR2 costimulation augments IL-2 responsiveness in T_{reg} progenitors and allows them to effectively respond to low and likely physiologically relevant levels of IL-2.

An alternative mechanism by which TNFRSF costimulation enhances T_{reg} development is through the induction of anti-apoptotic proteins. To address this, we determined whether blocking apoptosis via the addition of the pan-caspase inhibitor, z-VAD-fmk, had a similar

effect in promoting T_{reg} development to GITR-L–Fc. The addition of z-VAD-fmk significantly increased total cell recovery (p<0.02, data not shown); however, z-VAD-fmk did not enhance IL-2-dependent conversion of T_{reg} progenitors into mature T_{reg} cells (Fig. 3g). Thus, costimulation via GITR, OX40, and TNFR2 provide more than just pro-survival signals to developing T_{reg} cells.

GITR- or OX40-deficiency modestly blocks T_{reg} development

We next sought to determine if TNFRSF members drive T_{reg} differentiation *in vivo* in a cell-intrinsic manner. To examine this we created bone marrow chimeras in which a 50-50% mixture of CD45.1/SJL wild type congenic marrow was mixed with CD45.2 *Gitr* $^{-/-}$ (also known as *Tnfrsf18)* bone marrow, depleted of mature lymphocytes, and engrafted into sublethally irradiated *Rag2*$^{-/-}$ recipients. After 10-18 weeks of reconstitution, chimeric recipients were euthanized and we assayed T_{reg} development by flow cytometry. As shown in Fig. 4b, *Gitr* $^{-/-}$ cells gave rise to conventional CD4$^+$CD25$^-$Foxp3$^-$ thymocytes equally as well as their wild type counterparts, but were outcompeted by wild type cells by approximately 30% in generating mature CD25$^+$Foxp3$^+$ thymic T_{reg} cells. T_{reg} progenitor populations were also slightly reduced in GITR-deficient cells, although the reduction was significantly less than observed in mature Foxp3+ T_{reg} cells. Similar results were observed in splenic T_{reg} cells (data not shown). We carried out similar studies using *Ox40* $^{-/-}$ (also known as *Tnfrsf4*) mice; we observed a 50% reduction in the ability of *Ox40* $^{-/-}$ -derived cells to give rise to Foxp3$^+$ T_{reg} cells (Fig. 4b). The effect of TNFR2 on thymic T_{reg} development has not been examined as closely but as TNFR2-deficient mice are viable it is unlikely that they are devoid of T_{reg} cells. Thus, deletion of any one TNFRSF member alone imposes only a modest cell-intrinsic T_{reg} developmental block.

TNFRSF blockade inhibits T_{reg} development and maturation

We next sought to determine if TNFRSF function redundantly to drive T_{reg} development by blocking multiple ligands simultaneously. Thymic organ cultures (TOCs) are a widely utilized technique to study thymocyte development in vitro with minimal manipulation[31]. We harvested thymic lobes from 1 day-old Foxp3-GFP reporter mice and subjected them to pair-wise comparison in TOCs for 14 days supplemented with either irrelevant isotype control antibodies or neutralizing anti-GITR-L, OX40-L, and CD70 or anti-GITR-L, OX40-L, CD70 and TNFR2. Neutralization of either three or four distinct TNFRSF members resulted in a dramatic reduction in the percentage of mature CD25$^+$Foxp3$^+$ T_{reg} cells within CD4SP thymocytes (Fig. 5a,b).

We also evaluated the acquisition of T_{reg} maturation markers by Foxp3$^+$ cells from TOCs with or without neutralizing antibodies to GITR-L, OX40-L and CD70 and found that inhibition of this costimulatory pathway markedly reduced not only the efficiency of T_{reg} generation, but also the expression of CD25 and Foxp3 (Fig. 5c,d). We also stained for the extracellular AMP nucleotidase, CD73, and the folate receptor, FR4, both of which mark highly functional T_{reg} cells[32, 33], and found significant reductions in the expression of both in Foxp3$^+$ cells when costimulation via GITR-L, OX40-L, and CD70 were blocked (Fig. 5c,d). Although the numbers of T_{reg} cells recovered were lower in cultures treated with neutralizing antibodies to GITR-L, OX40-L, CD70 and TNFR2 the same effects on T_{reg}

maturation markers were observed. Thus, neutralization of select TNFSF members inhibits thymic T$_{reg}$ development and maturation.

TNFRSF members collectively drive T$_{reg}$ development

We next sought to determine whether inhibiting multiple TNFRSF members blocked T$_{reg}$ development *in vivo* in a cell-intrinsic manner. For these studies we focused on OX40, GITR, and TNFR2 as their expression correlated with TCR signal strength while CD27 did not (Fig. 2b). One difficulty with these studies is that the genes encoding GITR and OX40 are spaced a mere ~10 kb apart on mouse Chromosome 4. *Tnfr2* is encoded nearby on Chromosome 4 as well. This overall pattern is the same in humans, except that *GITR, OX40, and TNFR2* are located on Chromosome 1. In mice, *Tnfsf18 (*encoding GITR-L) and *Tnfsf4 (*OX40-L) are also located next to each other on Chromosome 1. Because of this genomic organization, conventional double- or triple-knockout strategies to account for biologic redundancy in this pathway were not feasible. Instead, we exploited the fact that TNFRSF members must multimerize to function and generated tailless, dominant negative forms of GITR and TNFR2.

Dominant negative GITR (dnGITR) and dominant negative TNFR2 (dnTNFR2) were linked to IRES-GFP (pMIGR vector) and IRES-Thy1.1 (pMITR vector) reporter constructs and administered to *Ox40 $^{-/-}$* bone marrow (CD45.2) via retroviral transduction. Transduced cells were mixed with congenically marked wild type marrow (CD45.1 or CD45.1 × CD45.2), engrafted into sublethally irradiated *Rag2 $^{-/-}$* recipients, and reconstituted for 10-12 weeks. This strategy produced chimeric mice in which 5 populations of bone marrow cells could compete to reconstitute the thymus: (1) wild type: CD45.1^{+}, (2) OX40-deficient*:* CD45.2^{+}, (3) OX40 plus GITR-deficient: CD45.2^{+}GFP^{+}, (4) OX40 plus TNFR2-deficient: CD45.2^{+}Thy1.1^{+}, and (5) OX40 plus GITR plus TNFR2-triple deficient (CD45.2^{+}GFP^{+}Thy1.1^{+}) (Fig. 6a,b). We then plotted the relative percentages of conventional CD4SP, T$_{reg}$ progenitors, and T$_{reg}$ cells within each of the gates shown in Figure 6b corresponding to increased expression of dnGITR, dnTNFR2, or both. T$_{reg}$ progenitors were not significantly affected by reduced GITR, OX40, and TNFR2 signaling (Supplementary Fig. 3). In contrast, superimposition of GITR- or TNFR2-deficiency in *Ox40$^{-/-}$* cells led to a striking dose-dependent abrogation of T$_{reg}$ development of >20 fold (Fig 6c,d). Importantly, we noted a further significant decrease in T$_{reg}$ cells derived from cells deficient in all three TNF receptors. In fact, *Ox40$^{-/-}$* cells expressing the highest levels of both dnGITR and dnTNFR2 completely lacked mature thymic T$_{reg}$ cells (Fig. 6c,d, and for a summary of all statistical comparisons, see Supplementary Table 1). Thus, costimulation via GITR, OX40, and TNFR2 is collectively required for T$_{reg}$ differentiation *in vivo*.

TNFRSF costimulation shapes T$_{reg}$ repertoire selection

The observation that TCR signal strength correlates with the expression of GITR, OX40, and TNFR2 in T$_{reg}$ progenitors suggests that these receptors may play an important role in biasing the T$_{reg}$ repertoire toward higher-affinity. To test this hypothesis, we generated Foxp3-RFP × Nur77-GFP reporter mice. We then sorted T$_{reg}$ progenitors and stimulated them with IL-2 and increasing doses of agonist GITR-L–Fc. Increasing GITR-L

concentration led to the development of a T_{reg} population with reduced expression of Nur77-GFP (Fig. 7a). This was not due to a loss of T_{reg} cells with high TCR signal strength, but rather inclusion of cells with lower Nur77-GFP expression. We found a clear negative correlation between GITR-L concentration and TCR signal strength within newly developed T_{reg} cells ($r^2 = 0.81$, Fig. 7b). These results demonstrate that altering the abundance of a TNFSF ligand directly affects selection of the T_{reg} repertoire.

A CD25−Foxp3^{lo} alternative T_{reg} progenitor population has also been described[34]. A key question is whether this putative T_{reg} progenitor is also affected by TNFRSF costimulation. We found that CD25−Foxp3^{lo} T_{reg} progenitors also express GITR and OX40 in direct proportion to TCR signal strength (Supplementary Fig. 4a-d). Stimulation of sorted CD25−Foxp3^{lo} T_{reg} progenitors in the presence of low dose IL-2 plus GITR-L–Fc dose-dependently enhanced maturation to CD25+Foxp3+ T_{reg} cells (Supplementary Fig. 4e). Furthermore, as GITR-L concentration was increased, the average Nur77-GFP expression decreased in cells that differentiated into CD25+Foxp3+ T_{reg} cells (Supplementary Fig. 4f). Thus, TNFRSF costimulation plays a critical role in the differentiation of all potential T_{reg} progenitors into mature T_{reg} cells, and competition for TNFSF ligands regulates TCR repertoire selection during T_{reg} development.

Discussion

T_{reg} development is a finely tuned process that is critical for preventing autoimmune disease. An intriguing feature of T_{reg} development is that this process discriminates between relative differences in TCR signal strength. T_{reg} cells with TCRs that recognize self-ligands with high-affinity predominate, although T_{reg} cells with lower-affinity TCRs can be found[35, 36]. The molecular program that accounts for this skewing process remains to be fully elucidated. One recent mechanism that has been proposed is TCR-induced expression of the NR4A family of transcription factors including NUR77, NOR1, and NURR1[37]. Expression of these transcription factors correlates with TCR signal strength[24] and these three transcription factors bind the *Foxp3* gene and are redundantly required for T_{reg} development[37]. This suggests that one potential mechanism to link TCR signal strength to T_{reg} development involves this transcription factor family. However, it is unlikely that this is the only mechanism that links TCR signal strength to T_{reg} development. For example, T_{reg} progenitors have clearly induced expression of NUR77 as documented by Nur77-GFP reporter mice yet they do not express Foxp3. In addition, in *Bim*−/− mice, many CD4SP thymocytes that have escaped deletion have high expression of NUR77 yet they do not differentiate into Foxp3+ T_{reg} cells[38]. TCR signals give rise to graded levels of NUR77 expression in thymocytes, yet Foxp3 is expressed in relative quantum increments with essentially no expression in T_{reg} progenitors and high levels in mature T_{reg} cells. If the effect of NUR77 family members on *Foxp3* transcription was truly linear then one might expect a continuum of Foxp3 expression in T_{reg} progenitors culminating in that seen in mature T_{reg} cells; that is not observed experimentally suggesting that other mechanisms must also contribute to the process of linking TCR signal strength to T_{reg} differentiation.

We propose that the TNFRSF members GITR, OX40 and TNFR2 also play a critical role in converting strong TCR signals into molecular pathways that drive T_{reg} differentiation. In

support of this hypothesis we observed that T_{reg} progenitors express much greater amounts of GITR, OX40, and TNFR2 relative to conventional $CD4^+CD25^-Foxp3^-$ thymocytes and that the level of expression of these receptors correlates directly with TCR signal strength as read out using Nur77-GFP reporter mice. This results in a competitive advantage as mature $Foxp3^+$ T_{reg} cells express even greater amounts of GITR and OX40. Blocking signaling via these receptors either by neutralizing antibodies or through combinations of gene knockouts and dominant negative receptors completely abrogates T_{reg} development. Thus, signaling through these receptors is collectively required for thymic T_{reg} development *in vivo*. Finally, the relative abundance of TNFSF ligands directly affects the TCR repertoire in developing T_{reg} cells. T_{reg} progenitors expressing the highest affinity TCRs, and hence the highest amounts of GITR, OX40, and TNFR2 can more effectively compete for their respective TNFSF ligands, and are thereby more likely to differentiate into mature T_{reg} cells.

The mechanism by which GITR, OX40 and TNFR2 promote T_{reg} development appears to primarily have effects on cell differentiation and not survival. GITR-L and OX40-L costimulation resulted in an ~10-fold increase in sensitivity to low doses of IL-2 that likely mimic what is available *in vivo*. This most likely reflects a direct effect on IL-2R signaling, as downstream STAT5 phosphorylation was also enhanced by costimulation with TNFSF agonists. Since GITR, OX40, and TNFR2 are known to signal via the NFκB pathway, we examined the expression of two NFκB-regulated genes that affect IL-2R signaling. We observed increased expression of CD25 on both T_{reg} progenitors and mature T_{reg} cells following costimulation with GITR-L, OX40-L, or TNF. In contrast, *Socs1* expression (which is induced by IL-2 signaling, and negatively regulated by NFκB via mIR-155[39]) was unaffected by GITR-L costimulation of T_{reg} progenitors (data not shown). When evaluating the effects of costimulation on mature T_{reg} cells, however, we observed that GITR-L reduced the *Socs1* expression induced by IL-2 stimulation by approximately 50% (data not shown). Thus, the predominant effect of GITR-L costimulation on enhancing T_{reg} progenitor sensitivity to IL-2 appears to be via CD25 induction.

An additional mechanism by which GITR, OX40, and TNFR2 could influence T_{reg} development is through the induction of anti-apoptotic genes[19, 21, 40, 41]. Such a mechanism has been proposed to account for the modest effect of CD27-CD70 on T_{reg} development[25]. We did observe that GITR, OX40, and TNFR2 costimulation weakly induced Bcl2 protein in T_{reg} progenitors (data not shown). However, blocking apoptosis with caspase inhibitors did not mimic the effect of GITR-L costimulation. Preliminary results with mice overexpressing Bcl2 indicate that ectopic anti-apoptotic gene expression does not prevent the defect in T_{reg} development imposed by TNFRSF blockade (data not shown). Thus, unlike CD27, which promotes cell survival, the induction of anti-apoptotic gene expression by GITR, OX40, or TNFR2 costimulation is not the dominant mechanism by which these TNFRSF drive T_{reg} differentiation.

It is unclear why three distinct TNFRSF members are required for T_{reg} differentiation. One possible explanation is that partial redundancy limits the chances of defects in T_{reg} development. A similar biological redundancy is observed for the NR4A transcription factor family in which neither NUR77, NOR1, or NURR1 is individually critical, but loss of all three stringently prevents T_{reg} development[37]. Alternatively, it is possible that selection for

multiple TNFRSF members on mature T_{reg} cells is important for their later function. For example, ligation of OX40 or GITR on mature T_{reg} cells has been shown to limit their suppressor function[18, 20]. In this regard, enhanced selection of T_{reg} progenitors that express GITR and OX40 in the thymus could function as a possible safeguard to shut down their suppressive function during inflammatory responses to pathogens. Other reports have suggested that expression of TNFRSF members is required to maintain survival of mature T_{reg} cells during inflammatory conditions[42, 43]. Finally, it is possible that higher expression of GITR and OX40 allows T_{reg} cells that most avidly recognize self-ligand to preferentially compete for IL-2 needed to sustain these cells in peripheral lymphoid organs.

In conclusion, our data support a model of T_{reg} differentiation in which developing thymocytes that bind MHC-II:self-peptide ligands with higher-affinity express greater amounts of GITR, OX40 and TNFR2. This elevated expression of select TNFRSF members on highly self-reactive T_{reg} progenitors results in a selective advantage that allows those cells to preferentially undergo maturation. Thus, upregulation of GITR, OX40, and TNFR2 by T_{reg} progenitors is a mechanism by which high-affinity TCR signals are translated into physical parameters that allow for more effective competition for IL-2 in a tightly regulated developmental niche. In agreement with previous reports[36], our model still allows for cells bearing TCRs with lower affinity for MHC-II:self-peptide complexes to emerge in the thymic T_{reg} population, but strongly favors T_{reg} differentiation in thymocytes receiving strong TCR signals since these cells most strongly upregulate TNFRSF receptors. This precisely matches the observed TCR affinity distribution in mature T_{reg} cells[24]. Thus, TNFSF ligand availability is an additional level of control by which thymic APCs define the T_{reg} developmental niche and couple TCR affinity to T_{reg} development.

Online Methods

Mice

All mice were housed in specific-pathogen free facilities at the University of Minnesota and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee. *Gitr* $^{-/-}$ mice were previously described[19] and provided by Drs. Carlo Riccardi and Ethan Shevach. *Ox40* $^{-/-}$, Foxp3-GFP, Foxp3-RFP, *Rag2* $^{-/-}$, and *Cd4*^{Cre} mice were purchased from the Jackson Laboratory (Bar Harbor, ME, stock numbers 012839, 006772, 008374, 008449, and 013234 respectively). *CD45.1* (B6.SJL) mice were purchased from the NCI (Bethesda, MD). Nur77-GFP BAC reporter mice[24], *Tak1* $^{FL/FL}$ and *Bcl2*-Tg mice were previously described[44, 45]. *Cd28*$^{-/-}$ and *Cd28*^{AYAA} knock-in mice were previously described[46]. All mice used were on the C57Bl/6 background, except for *Cd28*$^{-/-}$ and *Cd28*^{AYAA} mice and their wild type littermates, which were on a BALB/c background. Mice were randomly selected for experiments from our mouse facility in age-matched cohorts. The investigators were not blinded to genotype during data acquisition.

Tissue preparation and cell isolation

For analysis of thymocyte and T_{reg} development thymii and peripheral lymphoid organs were mechanically dissociated into PBS with 2% FBS and 2 mM EDTA, pH 7.4, using

frosted glass slides. Cell suspensions were passed through 70 μm filters and washed prior to staining. For analysis of thymic DC and epithelial cells (TEC), thymii were dissected into 5 mL complete RPMI supplemented with Collagenase D (120 Mandl U/mL) and DNase I (3 U/ml, both from Roche Applied Sciences, Indianapolis, IN) and diced into ~20 pieces and placed in a 37°C incubator for 30 minutes. Pieces were further homogenized by pipetting and the addition of EDTA (20 mM final concentration) which was immediately diluted 10-fold with buffer and the suspension filtered through 70 μm mesh. Thymic DC were magnetically enriched by labeling with anti-CD11C FITC (N418, eBioscience, San Diego, CA) and secondarily with anti-FITC microbeads (Miltenyi Biotec, Auburn, CA). Thymic epithelial cells were enriched by magnetic depletion of leukocytes using biotinylated anti-CD45 antibody (30F11, eBioscience) and streptavidin-conjugated microbeads (Miltenyi Biotec).

Flow cytometry and antibodies

All analysis was conducted in the Center for Immunology Flow Cytometry Core Facility using BD LSR II and Fortessa cytometers (BD, San Jose, CA). For surface staining, cells were stained for 20 minutes with 1:100 dilutions of fluorochrome-conjugated antibodies prior to washing and analysis or intracellular staining. Anti-mouse CD4 (GK1.5 or RM4-4), CD8 (53-6.7), CD25 (PC61.5), CD122 (TMb1), Foxp3 (FJK-16s), GITR (DTA-1), OX40 (OX86), TNFR2 (TR75-32), CD27 (LG.7F9), 4-1BB (17B5), GITR-L (YGL386), OX40-L (RML134L), CD70 (FR70), CD45 (30-F11), MHC Class II (I-A/I-E, M5/114.15.2), BP1 (6C3), Thy1.2 (30-H12 or 53.21), CD11C (N418), FR4 (eBio 12A5), CD73 (TY/11.8), CD103 (2E7), KLRG1 (2F1), and CD45R/B220 (RA3-6B2) were from eBioscience (San Diego, CA). Fluorescein labeled Ulex Europaeus Agglutinin I (UEA I) was purchased from Vector Laboratories (Burlingame, CA). FITC Anti-GFP was from Rockland Immunochemicals (Gilbertsville, PA). Intracellular detection of Foxp3 and P-STAT5 was performed as previously described[9].

Immunofluorescence microscopy

Tissues were frozen in 2-methylbutane surrounded by dry ice. Frozen blocks were cut into 7 μm thick sections and fixed in acetone. Nonspecific binding was blocked for 1 hour with 5% bovine serum albumin in PBS. Sections were then stained with hamster anti-CD11C conjugated-FITC; rabbit A488 conjugated anti-K5; UEA-1 conjugated-FITC; goat anti-OX40-L; rat anti-TNF PE (eBioscience); or rat anti-GITR-L PE (eBioscience). Signal from PE-conjugated anti-TNF and anti-GITR-L or irrelevant isotype controls was amplified with rabbit anti-PE (NB120-7011; Novus Biologicals) followed by staining with donkey anti-Rabbit Cy3 (Jackson Immunoresearch). Jackson Immunoresearch secondary antibodies conjugated to various fluorochromes were used for staining of unconjugated antibodies including Cy3–conjugated donkey anti-rat, Alexa-488-conjugated donkey anti-rabbit, and Cy3-conjugated donkey anti-goat. Sections were incubated for 1 hour in the dark at room temperature, and then washed with PBS 3 times prior to the next stain. After the final wash, slides were stained with DAPI (4,6-diamidino-2-phenylindole; Invitrogen) for 10 minutes before Fluoromount G was added with a cover slip. Tiled images were acquired with an automated Leica DM5500B microscope and analysis was done with Adobe Photoshop.

T_{reg} progenitor conversion assays

T_{reg} progenitors were isolated as previously described[9]. Briefly, 6-8 week old Foxp3-GFP thymii were dissected, dissociated, and pooled (up to 8 mice per experiment). CD4SP cells were enriched by magnetically depleting with biotinylated anti-CD8, CD11B, CD11C, CD19, CD45R/B220, MHC class II, NK1.1, and Ter119 antibodies (eBioscience) followed by secondary labeling with streptavidin-conjugated microbeads (Miltenyi Biotec). Enriched CD4SP cells were stained with fluorchrome-conjugated anti-CD4, CD25, and streptavidin prior to sorting $CD4^+CD25^+GFP^-$ cells using a BD Facs Aria sorter (BD Biosciences). Purified T_{reg} progenitors were incubated in complete RPMI and supplemented with human IL-2 (1 Unit/mL, from the NIH repository at Frederick National Laboratory) with or without 100 nM recombinant GITR-L–Fc, OX40-L–Fc (both from Imgenex, San Diego, CA), recombinant murine TNF (Peprotech, Rocky Hill, NJ), recombinant CD70-Fc (Sino Biologicals, Beijing, China), soluble CD70 (Enzo Life Sciences, Farmingdale, NY), agonist CD27 mAb (RM27-3E5)[47], or recombinant mouse CD30-L and 4-1BBL (the latter two of which were crosslinked using 5 ug/ml anti-poly His antibody, both from R&D Systems, Minneapolis, MN). After 72 hours, cells were harvested, stained with anti-CD4 and CD25 antibodies and analyzed by flow cytometry for the percentage of cells that express GFP after incubation.

Thymic organ cultures

Neonatal thymic organ cultures were performed as previously described[31]. Briefly, thymic lobes were harvested from 1-day-old Foxp3-GFP mice and set up in pairwise comparisons using isotype control or anti-TNFSF blocking antibodies. Neutralizing anti-OX40-L (RM134L) and anti-CD70 (FR70) were obtained from Bio X Cell (West Lebanon, NH). Neutralizing anti-GITR-L (Clone 337122) was from R&D Systems (Minneapolis, MN). Neutralizing anti-TNFR2 (Clone TR75-54) was previously described[48]. Thymic lobes were bisected and placed atop Millipore Cellulose Ester Gridded 0.45 μm filters (CAT HAWG01300) sitting on Gelfoam absorbable gelatin sponges (Pfizer, NY, NY). These filters were placed in 6 well plates with 2.1 mL of complete RPMI per well supplemented with 20 μg/mL of the indicated antibodies, and up to 6 bisected lobes placed on each filter. Medium and antibodies were replenished on day 7 and bisected lobes were collected and stained with primary antibodies for 20 minutes, washed, and analyzed by flow cytometry on day 14.

Mixed bone marrow chimeras

Rag2$^{-/-}$ recipient mice were sublethally irradiated 24 hours prior to engraftment using an X-ray irradiator (Radsource RS2000 Biological Irradiator, Radsource, Suwanee, GA) and administering two doses of 250 rads separated by a two-hour rest. Bone marrow was harvested from tibias and femurs of donor mice, dissociated into single cell suspensions, and magnetically depleted of contaminating mature T and B cells by labeling with biotinylated anti-CD3, CD4, CD8, CD19, and CD45R antibodies (eBioscience) and secondarily with streptavidin-conjugated microbeads (Miltenyi Biotec) prior to passage through magnetic columns. Cell suspensions were washed in PBS and injected intravenously via the tail vein. Chimeric recipients were engrafted for 10-18 weeks prior to analysis.

Preparation of dominant negative retroviral vectors

Dominant negative retroviral vectors were cloned into pMIGR[49] or a modified version of this vector in which Thy1.1 replaces GFP, hereafter called pMITR. Sequences encoding truncated TNFRSF members were prepared by amplifying the extracellular and transmembrane domains of GITR and TNFR2 from a murine cDNA library and inserting stop codons immediately after the transmembrane domain. Specific primers utilized were as follows: for GITR dominant negative (Forward CTGCTCGAGCTGACCATGGGGGGCATGGGCCAT, Reverse CTGGCGGCCGCCTGTCACAGGTCCTCCTCTGAGATCAGCTTCTGCATTGATGCCA TTTGCCTCCTCAGCTGCCATATG), and for TNFR2 dominant negative (Forward CTGCTCGAGCTGACCATGGCGCCCGCCGC, Reverse CTGGCGGCCGCCTGTCATTTGTCATCGTCATCCTTGTAGTCGGAGGGCTTCTTTT TCCTCTGCACC). Linear products were trimmed with *XhoI* and *NotI* and ligated into pMIGR or pMITR prior to transformation of chemically competent *E. coli* DH5α cells and plating on carbenicillin. LB starter cultures were prepared, digested, and picked for large culture. Mini- and maxipreps were performed using PureLink® kits according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Direct-Sanger sequencing was performed by the University of Minnesota Genomics Core facility and base calling was done by hand.

Bone marrow retroviral transduction

Subconfluent 293FT cells (ATCC, Manassas, VA) were transfected using Effectene reagent (Qiagen, Valencia, CA) with pMIGR-dominant negative GITR (dnGITR) or pMITR-dominant negative TNFRF2 (dnTNFR2) constructs in parallel with pHit60 and pHit123 packaging vectors for retrovirus production[50]. After 16 hours, sodium butyrate was added to the culture medium to a final concentration of 10 mM to enhance viral production. Eight hours later, medium was replaced to begin collecting retroviral particles. Supernatants were collected and medium replaced at 24 and 48 hours. After filtration through 0.45 μm filters, viral particles were centrifuged onto Retronectin-coated 6 well plates (Clontech Laboratories, Mountain View, CA) prior to overlaying $Ox40^{-/-}$ bone marrow cells at a density of 3 million cells per well. Bone marrows used for transduction had been isolated the previous day, depleted of T and B cells, and stimulated with 10 ng/mL IL-3 and IL-6, and 50 ng/ml SCF (Peprotech) to induce cell division. Medium and cytokines were replenished at the first and second retroviral transductions. Twenty-four hours after the second transduction, bone marrow was collected and $Ox40^{-/-}$ cells were mixed 80%/20% with congenically marked wild type bone marrow that had been stimulated identically in culture with cytokines. The mixture was washed in PBS and intravenously injected into sublethally irradiated $Rag1^{-/-}$ or $Rag2^{-/-}$ recipients and thymic T_{reg} was analyzed 10-12 weeks later. Bone marrow chimeric recipient mice that died due to a failure of engraftment were excluded from analysis.

Statistical analysis

P-values were generated using two-tailed T-tests; paired T-tests were used in the case of mixed bone marrow chimeras. Multiple sample comparisons were analyzed by one-way

ANOVA. P-values in figure 2 were calculated based on Pearson correlation coefficients. All values were calculated using Prism (GraphPad Software, LaJolla, CA). Sample sizes were selected to ensure a power of 80% for the detection of two-fold differences in figure 2d, 3b, 3c, 3g, 5a, and 6d. Sample sizes were selected to ensure a power of 80% for the detection of 20% differences in figure 3d, 4a, 4b, and 5d.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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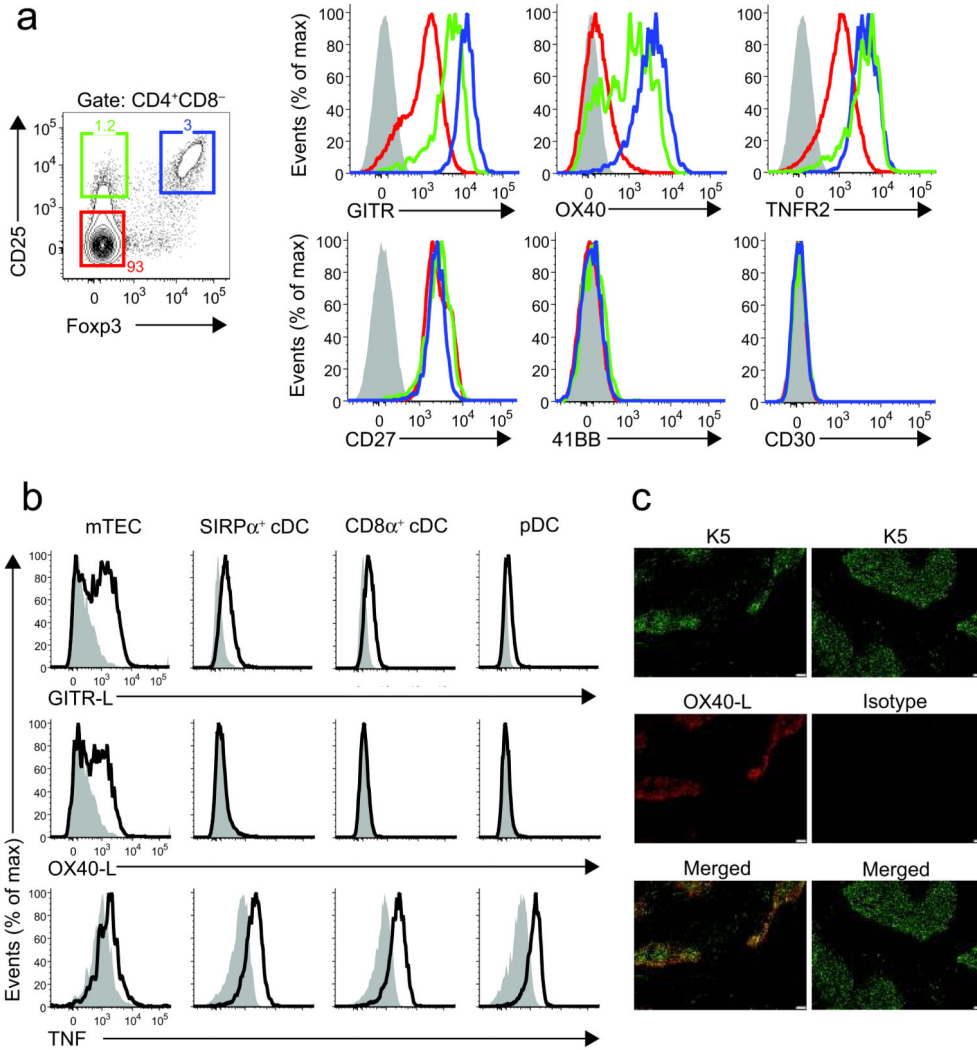
Figure 1. Expression of GITR, OX40, and TNFR2 on thymic T_{reg} progenitors and GITR-L, OX40-L, and TNF on APCs in the thymic medulla

(**a**) Thymocytes from adult (6-10 week old) female or male Foxp3-GFP mice were stained with antibodies against the indicated TNFRSF members in conjunction with antibodies against CD4, CD8, and CD25. In the left panel, conventional (non-T_{reg}) CD4SP thymocytes were gated as CD4$^+$CD8$^-$CD25$^-$Foxp3$^-$ (red lines), T_{reg} progenitors as CD4$^+$CD8$^-$CD25$^+$Foxp3$^-$ (green lines), and mature thymic T_{reg} cells were CD4$^+$CD8$^-$CD25$^+$Foxp3$^+$ (blue lines). The histograms to the right display the expression of TNFRSF within the indicated populations as compared to isotype-stained controls (gray shaded histograms). Data are representative of at least three separate experiments (n ≥ 3). (**b**) Expression analysis of the specific TNFSF ligands GITR-L, OX40-L, and TNF was conducted by flow cytometry on collagenase- and DNase I-digested thymii harvested from 6-8 week old male or female mice, which were magnetically depleted with anti-CD45 for enrichment of mTEC or enriched with anti-CD11C for DCs. Histograms show the expression of GITR-L (top), OX40-L (middle), and total TNF (membrane + intracellular, bottom) within the indicated populations gated as follows: mTEC (CD45$^-$EpCAM$^+$UEA1$^+$),

SIRPα$^+$ cDC (CD11c$^+$B220$^-$SIRPα$^+$CD8α$^-$), CD8α$^+$ cDC (CD11c$^+$B220$^-$SIRPα$^-$CD8α$^+$), and pDC (CD11c$^+$B220$^+$). Data are representative of thymic DC or mTEC isolations pooled from 3-4 mice in at least three separate experiments (n   3). (**c**) Frozen thymic sections from 6-12 week old female or male mice were stained with anti-Keratin 5 (K5-FITC, green), anti-OX40-L or isotype control (PE, red), and corresponding images were merged (yellow) to evaluate the spatial distribution of TNFSF ligands within the thymus. (10X magnification, white bars represent 100 um; n=3, one of three independent experiments).
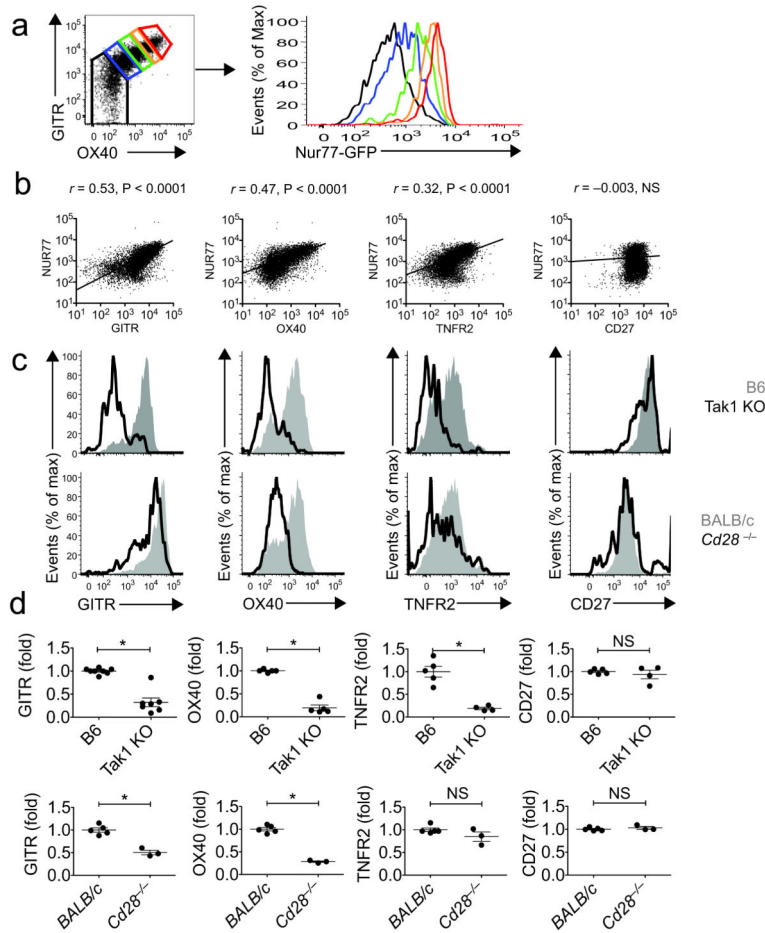
Figure 2. T_reg progenitors express select TNFRSF members in direct proportion to TCR signal strength

(**a**) Six-to-twelve week old female or male Nur77-GFP reporter mice were used to evaluate the role of TCR signal strength on the expression of TNFRSF members. T_reg progenitors were plotted on the basis of their expression of GITR and OX40 (left), and quartile gates representing the spectrum of expression of these receptors were evaluated for Nur77-GFP expression in the corresponding histograms (right). Data are representative of four separate experiments (n = 4). (**b**) Raw values for GITR, OX40, TNFR2, and CD27 on T_reg progenitors were plotted versus Nur77-GFP expression. Lines represent the correlation between x and y variables and were used to calculate Pearson correlation coefficients (*r*). Correlation data for GITR are representative of 8 separate experiments (n = 8), for OX40 from 4 experiments (n = 4), for TNFR2 from 3 experiments (n = 3), and for CD27 from 3 experiments (n = 3). (**c**) Histograms derived by gating on T_reg progenitors from *Cd4*^Cre × *Tak1*^FL/FL mice (abbreviated as Tak1 KO, black histograms) and their wild type littermates (B6 background; gray shaded histograms; top panel) or *Cd28*^−/− mice (black histograms) and their wild type littermates (BALB/c; gray shaded histograms; lower panel) are plotted demonstrating the expression of GITR, OX40, TNFR2, and CD27. (**d**) Cumulative data show the relative expression of GITR, OX40, TNFR2, and CD27 on T_reg progenitors from TAK1-deficient and CD28-deficient mice in comparison to wild type littermates. Raw MFI

values obtained from each mouse after gating on CD25$^+$Foxp3$^-$ T$_{reg}$ progenitors were divided by the average MFI obtained from all wild type mice for normalization amongst separate experiments (mean ± SEM, for Tak1 KO experiments n 4 from at least two separate experiments, and for *Cd28*$^{-/-}$ n 3 from one experiment on the BALB/c background and n 3 from one experiment on the B6 background, see Supplementary Fig. 2, * P < 0.001).
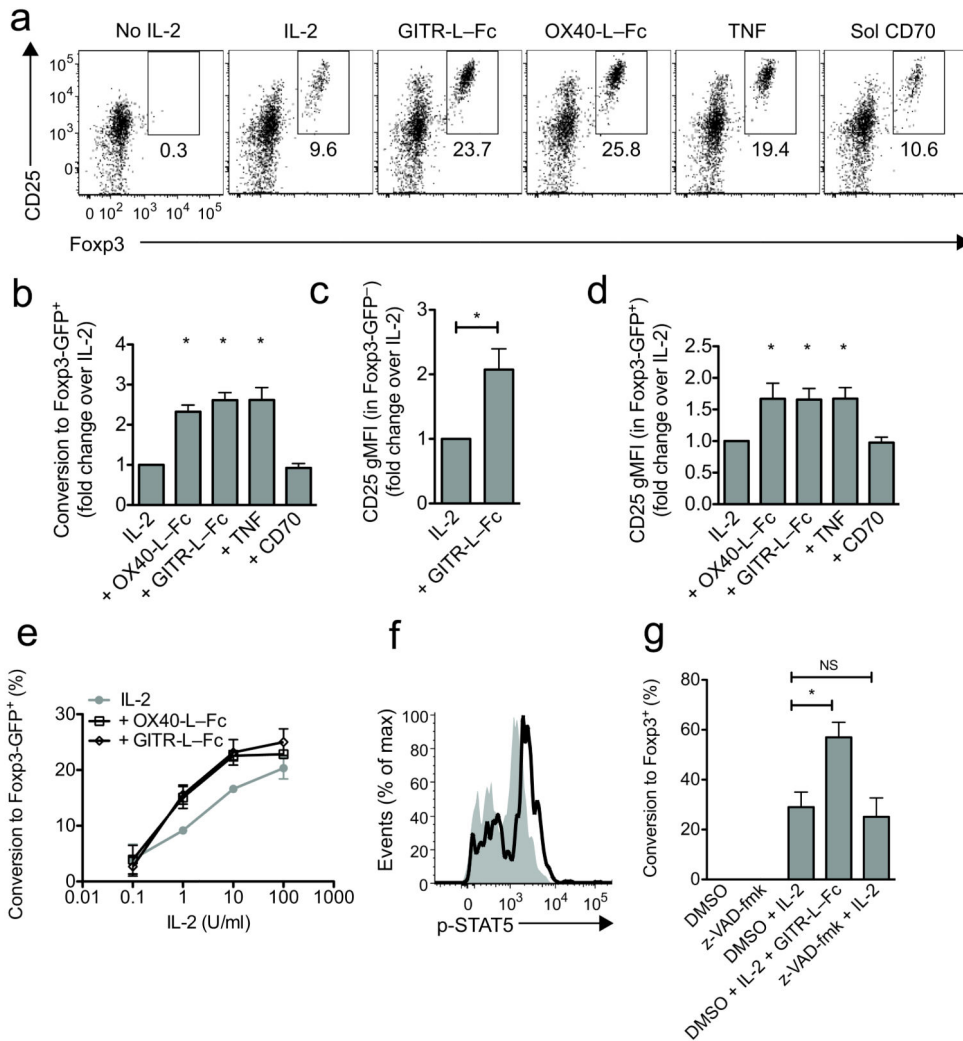
Figure 3. TNFRSF agonists enhance conversion of T$_{reg}$ progenitors to Foxp3$^+$ T$_{reg}$ cells
(**a,b**) Thymii from six- to eight-week old female or male Foxp3-GFP mice were harvested, pooled, and depleted of CD8SP, DP, and non-T cells prior to sorting purified CD4$^+$CD25$^+$Foxp3-GFP$^-$ T$_{reg}$ progenitors. Sorted cells were incubated in culture with or without IL-2 (1 U/mL) and 100 nM of the indicated TNFRSF agonists for 72 hours prior to flow cytometric analysis to determine the fraction of cells that acquired Foxp3-GFP during the course of the incubation (mean ± SEM from three independent experiments, n = 3, * P < 0.05). (**c**) The expression of CD25 amongst Foxp3-GFP$^-$ T$_{reg}$ progenitors after stimulation with IL-2 and GITR-L–Fc is plotted as fold change in geometric mean fluorescence intensity (gMFI) over IL-2 treatment alone (mean ± SEM derived from seven independent experiments, n = 7, * p < 0.05). (**d**) CD25 expression was measured as gMFI in the Foxp3-GFP$^+$ gate after a 72h stimulation with IL-2 and GITR-L–Fc (mean ± SEM from three independent experiments, n = 3, * P < 0.05). (**e**) The efficiency of T$_{reg}$ progenitor maturation with or without TNFRSF costimulation in response to a range of IL-2 concentrations is shown (mean ± SEM from three independent experiments, n = 3). (**f**) Intracellular staining for p-STAT5 was performed after stimulating sorted T$_{reg}$ progenitors with IL-2 and/or

TNFRSF agonists for 24 hours. Data are representative of one of two independent experiments (n = 2). (**g**) Shown are the percentages of sorted T_{reg} progenitors that converted to Foxp3-GFP$^+$ T_{reg} cells during a 72h incubation with IL-2 and GITR-L–Fc or IL-2 and the pan-caspase inhibitor, z-VAD-FMK, using DMSO (v/v) as a vehicle control (mean ± SEM derived from three independent experiments, n = 3, * P < 0.05).
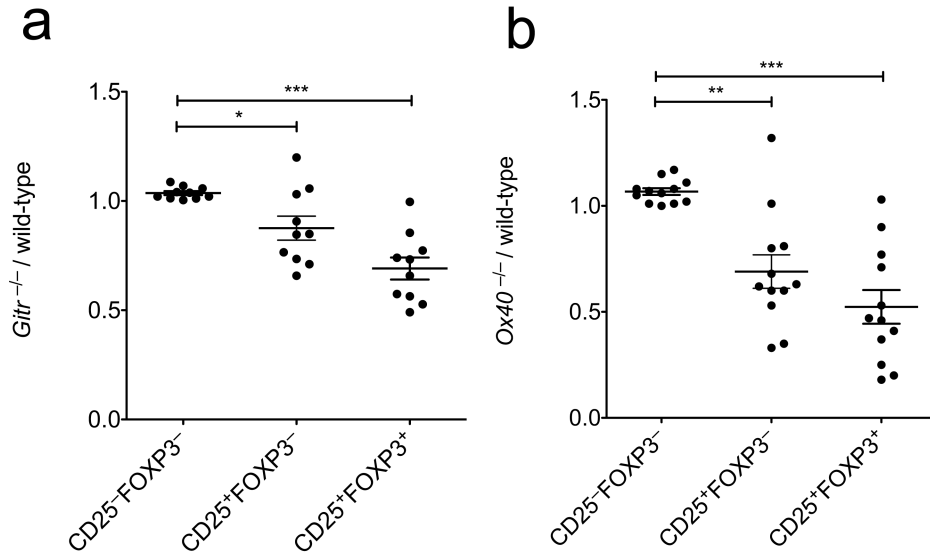
Figure 4. GITR- or OX40-deficiency imposes a modest cell-intrinsic thymic T$_{reg}$ developmental block
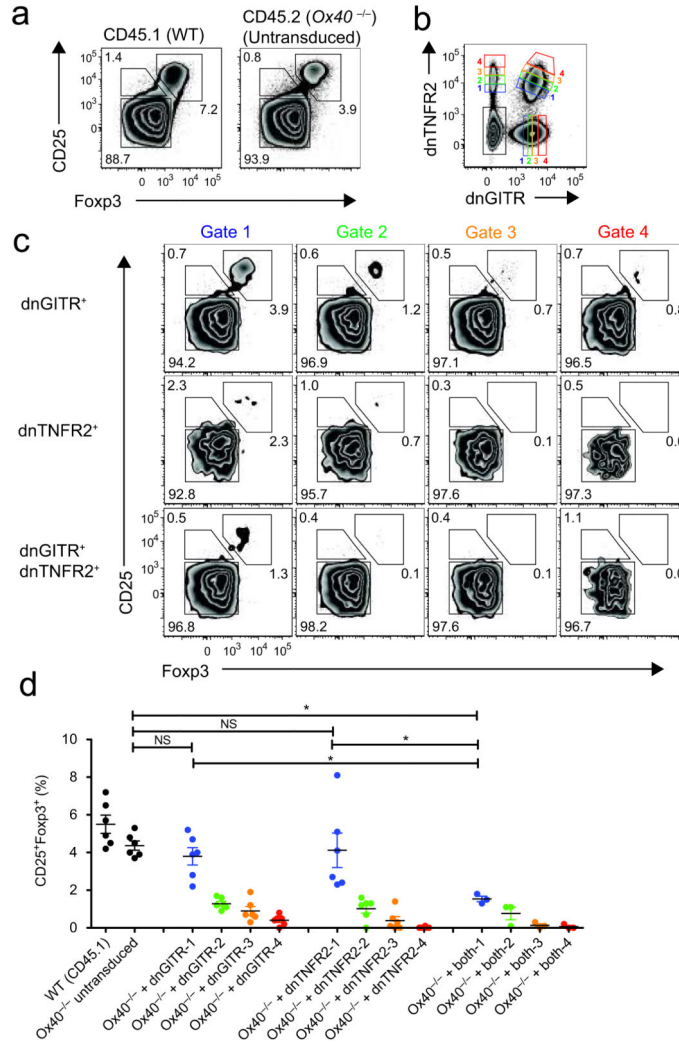
Bone marrow was harvested from 8-12 week old female or male (**a**) *Gitr* $^{-/-}$ or (**b**) *Ox40* $^{-/-}$ mice (CD45.2; also known as *Tnfrsf18* and *Tnfrsf4*, respectively) and age- and sex-matched congenic recipients and depleted of mature lymphocytes. Donor cells were mixed prior to engraftment into sublethally irradiated adult *Rag2* $^{-/-}$ mice by IV injection. Ten to 18 weeks later thymii and spleen were analyzed for T$_{reg}$ development and homeostasis by flow cytometry. (**a,b**) Shown are the percentages of CD45.2$^+$ vs. CD45.1$^+$ cells occupying gates that identify conventional T cells (CD25$^-$Foxp3$^-$), T$_{reg}$ progenitors (CD25$^+$Foxp3$^-$), and mature thymic T$_{reg}$ cells (CD25$^+$Foxp3$^+$) normalized to the ratio of CD45.2$^+$ vs. CD45.1$^+$ within total CD4SP thymocytes. Results in panel **a** are from 10 individual chimeras from two independent experiments; results in panel **b** are from 12 individual chimeras from three separate experiments (mean ± SEM, P < 0.05 values calculated by ANOVA with Bonferroni's multiple comparison test).
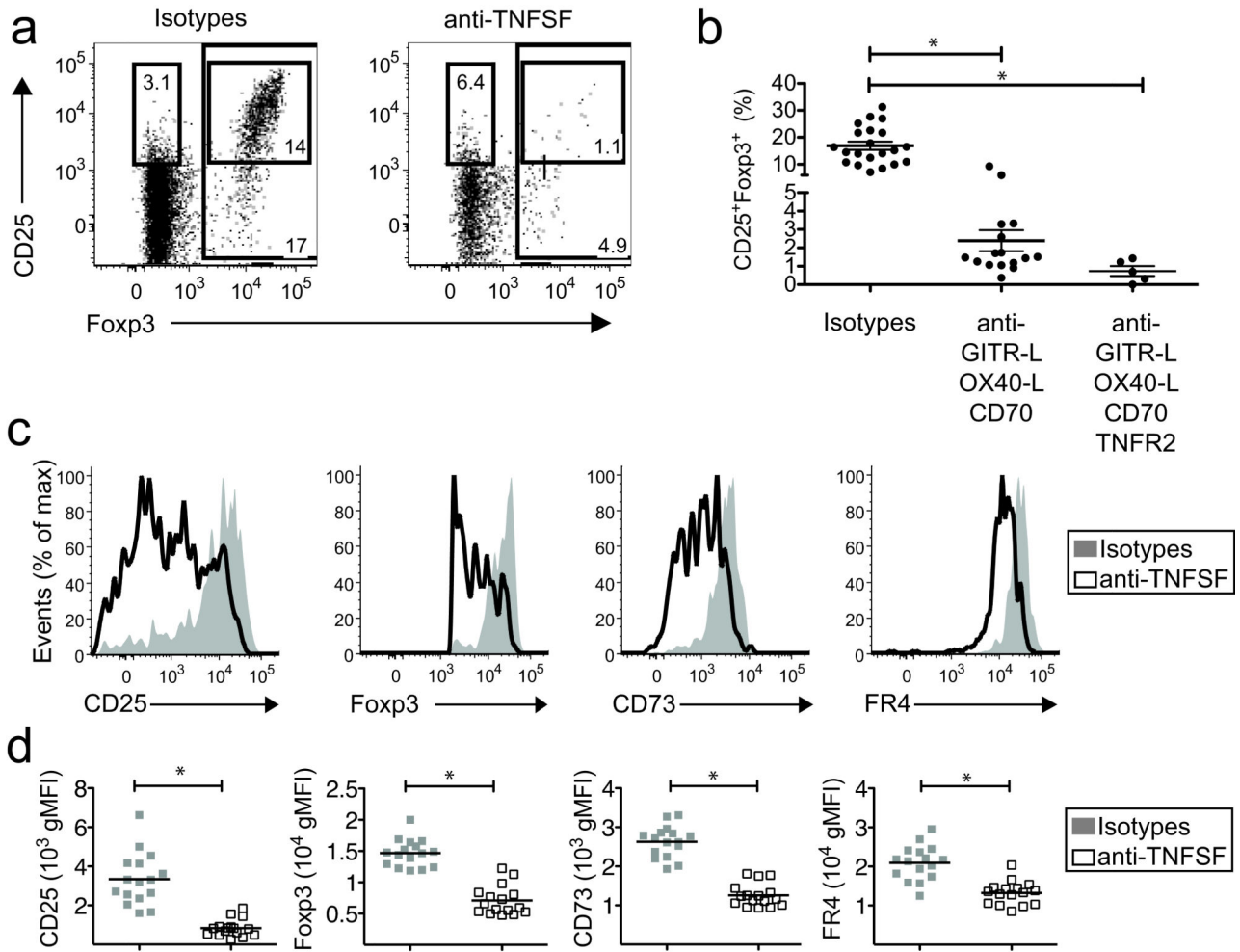
Figure 5. Antibody neutralization of TNFSF members in thymic organ cultures inhibits T_reg development and the acquisition of maturation markers

Neonatal Foxp3-GFP thymic lobes from one day-old pups were separated and plated in organ cultures with isotype controls or neutralizing antibodies to GITR-L, OX40-L, and CD70, or the combination of anti-GITR-L, OX40-L, CD70, and TNFR2. After 14 days, lobes were dissociated and T_reg development was analyzed by flow cytometry. (**a**) A representative comparison of CD4SP thymocytes from isotype- vs. antibody-treated thymic lobes is shown. (**b**) Shown are the percentages of CD25+Foxp3+ T_reg cells amongst total CD4SP thymocytes (mean ± SEM, n=21 over four experiments for isotype controls, n=16 over three experiments for anti-GITR-L, OX40-L, and TNF; n=5 over two experiments for anti-GITR-L, OX40-L, CD70, and TNFR2; * p < 0.05 calculated by ANOVA with Bonferroni's multiple comparison test). (**c,d**) The expression of CD25, Foxp3, CD73, and FR4 expression in CD4+Foxp3+ cells from TOCs treated with isotype control antibodies- (solid gray) or TNFSF neutralizing Abs treated (open black) is displayed in the histograms and corresponding bar graphs below (n=16 from three independent experiments, mean ± SEM). P-values were generated using paired, two-tailed T tests (* P < 0.0001).

Figure 6. GITR, OX40, and TNFR2 redundantly drive T_reg development in vivo
(**a**) Bone marrow from 8-12 week old male or female CD45.2+ *Ox40* −/− mice was harvested and prestimulated with IL-3, IL-6, and SCF prior to retroviral transduction with vectors encoding dominant negative GITR (dnGITR) and TNFR2 (dnTNFR2). Cells were mixed with congenically marked CD45.1+ control marrow and engrafted into adult sublethally irradiated *Rag*-deficient hosts for 10-12 weeks prior to analysis by flow cytometry. Representative flow cytometry plots derived by gating on CD45.1 WT or CD45.2 (*Ox40*−/−) cells which were negative for GFP and Thy1.1 (untransduced) show the percentages of CD25−Foxp3− conventional CD4SP cells, CD25+Foxp3− T_reg progenitors, and CD25+Foxp3+ T_reg cells amongst CD4SP thymocytes. (**b**) To evaluate dose-dependent effects of inhibiting multiple TNFRSF on T_reg development, *Ox40*−/− (CD45.2+) thymocytes were plotted for their expression of dnGITR (GFP) and dnTNFR2 (Thy1.1) and gates were drawn on cells expressing increasing levels of GFP, Thy1.1, or both. Gates are labeled 1 through 4 and colored in blue, green, orange, and red, respectively. (**c**) Cells derived from gates in panel (**b**), representing increasing expression of dnGITR, dnTNFR2, or both, were evaluated for CD25 and Foxp3 expression to determine the frequencies of conventional
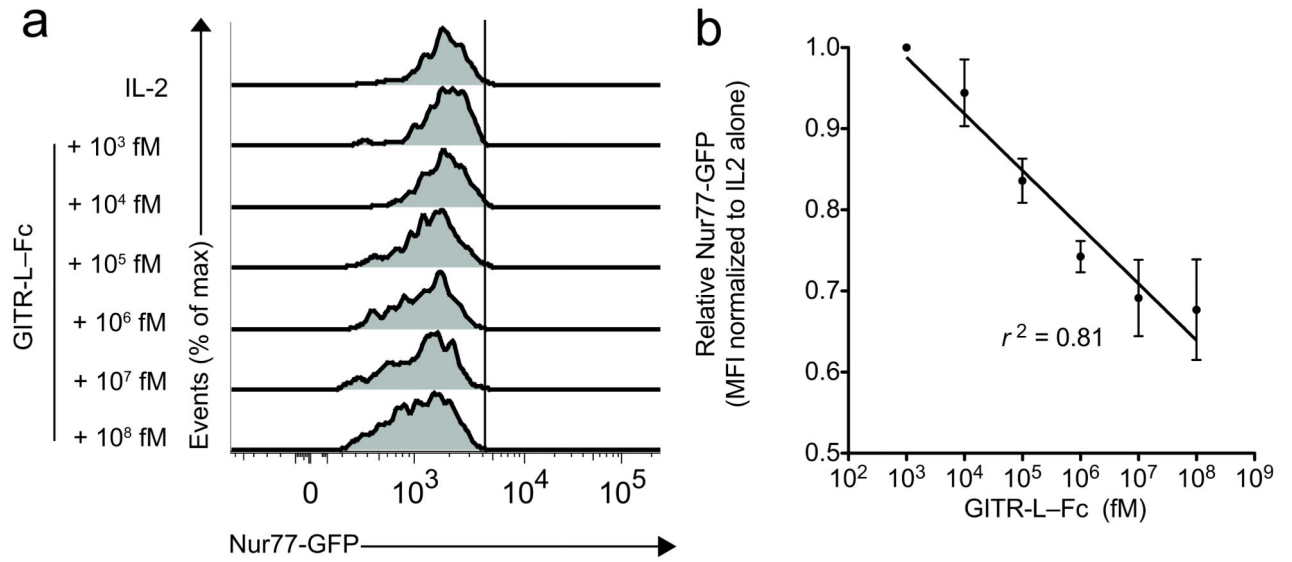
CD4SP, T_{reg} progenitors, and mature T_{reg} cells within each gate (**d**) Cumulative data from one of three independent experiments are shown in the scatter plot below (mean ± SEM, n=6, * P < 0.05 as determined by 1-way ANOVA using Bonferroni's multiple comparison test). For a summary table of all statistical comparisons see Supplementary Table 1.

Figure 7. Excess TNFSF ligand broadens the T_{reg} repertoire to contain a greater fraction of cells with a lower affinity for self

(**a**) T_{reg} progenitors from 6-8 week old female or male Foxp3-RFP × Nur77-GFP dual reporter mice were isolated by cell sorting and stimulated in culture for 72h with 1 U/ml IL-2 and increasing concentrations of agonist GITR-L–Fc. Shown are stacked histograms displaying Nur77-GFP levels amongst converted Foxp3-RFP$^+$ T_{reg} cells. (**b**) Cumulative data from three independent experiments (n = 3) show a dose-dependent relationship between GITR-L–Fc concentration (x-axis) and the Nur77-GFP MFI (y-axis) within converted Foxp3-RFP$^+$ T_{reg} cells (as normalized to the value obtained with IL-2 treatment alone; mean ± SEM, n=3).