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Modulation of Treg cells/T effector function by GITR signaling is context–dependent

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Abstract

Treg cells express high levels of the glucocorticoid-induced tumor necrosis factor-related receptor (GITR), while resting conventional T (Tconv) cells express low levels that are increased upon activation. Manipulation of GITR/GITR-Ligand (GITR-L) interactions results in enhancement of immune responses, but it remains unclear whether this enhancement is secondary to costimulation of Tconv cells or to reversal of Treg-cell-mediated suppression. Here, we used a nondepleting Fc-GITR-L and combinations of WT and GITR KO Treg cells and Tconv cells to reexamine the effects of GITR stimulation on each subpopulation in both unmanipulated mice and mice with inflammatory bowel disease. Treatment of mice with Fc-GITR-L resulted in significant expansion of Treg cells and a modest expansion of Tconv cells. When RAGKO mice were reconstituted with Tconv cells alone, GITR-L resulted in Tconv-cell expansion and severe inflammatory bowel disease. The protective effect of Treg cells was lost in the presence of Fc-GITR-L, secondary to death of the Treg cells. When RAG KO mice were reconstituted with Treg cells alone, the transferred cells expanded normally, and Fc-GITR-L treatment resulted in a loss of Foxp3 expression, but the ex-Treg cells did not cause any pathology. The effects of GITR activation are complex and depend on the host environment and the activation state of the Treg cells and T effector cells.

Keywords

Glucocorticoid-induced tumor necrosis factor-related receptor (GITR); Inflammatory bowel disease; Treg cell; T effector cells

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Introduction

The glucocorticoid-induced tumor necrosis factor-related receptor (GITR), a member of the TNF receptor superfamily (TNFRSF) is expressed at high levels on the majority of freshly explanted Foxp3$^+$ Treg cells, activated CD4$^+$ and CD8$^+$ T effector (Teff) cells [1] and at low levels on other cell types including B cells, NK cells, macrophages, dendritic cells, eosinophils, basophils, and mast cells [2]. The GITR ligand (GITR-L) is also widely expressed in the immune system and can be detected on basal levels on dendritic cells, B cells, monocytes, macrophages, with particularly high expression on plasmacytoid DCs [3] and its expression is transiently upregulated during inflammatory responses. Experiments using anti-GITR agonistic antibodies initially suggested that GITR played a critical role in the function of Treg cells, as engagement of the GITR by the agonist antibody appeared to reverse the suppressive effects of Treg cells in vitro [1, 2]. Subsequent studies using combinations of GITR sufficient and KO Treg cells and Teff cells in vitro demonstrated that the abrogation of suppression was secondary to engagement of the GITR on Teff cells rather than Treg cells, thereby rendering the Teff cells resistant to suppression [3]. Other studies in vitro have demonstrated that triggering of the GITR only on Teff cells by either agonistic antibody, soluble GITR-L or cells transfected with GITR-L enhanced both CD4$^+$ and CD8$^+$ T-cell proliferation to suboptimal anti-CD3 stimulation, enhanced cell-cycle progression, augmented cytokine production, and rescued anti-CD3 treated T cells from apoptosis [3–5]. More recent studies have also demonstrated that P815 cells transfected with GITR-L were capable of augmenting Treg-cell proliferation in vitro, enhancing IL-10 production, and augmenting Treg-cell suppressive capacity [5]. The GITR is not essential for Treg-cell function, as Treg cells from GITR KO mice display a normal capacity to suppress T-cell proliferation in vitro [3].

The GITR has been implicated in the regulation of both adaptive and innate immune responses in vivo. In general, engagement of the GITR by agonistic antibodies enhances immune responses including anti-viral specific CD4$^+$ and CD8$^+$ T cells responses in both acute and chronic infection [6, 7]. Most importantly, engagement of the GITR resulted in potent anti-tumor responses including eradication of established Meth-A sarcomas [8], poorly immunogenic B16 melanoma [9], and CT26 colon tumors [10]. Conversely, inhibition of GITR/GITR-L interactions by administration of soluble GITR-Fc resulted in prolongation of allograft survival potentially by preventing GITR-L-mediated reversal of Treg-cell-mediated suppression [11]. GITR knockout mice and mice treated with a blocking GITR-Fc had reduced inflammation, tissue damage, and reduced mortality in a model multiple organ failure [12]. While the costimulatory effects of GITR engagement on Teff cells are clear, controversial results have been reported on the effects of GITR engagement on Treg cells in vivo [11]. Some studies have demonstrated enhancement of Treg-cell numbers following treatment of mice with recombinant Fc-GITR-L [13] and mice expressing a GITR-L transgene in B cells had an increase in the ratio of Treg cells/Tconv cells and a delay in the onset of experimental autoimmune encephalitis [14]. Conversely, several studies in tumor models have described a decrease in the percentage of Foxp3$^+$ T cells in the tumor, as well as a redistribution of the intracellular localization of Foxp3 [15].

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However, interpretation of some of these studies that used anti-GITR mAbs is complicated as administration of anti-GITR in vivo can result in depletion of Treg cells [16].

In the present study, we have used a nondepleting, recombinant Fc-GITR-L and combinations of GITR WT and GITR KO Treg cells and Teff cells to reexamine the effects of GITR stimulation on each subpopulation in both unmanipulated mice and in a well-characterized model of inflammatory bowel disease (IBD). We demonstrate that the effects of that Fc-GITR-L-induced GITR signaling are complex and depend on the physiologic environment in the host as well as the activation state of the Treg cells and Teff cells. The implications of these results regarding the therapeutic manipulation of the immune response by members of the TNFRSF are discussed.

Results

Engagement of GITR stimulates the expansion of both Foxp3$^+$ and Foxp3$^-$ T cells in naïve mice

Previous studies have demonstrated that engagement of the GITR provides a costimulatory signal for activation of the proliferation of both CD4$^+$ and CD8$^+$ Foxp3$^-$ T cells in vitro [2, 3], while engagement of the GITR on Foxp3$^+$ Treg cells in vitro stimulated their proliferation in the presence of IL-2, but in the absence of TCR stimulation [1]. To assess the effect of GITR engagement in vivo, we administered Fc-GITR-L, a nondepleting soluble recombinant protein dimer that has been shown to enhance tumor immunity [17] or human IgG1 as a control to unmanipulated mice. Fc-GITR-L administration in the absence of any other exogenous stimulation significantly increased Foxp3$^+$ T-cell frequency and absolute numbers on day 3 after treatment (Fig. 1A–C). The increase in Treg-cell frequency was also reflected in enhanced incorporation of BrdU (Supporting Information Fig. 1A). A modest increase in the absolute numbers of Tconv cells was also seen (Fig. 1D). A similar enhancement in Treg cells was seen in mice treated with a different preparation of Fc-GITR-L [13], but these authors did not observe any increase in Tconv cells. To determine if GITR stimulation modulated Treg-cell function, we purified CD4$^+$CD25$^+$T cells from Fc-GITR-L and IgG1-injected mice and assessed their suppressive capacity in vitro (Supporting Information Fig. 1B). Treg cells from Fc-GITR-L-treated mice were as suppressive as Treg cells from control human IgG1-treated mice. The increase in Treg cells was transient and the percentage of Foxp3$^+$ T cells returned to normal by day 9 after treatment (Supporting Information Fig. 1C).

Fc-GITR-L exacerbates IBD by enhancing the expansion of pathogenic T cells

Previous studies suggested that treatment of mice with an agonist anti-GITR mAb, following anti-CD25 depletion of Treg cells, was capable of enhancing the pathogenicity of autoantigen-specific T cells in an experimental autoimmune encephalomyelitis model [18]. One problem with this approach is that Treg-cell depletion is usually incomplete and Treg cells rapidly repopulate the treated animals [19]. To more directly address the effects of GITR stimulation on Teff cell numbers and function, we used the IBD model [20] and transferred CD4$^+$CD45RB^{hi} Foxp3$^-$ T cells into RAG KO mice followed by weekly treatment with Fc-GITR-L (100 μg/mouse i.v.). Mice treated with Fc-GITR-L exhibited a

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markedly enhanced loss of weight compared with mice that just received $CD4^+CD45RB^{hi}$ T cells (Fig. 2A). The percentage of $CD4^+$ T cells secreting IFN-γ was similar in treated and control animals (Fig. 2B and D), but the absolute number of $CD4^+$ T cells secreting IFN-γ was markedly increased in the mesenteric LN (Fig. 2C). In contrast, we observed no changes in either the percentages or absolute numbers of IL-17-producing T cells (Fig. 2E and F). Teff-cell expansion was also reflected in enhanced Ki67 staining in the treated mice (Fig. 2G and H). Thus, engagement of the GITR by GITR-L in vivo has no effect on T-cell differentiation, but significantly augments the absolute number of pathogenic IFN-γ producing T cells and disease severity. Our results are similar to the effects of GITR engagement that have been reported [21] on $CD8^+$ Teff cells in a viral model where GITR engagement increased $CD8^+$ T-cell expansion without enhancing their effector functions. Small percentages of $Foxp3^+$ iTreg cells were observed in mice that received $CD4^+CD45RB^{hi}$ $Foxp3^-$ T cells, but the percentages were the same in untreated or GITR-L-treated mice (data not shown).

The GITR is also expressed on APCs and NK cells at a low levels [2] and it has been suggested [22, 23] that some of the effects of GITR engagement in vivo may be secondary to modulation of innate immune functions. To address this issue, we transferred $CD4^+CD45RB^{hi}$ T cells from $GITR^{-/-}$ mice to $RAG^{-/-}$ mice (Supporting Information Fig. 2A). $CD4^+CD45RB^{hi}$ T cells from $GITR^{-/-}$ mice were as efficient in inducing weight lost as $CD4^+CD45RB^{hi}$ T cells from WT mice (Fig. 2A compared with Supporting Information Fig. 2A). However, treatment with Fc-GITR-L did not exacerbate weight loss or increase the absolute number of $CD4^+$ T cells secreting IFN-γ in the mesenteric LN (Supporting Information Fig. 2B). This study demonstrates that the effects of GITR-L administration are mediated directly on Teff cells and not indirectly on cells of the innate immune system.

Engagement of the GITR induces Treg-cell loss in the IBD model

As Fc-GITR-L treatment was capable of primarily expanding Treg cells in normal unmanipulated mice and could also enhance Teff-cell numbers in the absence of Treg cells, it was of interest to determine which one or these effects predominated in the IBD model. We transferred $CD4^+CD45RB^{hi}GFP^-$ T cells (4×10^5) from Foxp3-GFP knock in mice together with $CD4^+GFP^+$ Treg cells (2×10^5) into RAG KO mice. Mice treated with Fc-GITR-L exhibited weight loss, while untreated mice were, as expected, protected from IBD (Fig. 3A). Surprisingly, both the percentages and the absolute number of $Foxp3^+$ T cells in Fc-GITR-L-treated mice were decreased in the mesenteric LN but this difference was not statistically significant (Fig. 3B). We did not rely on GFP expression to detect $Foxp3^+$ T cells and in all studies performed intracellular staining for Foxp3 expression.

To determine if the decrease in Treg-cell frequency was secondary to a direct engagement of GITR on Treg cells or secondary to potent bystander T-cell activation of Teff cells, we transferred $CD45RB^{hi}CD4^+$T cells (5×10^5) purified from $GITR^{-/-}$ mice together with wild-type $CD4^+CD25^+$ Treg cells cells (2×10^5) into $RAG^{-/-}$ mice. We distinguished Treg cells from Teff cells based on GITR expression. $CD45RB^{hi}$ $GITR^{-/-}$ $CD4^+$ T cells induced weight loss that was reversed by cotransfer of $GITR^{+/+}$ Treg cells (Fig. 4A). Surprisingly, when Fc-GITR-L was administered, the protective effect of the $GITR^{+/+}$ Treg cells was lost

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and the recipients developed significant weight loss (Fig. 4A). The percentage and absolute number of $GITR^{+/+}$ $Foxp3^+$ T cells in the mesenteric LN were dramatically decreased in Fc-GITR-L injected group (Fig. 4B–D). This loss of $Foxp3^+$ T cells was not secondary to loss of Foxp3 expression, as the absolute number of $Foxp3^-GITR^{+/+}$ T cells was comparable with that of the untreated group (data not shown) and therefore likely represents death of the $Foxp3^+$ population in the GITR-L-Fc-treated mice. Although the absolute number of $GITR^{-/-}$ Teff cells in the mesenteric LN was comparable in Treg-cell treated mice in the presence and absence of Fc-GITR-L (Fig. 4E), the percentage of IFN-γ-secreting cells in the mesenteric LN was significantly increased (Fig. 4F) suggesting that under these conditions loss of Treg-cell suppressor function results in an enhancement of Teff-cell differentiation. As a negative control, we cotransferred $CD45RB^{hi}GITR^{-/-}CD4^+$ T cells and $CD4^+CD25^+GITR^{-/-}$ Treg cells into $RAG^{-/-}$ mice. Treg-cell function was not altered by treatment of these recipients with FcGITR-L, as the treated mice did not lose weight and the absolute number of $Foxp3^+$ T cells was comparable between the control and treated mice (Supporting Information Fig. 3A and B). Thus, the effects of GITR engagement on Treg cells in this model of IBD differ markedly from the effects of GITR engagement in normal mice where GITR stimulation leads to Treg-cell expansion.

Fc-GITR-L stimulation downmodulates Foxp3 expression under lymphopenic conditions

It was also of interest to examine the fate of GITR engagement on Treg cells in the absence of Teff cells. When $Foxp3^+CD4^+$ T cells were sorted from Foxp3-GFP knock in mice and transferred to RAG KO mice, comparable expansion (>20×) of the transferred $CD4^+$ T cells was observed at either 4 (Fig. 5A) or 10 weeks (data not shown) in mice treated with Fc-GITR-L or not treated. However, the absolute number and the frequency of cells retaining Foxp3 expression was significantly decreased in the mLN, but not the spleen, in Fc-GITR-L-treated mice (Fig. 5B and C). Since the total number of $CD4^+$ T cells is unchanged, this result suggests that GITR engagement under lymphopenic, IL-2 deprivation conditions results in loss of Foxp3 expression. However, the level of expression Foxp3 (MFI treated = 6438 and untreated = 6311) is similar in the remaining $Foxp3^+$ T cells (Fig. 5B). An alternative explanation is that the Treg-cell populations in both treated and untreated mice are losing Foxp3 at the same rate in the lymphopenic environment, but that Treg cells that have lost Foxp3 in the treated animals are then stimulated to proliferate at a greater rate similar to the effect of Fc-GITR-L in mice receiving Teff cells alone (Fig. 2C). However, the percentages of $Foxp3^-$ $Ki67^+$ cells were similar in the control and Fc-GITR-L-treated mice (Supporting Information Fig. 4A and B). This process may also be accompanied by Treg-cell death, as seen in Fc-GITR-L-treated RAG KO mice reconstituted with GITR KO Teff cells and WT Treg cells (Fig. 4C). Indeed, we did observe a higher incidence of death only of the $Foxp3^+$ T cells in GITR-L-Fc-treated mice than the controls particularly in the mesenteric LN (Supporting Information Fig. 4C, D).

One possibility is that the $Foxp3^+$ T cells that have lost expression of Foxp3 and can be termed ex-Treg cells [24] have been converted to pathogenic Teff cells. However, none of the $RAG^{-/-}$ recipients of Treg cells lost weight during the 8 weeks of treatment with Fc-GITR-L (Fig. 5D). The frequency of $CD4^+$ T cells producing IFN-γ was similar in the ex-Treg-cell populations in treated and nontreated groups (Fig. 5E). A significant increase in

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IL-17 producing ex-Treg cells was observed in the mLN of GITR-L-treated mice (Fig. 5F). The remaining Foxp3+ T cells contained very low (<1%) levels of IFN-γ-producing cells or IL-17 (<0.5%) producing T cells and their frequency was comparable between the treated and untreated groups (data not shown).

Discussion

There is little doubt that modulation of the numbers and/or function of Foxp3+ Treg cells represent a major goal for the therapy of autoimmune diseases. Members of the TNFRSF play a diverse role in fine-tuning immune responses and several members are preferentially expressed on Foxp3+ Treg cells including the GITR (TNFRSF18), OX40 (TNFRSF4) [25], and DR3 (TNFRSF25) [26]. One major issue that remains unresolved is whether therapeutic targeting of TNFRSF members can be used to enhance Treg-cell function in vivo and whether this approach can be used as an alternative to IL-2 treatment [27] or Treg-cell cellular biotherapy [28]. Although some studies have demonstrated the selective effect of agonist mAbs or soluble ligands to these receptors on Treg-cell function [13] in the mouse, interpretation of most of these studies is complicated because these reagents also exert potent costimulatory effects on Teff cells and some of the reagents may result in Treg-cell depletion [16]. Some of the latter studies have probably been misinterpreted as demonstrating reversal of Treg-cell suppressor function secondary to engagement of the GITR on Treg cells.

In order to dissect the role of the GITR in Treg cell/Teff cell function, we have analyzed the effects of GITR stimulation by soluble Fc-GITR-L under a number of experimental conditions. In healthy, unmanipulated mice Fc-GITR-L treatment resulted in a short-term expansion of Treg cells accompanied by a modest enhancement of Tconv cells. In contrast, in the absence of Treg cells, Fc-GITR-L resulted in marked enhancement of the numbers of Teff cells in the IBD model, but had little effect on their differentiation. In the presence of both Teff and Treg cells in the IBD model, the effects of Fc-GITR-L treatment on Treg cells were much more complex. In the presence of WT Teff cells and WT Treg cells, administration of Fc-GITR-L resulted in a moderate decrease in the numbers of the Treg cells and in their suppressive function. However, when GITR KO Teff cells were cotransferred with WT Treg cells and the recipients treated with Fc-GITR-L, there was a dramatic decrease in the numbers of Treg cells and a loss of their suppressive function. One caveat in the interpretation of the IBD experiments is that they were all performed in immunodeficient mice and both the Teff cells and the Treg cells undergo marked homeostatic proliferation under these conditions. Nevertheless, this experimental protocol allowed us to define specific effects of GITR engagement on both subpopulations and to exclude any effect of GITR-L on cells of the innate immune system.

In general, GITR-L treatment augmented the number of IFN-γ-producing cells, but had no effect of the number of IL-17-producing cells. The role of IL-17 in the pathogenesis of IBD remains controversial [29]. In some studies, we have observed an increase in IL-17-producing cells under conditions where Treg cells have had a therapeutic effect. It is possible that these cells represent protective Th17 cells [30]. When WT Treg cells were transferred to RAG KO mice in the absence of Teff cells, Fc-GITR-L treatment resulted

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in an almost complete loss of Foxp3 expression, but the ex-Treg cells persisted and did not induce disease during the 8 weeks of the study. It is likely that the failure to observe disease during this time period was secondary to the persistence of some Treg cells that maintained Foxp3 expression. A similar absence of disease induction was seen in another study in which Foxp3^{+} T cells were transferred to RAG$^{-/-}$ recipients [31]. While 50% of the cells lost expression of Foxp3, the recipients did not develop IBD. However, when the Foxp3^{-} cells were isolated and transferred to secondary RAG$^{-/-}$ mice, the recipients did develop tissue inflammation.

Taken together, GITR activation on Treg cells can have different outcomes depending on the experimental context ranging from expansion in normal mice to death in the IBD model. This dual action of GITR engagement on Treg cells is not unexpected, as similar to other members of the TNFRSF, GITR might activate more than one signaling pathway. Activation of the NF-κB pathway may result in Treg-cell expansion [32], while GITR signaling via Siva may result in apoptosis [33]. It also remains possible that the rapid induction of Treg-cell proliferation in a highly proinflammatory environment may result in activation-induced cell death via FAS/FAS-L or TNF/TNFR. Taken together, the translation of studies of GITR function in the mouse model to the use of Fc-GITR-L or agonist mAbs in man should be undertaken with caution depending on the disease (autoimmunity versus tumor immunity) under study and the immune status of the host.

Materials and methods

Animals

C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD). Foxp3-GFP mice were obtained from Dr. V.J. Kuchroo (Harvard University, Boston, MA) and maintained by Taconic Farms (Germantown, NY) under contract by NIAID. RAG$^{-/-}$ mice obtained from Taconic Farms. *GITR*$^{+/-}$ embryos (Sv129 × B6) were provided by C. Ricarrdi (Perugia University Medical School, Perugia, Italy). Rederived *GITR*$^{+/-}$ mice were backcrossed once with C57BL/6 mice, and the resulting progeny were screened for the mutant allele by PCR. The identified *GITR*$^{+/-}$ progeny were then intercrossed to generate *GITR*$^{-/-}$ mice. All mice were bred and housed at National Institutes of Health/ National Institute of Allergy and Infectious Diseases facilities under specific pathogen-free conditions. All studies were approved by the Animal Care and Use Committee of the NIAID.

Media, mAbs, and reagents

Fc-GITR-L, construct #178–14, was prepared as previously described [15]. Anti-CD4 V-500 and PE-Cy5, anti-CD25 PE, anti-GITR-PE, anti-CD44 Alexa Fluor 700, CD45.2 allophycocyanin-eFluor 780, anti-CD45.1 PE-Cy7, fixable viability dye allophycocyanin-eFluor 780 and eFluor 450, anti-Foxp3 PE, eFlour 450 and allophycocyanin, ant-IL-17 Alexa Fluor 647 and anti-IFN-γ PE-Cy7 were purchased from (eBioscience, San Diego, CA). PE–anti-CD25 (PC61), anti-GITR PE-Cy7, anti-CD45RB FITC and PE, anti-Ki67 PE and anti-BrdU FITC were purchased from BD Biosciences (San Jose, CA). Intracellular staining was performed with the Foxp3 staining buffer kit, according to the manufacturer's

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protocol (eBioscience or BD Biosciences). CD4 microbeads were purchased from Miltenyi Biotec (Auburn, CA). Flow cytometry analysis was performed using FlowJo software.

Purification of T-cell subsets

Peripheral LNs and spleens were harvested from 8-week-old female mice. $CD4^+$ T cells where enriched by Automacs using CD4 microbeads, labeled with anti-CD4 PE-Cy5, anti-CD25 PE, and CD45RB FITC or anti-CD4 PE-Cy5 and anti-CD45RB PE and purified by cell sorting. The purity of $CD4^+CD25^-CD45RB^{hi}$, $CD4^+CD25^+$, $CD4^+GFP^-CD45RB^{hi}$, $CD4^+GFP^+$ cells was >98%.

T-cell reconstitution and Fc-GITR-L treatment

RAG KO mice were injected i.v. with sorted $CD4^+$ T-cell subpopulations in PBS. Mice received 5×10^5 $CD4^+CD45RB^{high}$ from WT GITR or GITR KO mice alone or in combination with 2×10^5 $CD4^+$ GFP^+ GITR WT, $CD4^+$ $CD25^+$ GITR WT, or $CD4^+$ $CD25^+$ GITR KO cells; one group of mice received 2×10^5 $CD4^+$ GFP^+ GITR WT alone. Fc-GITR-L (200 μg) was injected i.v. one day after T-cell reconstitution, and then once weekly until the study was terminated. Mice were weighed weekly basis.

Suppression assay

$CD4^+CD25^-$T cells and $CD4^+CD25^+$ T cells were purified by cell sorting; postsort purity was >98%. Suppression assays were performed as previously described [3].

Statistical analysis

Statistical studies were compared using Mann–Whitney *U* test, and differences were considered statistically significant with $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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Abbreviations:

GITR	glucocorticoid-induced tumor necrosis factor-related receptor
GITR-L	glucocorticoid-induced tumor necrosis factor-related receptor ligand
IBD	inflammatory bowel disease
Tconv	conventional T
Teff	effector T
TNFRSF	tumor necrosis factor receptor superfamily

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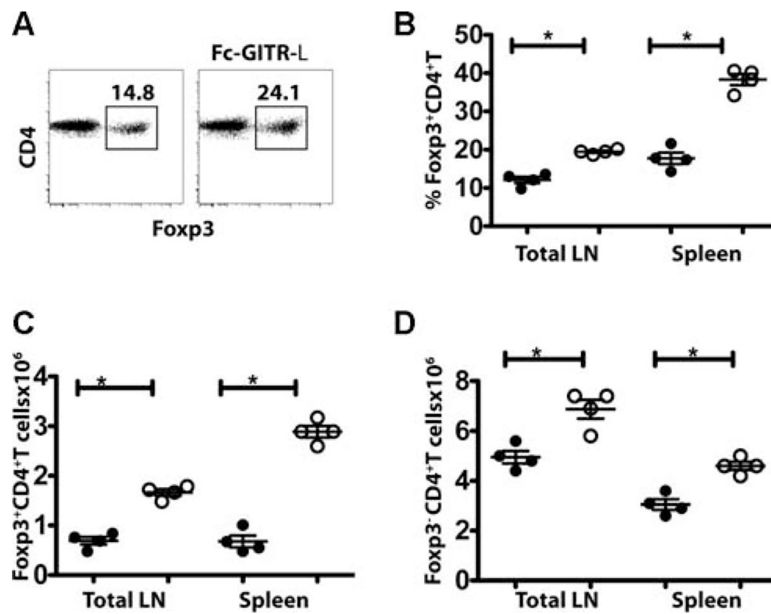

Figure 1.

Fc-GITR-L expands the absolute number of Treg cells and Tconv cells. C57BL/6J mice were injected with human IgG1 (solid circles) or Fc-GITR-L i.p. (open circles). At day 3, total LN and spleen cells were harvested. (A) Dot plot representing the percentage of Foxp3^{+} T cells in CD4^{+} gate. (B) The percentage of Foxp3^{+} T cells in CD4^{+} gate is shown. (C) The absolute number of Foxp3^{+} T cells is shown. (D) The absolute number of Foxp3^{-} T cells is shown. Asterisks indicate statistical significance determined by Mann–Whitney *U* test ($*p = 0.02$). (A–D) Data are shown as mean ± SEM of four mice per group and are from one experiment representative of four independent experiments performed.

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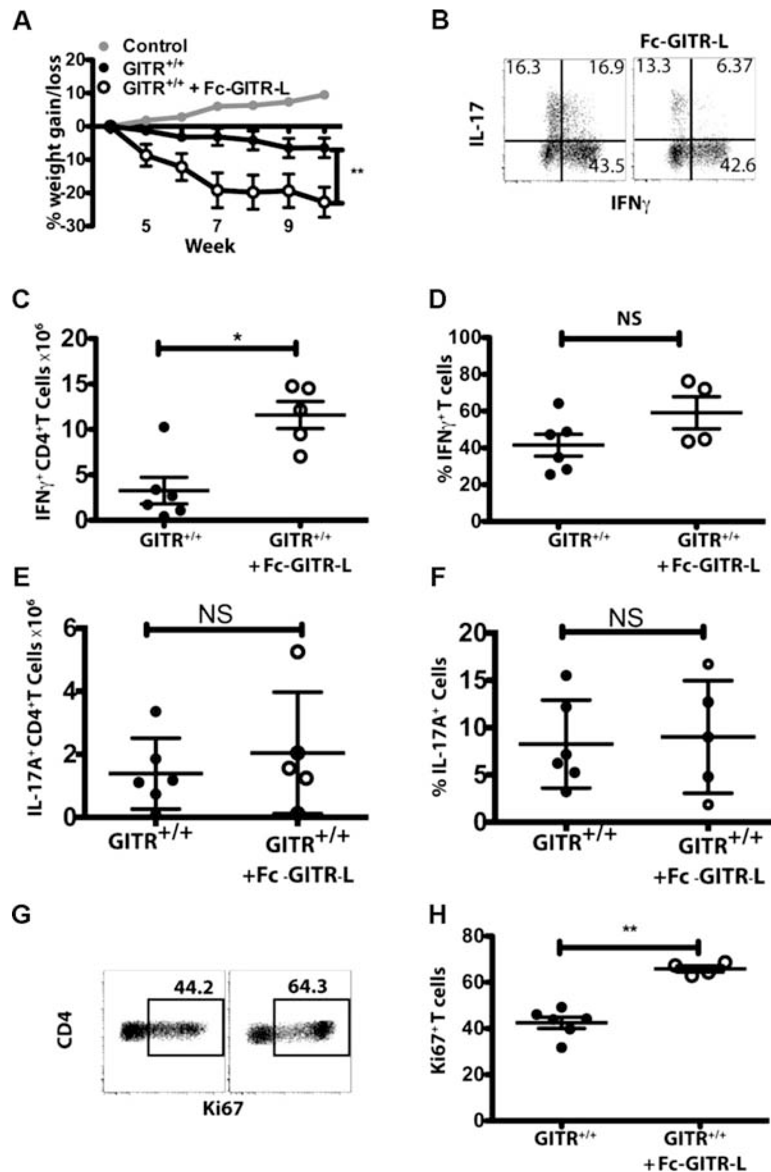

Figure 2.

Fc-GITR-L exacerbates IBD by inducing Teff-cell proliferation. RAG KO mice were reconstituted GITR$^{+/+}$Foxp3^{-}CD45RB^{high} T cells and not treated (solid circles) or treated with Fc-GITR-L weekly (open circles). Control mice were nonreconstituted RAG$^{-/-}$ mice (gray-filled circles). (A) The percentage of weight gain or loss is shown as the mean ± SEM. Asterisks indicate statistical significance determined by Mann–Whitney *U* test (**p = 0.007). (B) A representative dot plot of IFN-γ versus IL-17 expression in the mesenteric LN. (C) The absolute number of IFN-γ-producing cells in the mesenteric LN (*p< 0.01). (D) The percentage of IFN-γ–producing CD4^{+} T cells in the mesenteric LN is shown. (E) The absolute number and (F) percentage of IL-17A-producing cells in the mesenteric LN in untreated and GITR-L treated mice are shown. (G) A representative dot plot of Ki67 expression in the CD4^{+} T-cell gate in the mesenteric LN is shown. (H) The percentage of Ki67^{+}T cells among CD4^{+} T cells in the mesenteric LNis shown (**p<0.009).(A–H)

Dataareshown as mean ± SEM of five to six mice per group and are from one experiment representative of three independent experiments performed.

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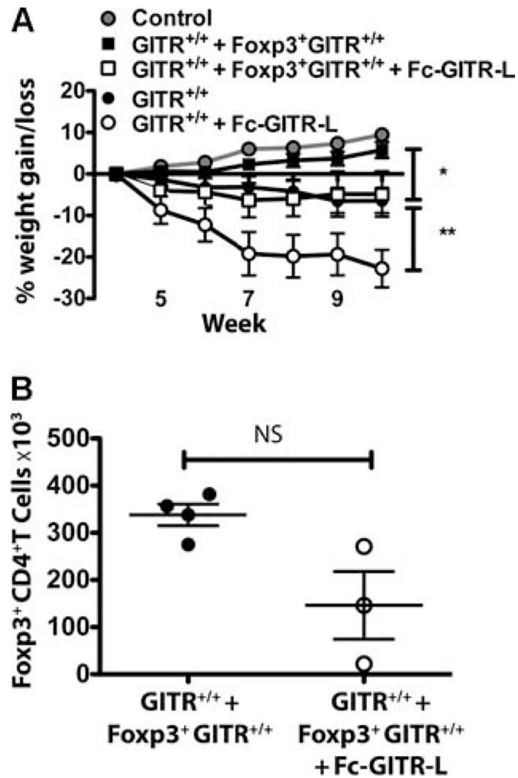

Figure 3.

Fc-GITR-L treatment modulates Treg-cell-mediated suppression. RAG KO mice were reconstituted with $GITR^{+/+}$ $Foxp3^{-}CD45RB^{high}$ T cells from Foxp3-GFP mice and not treated (closed circles) or treated with Fc-GITR-L (open circles). Separate groups of RAG KO mice were reconstituted with WT GITR $Foxp3^{-}CD45RB^{high}$ T cells together with WT GITR $Foxp3^{+}$ cells from Foxp3-GFP mice and not treated (solid squares) or treated with Fc-GITR-L weekly (open squares). A control group consisted of nonreconstituted RAG KO mice (gray-filled circles). (A) The percentage of weight gain or loss is shown. Asterisks indicate statistical significance determined by Mann–Whitney U test ($*p < 0.02$) for untreated mice reconstituted with $GITR^{+/+}$ $Foxp3^{-}CD45RB^{high}$ T cells together with $GITR^{+/+}$ $Foxp3^{+}$ T cells versus the mice that received both combination of cells and treated with Fc-GITR-L. ($**p < 0.007$) for Fc-GITR-L treated mice reconstituted with $GITR^{+/+}$ $Foxp3^{-}CD45RB^{high}$ T cells alone versus the Fc-GITR-L treated mice group reconstituted with $GITR^{+/+}$ $Foxp3^{-}CD45RB^{high}$ T cells together with $GITR^{+/+}$ $Foxp3^{+}$ T cells. (B) The absolute number of $Foxp3^{+}$ T cells in the mesenteric LN in the indicated group is shown. (A and B) Data are shown as mean ± SEM of four to five mice per group and are from one experiment representative of two independent experiments performed.

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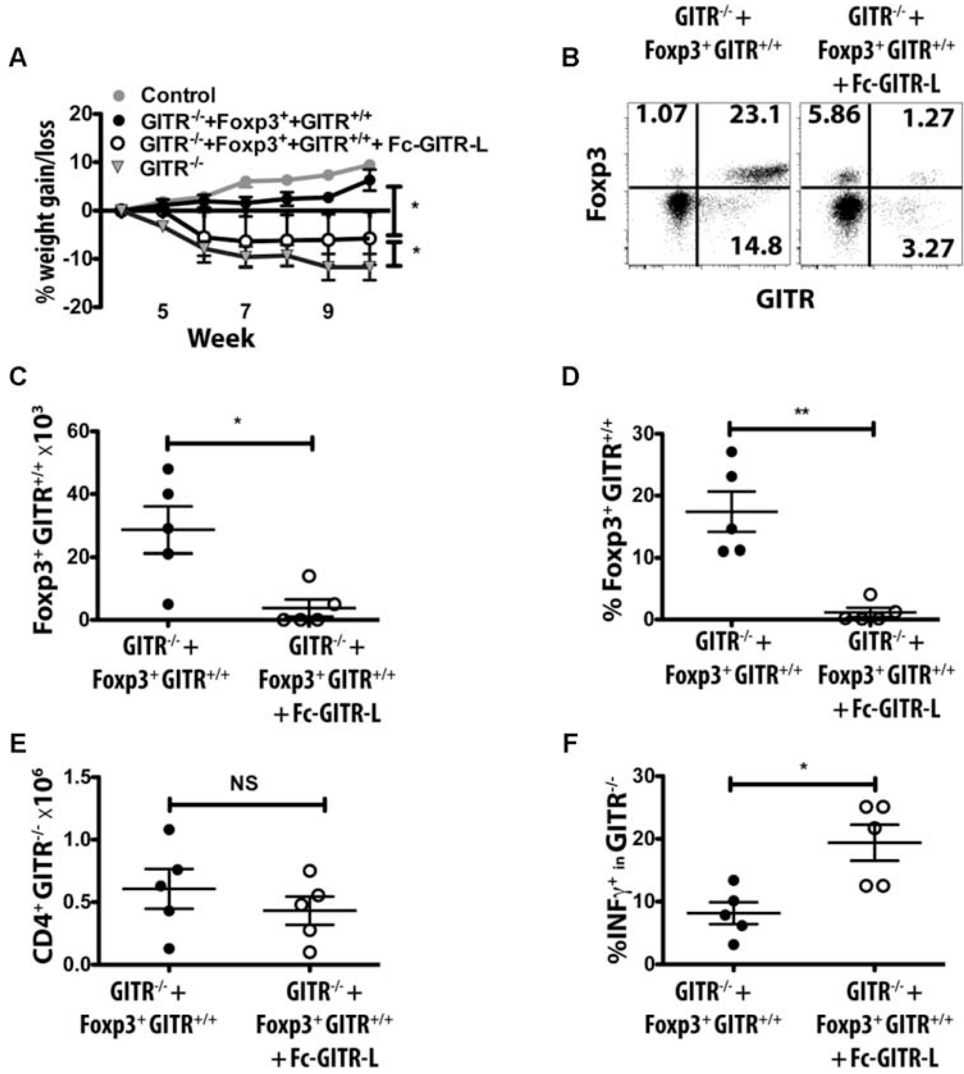

Figure 4.

Fc-GITR-L treatment results in a loss of Treg cells in IBD. $RAG^{-/-}$ mice were reconstituted with $GITR^{-/-}CD45RB^{high}CD4^{+}$ T cells and $GITR^{+/+}$ $CD4^{+}$ $CD25^{+}$ T cells and not treated (solid circles) or treated with Fc-GITR-L weekly (open circles). One group of animals received $GITR^{-/-}$ $CD45RB^{high}$ T cells alone (solid triangles). The control mice were nonreconstituted RAG KO mice (gray-filled circles). (A) The percentage of weight gain or loss is shown as mean ± SEM. Asterisks indicate statistical significance determined by Mann–Whitney *U* test (*$p = 0.02$) for Fc-GITR-L treated mice reconstituted with $GITR^{-/-}Foxp3^{-}CD45RB^{high}$ T cells together with $GITR^{+/+}Foxp3^{+}$ T cells versus the untreated mice reconstituted with a similar combination of cells. (*$p = 0.02$) for Fc-GITR-L treated mice reconstituted with $GITR^{-/-}$ $Foxp3^{-}CD45RB^{high}$ T cells together with $GITR^{+/+}$ $Foxp3^{+}$ T cells versus the Fc-GITR-L treated mice reconstituted with $GITR^{-/-}$ $Foxp3^{-}CD45RB^{high}$ T cells. (B) A representative dot plot representing Foxp3 versus GITR expression in the $CD4^{+}$ T-cell gate in the mesenteric LN is shown. (C) The absolute number of $GITR^{+/+}$ $Foxp3^{+}$ T cells in the mesenteric LN is shown. (*$p = 0.02$). (D) The percentage of $GITR^{+/+}$ $Foxp3^{+}$among $CD4^{+}$ T cells is shown. (**$p = 0.007$). (E) The absolute number of $GITR^{-/-}$

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$CD4^+$ T cells in the mesenteric LN is shown. (F) The percentage of IFN-γ-producing cells among $CD4^+$ T cells in the mesenteric LN is shown (*$p = 0.03$). (A–F) The data are shown as mean ± SEM of five mice per group and are from one experiment representative of two independent experiments performed.

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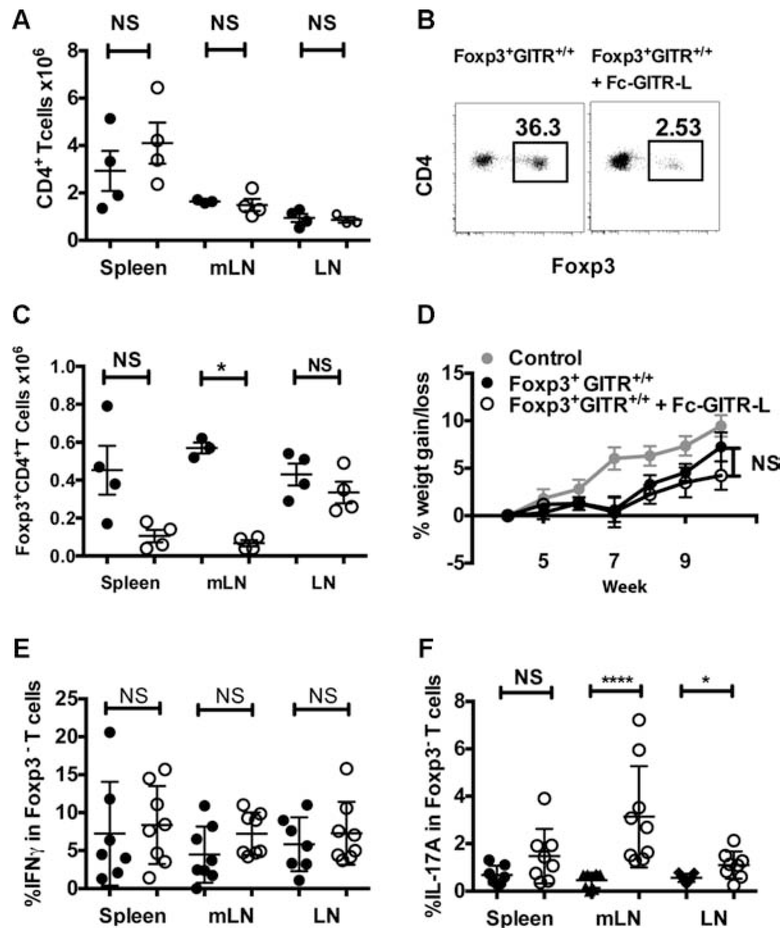

Figure 5.

Fc-GITR-L enhances loss of Foxp3 expression under lymphopenic conditions. $RAG^{-/-}$ mice were reconstituted with $GITR^{+/+}$ $CD4^+$ $Foxp3^+$ T cells and not treated (solid circles) or treated with Fc-GITR-L weekly (open circles). All analyses were done at week 4 after transfer. (A) The absolute number $CD4^+$ T cells in the spleen, mesenteric, and peripheral LN is shown. (B) A representative dot plot representing CD4 versus Foxp3 expression in $CD4^+$ T-cell gate is shown. (C) The absolute number $Foxp3^+CD4^+$ T cells in the spleen, mesenteric, and peripheral LN is shown. Asterisks indicate statistical significance determined by Mann–Whitney U test (*$p = 0.02$). (D) The percentage of weight gain or loss is shown. (E) The percentage of IFN-γ-producing cells in the $Foxp3^-$ gate is shown. (F) The percentage of IL-17A-producing cells in the $Foxp3^-$ gate is shown (****$p = 0.0001$) (*$p = 0.04$). (A–C) The data are shown as mean ± SEM of four mice per group and representative of two independent experiments. (E and F) The data are shown as mean ± SEM of eight mice per pooled from two independent experiments.