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Glucocorticoid-Induced Tumor Necrosis Factor Receptor Family-Related Protein Regulates CD4⁺ T Cell-Mediated Colitis in Mice

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Abstract

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Author Contributions: GL performed all IBD inducing experiments, DC and monocyte subsets fractionation, *in vitro* DC functional assays; CD helped processing samples and editing the manuscript; SBB performed the CBA cytokine assays and analysis; PE developed several anti-GITR-L monoclonal antibodies for FACS analysis; RM generated GITR-L deficient mice; RWH helped discuss and write manuscript; AKB helped assessing histology score; CT is the major organizer of this work and designed the experiments with GL. All authors declare no conflict of interest.

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Background & Aims—The glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR; also called TNFRSF18 or CD357) regulates the T-cell mediated immune response and is present on surfaces of T regulatory (Treg) and activated CD4⁺ T cells. We investigated the roles of GITR in the development of colitis in mice.

Method—Chronic enterocolitis was induced by the transfer of wild-type or *GITR*^{−/−} CD4⁺ T cells to *GITR*^{−/−} × *Rag*^{−/−} or *Rag*^{−/−} mice. We determined colitis severity using the disease activity index, measured levels of inflammatory cytokines, T cells, and dendritic cells, and performed histologic analysis of colon samples.

Results—Transfer of non-fractionated CD4⁺ cells from wild-type or *GITR*^{−/−} donors induced colitis in *GITR*^{−/−} × *Rag*^{−/−} but not in *Rag*^{−/−} mice. Among mice with transfer-induced colitis, the percentage of Treg and T-helper (Th)17 cells was reduced but that of Th1 cells increased. Treg cells failed to prevent colitis in *GITR*^{−/−} × *Rag*^{−/−} recipients; this was not the result of aberrant function of *GITR*^{−/−} Treg or T effector cells, but resulted from an imbalance between the numbers of tolerogenic CD103⁺ and PDCA1⁺ plasmacytoid dendritic cells in *GITR*^{−/−} mice. This imbalance impaired Treg cell development and expanded the Th1 population in *GITR*^{−/−} × *Rag*^{−/−} mice following transfer of non-fractionated CD4⁺ cells.

Conclusions—GITR is not required on the surface of Treg and T effector cells to induce colitis in mice; interactions between GITR and its ligand are not required for colitis induction. GITR instead appears to control dendritic cell and monocyte development; in its absence, mice develop aggravated chronic enterocolitis, via an imbalance of colitogenic Th1 cells and Treg cells.

Keywords

TNF; immune regulation; inflammation; IBD

Introduction

Inflammatory bowel diseases (IBD), which are divided into Crohn's disease and ulcerative colitis, are idiopathic diseases. Some phenotypes of Crohn's diseases can be mimicked by the transfer of CD4⁺ CD45RB^{hi} naïve T cells into recombination activating gene deficient (*Rag*^{−/−}) mice, which is preventable by cotransfer of CD4⁺CD25⁺ regulatory T cells.

As a TNF receptor superfamily member, GITR may regulate T cell-mediated immune responses due to its constitutive expression on CD4⁺CD25⁺ Treg cells and a low but inducible expression on CD4⁺CD25[−] Teff cells¹. However, it is not conclusive yet whether signaling through GITR will break or enhance immunological tolerance¹⁻⁴.

GITR is also expressed in antigen presenting cells including monocytes, macrophages, neutrophils, and DCs *et al*⁵. The expression levels of TLR-2, TLR-4, CD40 and CD80 in DCs were affected by GITR deficiency when exposed to *Candida albicans*⁶. GITR deficiency protects mice from TNBS-induced colitis⁷, suggesting an involvement of GITR signaling in regulating the function of antigen presenting cells (APCs) and the development of colitis.

GITR-L, the non-promiscuous ligand of GITR, is highly expressed in plasmacytoid dendritic cells (pDCs)⁸, macrophages and langerhans dendritic cells^{9, 10}. Engagement of GITR ligand (GITR-L or TNFSF18) by GITR in pDCs activates an inhibitory enzyme indoleamine 2,3 dioxygenase (IDO), which protects mice against allergic bronchopulmonary aspergillosis⁸. Recombinant soluble GITR induces production of inducible nitric oxide synthase, cyclooxygenase-2, and matrix metalloproteinase-9 in the macrophage cell line RAW264.7 and in murine peritoneal macrophages^{11, 12}. GITR-L engagement by a monoclonal antibody blocks the migration of langerhans dendritic cells to the draining lymph node⁹. These reports

indicate that GITR/GITR-L interaction not only induces a costimulatory signal for both Teff and Treg cells but also affects the function of APCs.

Although GITR/GITR-L signaling is potentially implicated in regulating T cell- and APC-mediated immune responses, *GITR*^{-/-} mice do not have a dramatic phenotype reminiscent to another TNFR family member (HEVM)¹³. This may reflect the balance between Treg cells, Teff cells and their interaction with APCs, which regulate immune responses oppositely.

To dissect the precise mechanisms how GITR on the surface of Treg cells, effector T cells and antigen presenting cells acts in tolerance induction *in vivo*, we employed a well-known model of chronic enterocolitis. To this end, we evaluated the pathogenesis of colitis upon the transfer of un-fractionated CD4⁺ T cells or combinations of GITR-deficient, GITR-L-deficient and -sufficient CD4⁺ T cell subsets into *Rag*^{-/-} or *GITR*^{-/-}*xRag*^{-/-} recipients. Unexpectedly, the outcomes of these studies demonstrate that the presence of GITR on the surface of dendritic cells and macrophages is requisite for controlling colitis. Upon transfer of non-fractionated CD4⁺ cells into *GITR*^{-/-}*xRag*^{-/-} mice, disease develops because of an imbalance between Treg and Th1 cell proliferation in the lamina propria and MLN.

Materials and Methods

Mice

B6129SF1, C57BL/6, B6.PL-Thy1^a/CyJ, *Rag*^{-/-} (Recombination Activating Gene 1, *Rag*-1^{tm1mom}/J) and OTII-Tg transgenic mice [C57BL/6-Tg(TCR α TCR β)425Cbn/J] were purchased from the Jackson Laboratory. *GITR*^{-/-} mice were provided by Dr. C. Riccardi and Dr. P.P. Pandolfi¹⁴. FoxP3-IRES-EGFP knock-in C57BL/6 mice were generously provided by Dr. V. Kuchroo¹⁵. *GITR*^{-/-} mice were crossed with *Rag*^{-/-} mice to generate *GITR*^{-/-}*xRag*^{-/-} double knockout mice. F₂ mice were interbred and used for experiments. *GITR*^{-/-} C57BL/6 mice were generated as described in Supplement Figure S1. All animals were housed in the Center for Life Science animal facility of Beth Israel Deaconess Medical Center. The experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at BIDMC.

Antibodies

Anti-CD3s-Biotin, CD11b-Pacific Blue, CD4-PE(FITC), CCR7-PE, GITR-PE, IDO and Streptavidin-PerCP were from Biolegend (San Diego, USA). Anti-FoxP3-FITC, IL-17A-PE and GITR-L-PE were products of eBioscience (San Jose, USA). Anti-CCR9-Allophycocyanin was from R&D (Minneapolis, USA). Anti-CD25-PE, CD115-PE, CD11c-PE, CD45RB-FITC, Ly6C-PerCP, CD103-APC, TCR $\gamma\beta$ 5-Biotin and IFN γ -PE were products from BD Biosciences (San Jose, USA). Anti-PDCA1-FITC was purchased from Miltenyi Biotec (Auburn, USA).

Induction and assessment of colitis

Briefly, CD4⁺CD45RB^{hi}, CD4⁺CD25⁺ or CD4⁺ T cells were sorted by FACS and *i.p.* injected into *GITR*^{-/-}*xRag*^{-/-} or *Rag*^{-/-} recipients. Recipient mice were analyzed for disease activity index (DAI) upon the first observance of diarrhea as previously described¹⁶. Mice were checked on a daily basis and euthanized if moribund. Histology grades were assigned in a blinded fashion by a pathologist (A.K.B). Lamina propria cells were isolated from colon for analyzing the cellularity. Colon from each mouse was incubated in RPMI medium for 24 hours. Supernatants were collected for cytokine analysis.

Isolation and analysis of lamina propria cells

Lamina propria cells were isolated as previously described¹⁷. Briefly, after disruption of epithelial cells from the mucosa in HBSS/EDTA buffer, collagenase D and DNase were used to dissociate lamina propria cells of colon pieces. The cells were then purified using gradient centrifugation.

In vitro CD4⁺ T cell proliferation and cytokine assays

CD11c⁺ DCs were isolated from *wt* or *GITR*^{-/-} spleens or MLNs using a CD11c⁺ DC isolation kit (Miltenyi Biotec). DCs primed with 2mg/ml chicken egg ovalbumin were irradiated with an X-ray irradiator (3000 Rad) and used to activate OTII-Tg CD4⁺ T cells labeled with CFSE (at 5:1 ratio) for 72 hours. TCRvβ5⁺ cells were compared for the times of proliferation and the percentage of IFN-γ expressing cells. Supernatants were collected to assess the production of cytokines.

IDO Assay

Colon of *GITR*^{-/-}*xRag*^{-/-} and *Rag*^{-/-} mouse were mashed in PBS supplemented with PMSF and protease inhibitors as described before¹⁸. IDO protein levels were measured by immunoblotting with an αIDO antibody. IDO activity was quantified by the production of L-kynurenine¹⁹.

Cytokine production

Cytokines in cell or colon cultures were analyzed using cytometric bead array Mouse Inflammatory Kits (BD Biosciences, #552364).

Statistical analysis

All data were analyzed with the Prism 4.0c software (GraphPad, San Diego, CA) and presented as the mean values ± Standard Deviation (SD). Statistical comparisons were performed by two-tailed Student's *t* test. Values *P* < .05 were considered to be statistically significant.

Results

Transfer of non-fractionated CD4⁺ T cells into *GITR*^{-/-}*xRag*^{-/-} mice induces colitis

To address the role of GITR/GITR-L interactions in suppression of colitis, we transferred combinations of GITR-deficient or GITR-sufficient CD4⁺ cell subsets into either *GITR*^{-/-}*xRag*^{-/-} or *Rag*^{-/-} recipients. Surprisingly, when non-fractionated *wt* CD4⁺ T cells were transferred into *GITR*^{-/-}*xRag*^{-/-} recipients, an aggressive chronic enterocolitis developed. In contrast, weight loss, disease activity indexes (DAI) and histology scores were low in *Rag*^{-/-} mice kept in the same cage [Figure 1 A-D]. Pro-inflammatory cytokines produced by *ex vivo* colon cultures (Figure 1E) or *in vitro* cultures of MLN CD4⁺ T cells demonstrated a preference for a Th1 type inflammation, while the number of IL-17A producing CD4⁺ T cells was reduced (Figure 1F).

To assess whether GITR on the surface of donor CD4⁺ T cells was involved in the CD4⁺ T cell induced colitis, *GITR*^{-/-} CD4⁺ T cells were injected. Again, upon transfer into *GITR*^{-/-}*xRag*^{-/-} recipients colitis developed (Figure 2A-B and Supplement Figure S2A), and the same pattern of proinflammatory cytokines was detected in colon samples (Figure 2C). In contrast, upon transfer into *Rag*^{-/-} mice, no colitis developed. We conclude that although the donor cell population contained both Tregs and colitis inducing CD4⁺ cells²⁰⁻²², in the absence of GITR in the recipient mouse suppression appeared to be reduced and a Th1-driven disease prevailed.

The percentage of CD4⁺FoxP3⁺ T cells is significantly lower in *GITR*^{-/-}*xRag*^{-/-} recipients than that in *Rag*^{-/-} mice after transfer of non-fractionated CD4⁺ T cells (Supplement Figure S3), suggesting an imbalance between Teff and Treg cells may be involved in the development of colitis after the transfer of non-fractionated CD4⁺ T cells into *GITR*^{-/-}*xRag*^{-/-} recipients.

Impaired Treg activity upon transfer into *GITR*^{-/-}*x Rag*^{-/-} mice

As neither *GITR*^{-/-} nor *GITR*^{-/-}*xRag*^{-/-} mice develop colitis spontaneously (Supplement Figures S4 and S5), the disease may have developed due to either impaired interactions between Treg and CD45RB^{hi} cells or between these cells and a cell in the recipient mouse. We then assessed the influence of GITR-deficiency on the function of colitis-inducing CD45RB^{hi} cells and colitis-preventing Treg cells in *GITR*^{-/-}*x Rag*^{-/-} as well as in *Rag*^{-/-} recipients.

As expected from the previous experiments, GITR-deficient CD45RB^{hi} cells induced colitis as well as GITR-sufficient CD45RB^{hi} cells in *GITR*^{-/-}*xRag*^{-/-} recipients. Whilst neither *GITR*^{+/+} nor *GITR*^{-/-} CD25⁺ Treg cells could suppress colitis (Figure 3A-B and Supplement Figure S2B). In contrast, the absence of GITR did not have an effect on the colitis-inhibiting activity of Treg cells (Figure 3C-D and Supplement Figure S2C), nor did the absence of GITR affect the colitis-inducing activity of Teff cells (Supplement Figure S6) in the *Rag*^{-/-} recipients.

Furthermore, CD4⁺CD45RB^{hi} T cells alone induced a faster weight loss (as early as week 4) and a more severe inflammation in the colon of *GITR*^{-/-}*xRag*^{-/-} recipients than that of *Rag*^{-/-} recipients (Figure 3E-F). Conversely, CD4⁺FoxP3⁺ Treg cells alone did not induce colitis either in *GITR*^{-/-}*xRag*^{-/-} or in *Rag*^{-/-} recipients (analyzed at week 9). The percentage of donor cells that lost FoxP3 expression was comparable in both donors (Supplement Figure S7). Colitis induced by the transfer of non-fractionated CD4⁺ T cells in *GITR*^{-/-}*xRag*^{-/-} recipients depends on the naïve T cells but not the Treg cells.

To elucidate the fate of naïve T cells and the Treg cells after their transfer into *GITR*^{-/-}*xRag*^{-/-} recipients, naïve CD4⁺CD45RB^{hi} cells from Thy1.1⁺ C57BL/6 congenic mice were mixed with CD4⁺FoxP3⁺ Treg cells FACS sorted from Thy1.2⁺ FoxP3-IRES-GFP reporter mice at 9:1 ratio and adoptively transferred to *GITR*^{-/-}*xRag*^{-/-} or in *Rag*^{-/-} recipients. As shown in Figure 4A-B, a mix of Thy1.1⁺ naïve T cells and Thy1.2⁺ Treg cells induced an accelerated weight loss and severe colitis in *GITR*^{-/-}*xRag*^{-/-} but not in *Rag*^{-/-} recipients. The percentage of Thy1.1⁺ naïve T cells was significantly increased, whilst the percentage of Thy1.2⁺FoxP3(GFP)⁺ Treg cells was reduced particularly in the mesenteric lymph node and in the lamina propria of *GITR*^{-/-}*xRag*^{-/-} recipients as compared to that of *Rag*^{-/-} recipients (Figure 4C-D). Thy1.1⁺FoxP3⁺ cells, which represent the inducible Treg cells converted from naïve T cells in the peripheral lymphoid organ, was also lower in *GITR*^{-/-}*xRag*^{-/-} recipients than in *Rag*^{-/-} recipients (Figure 4E). Surprisingly, Treg-mediated suppression is not ameliorated *in vitro* by the presence of *GITR*^{-/-}*xRag*^{-/-} splenocytes as APCs (Supplement Figure S8), suggesting an *in vitro* suppression may not mimic the condition *in vivo*.

We conclude that the expression of GITR is neither required for the colitis-inducing activity of CD45RB^{hi} T cells nor for the suppressor function of Treg cells in *Rag*^{-/-} recipients. However, CD4⁺ CD45RB^{hi} T cells are refractory to the inhibition either by GITR-deficient or GITR-sufficient Treg cells and induce colitis in *GITR*^{-/-}*xRag*^{-/-} recipients.

Treg cells isolated from GITR-L-deficient mice do not ameliorate disease upon co-transfer with CD45RB^{hi} T cells into *GITR*^{-/-}*xRag*^{-/-} mice

The loss of their ability to ameliorate colitis upon the transfer of Treg into *GITR*^{-/-}*xRag*^{-/-} mice could also be due to an inability of GITR-L on the surface of Treg cells to appropriately interact with a GITR⁺ cell in the recipient mouse. To evaluate this possibility, we first analyzed by monoclonal antibody staining and PCR whether T cells express GITR-L²³. Although none of these analyses detected GITR-L in CD4⁺ T cells (Supplement Figure S9), it was conceivable that this cell surface ligand could be expressed at some stage during activation *in vivo*. We therefore transferred combinations of *GITR*^{-/-} CD45RB^{lo} and CD45RB^{hi} cells into *Rag*^{-/-} recipients. As shown in Figure 5 and Supplement Figure S2D, *GITR*^{-/-} Tregs ameliorate CD45RB^{hi} induced colitis in *Rag*^{-/-} mice. Thus, if GITR-L were to be fleetingly expressed on the surface of CD4⁺ T cells, its absence did not impair their ability to induce or suppress chronic enterocolitis in the *Rag*^{-/-} mouse. By contrast, upon co-transferring *GITR*^{-/-} CD45RB^{lo} cells together with CD45RB^{hi} cells into *GITR*^{-/-}*xRag*^{-/-} recipients disease developed.

We conclude that the impaired Treg function observed after transfer into *GITR*^{-/-}*xRag*^{-/-} mice was not caused by the absence of GITR-L on CD4⁺ T cells. More importantly, the experiments convincingly demonstrate that the absence of GITR in the recipient *Rag*^{-/-} mouse affects the suppressive effect by Tregs and this effect is not due to a direct interaction between two T cell subsets or between a GITR⁺ APC and a GITR-L⁺ T cell. It is therefore more likely that the absence of GITR affects an APC-intrinsic functions.

Expression of GITR on the surface of dendritic cells and macrophages

Development of colitis in *Rag*^{-/-} mice requires an interplay in the lamina propria and mesenteric lymph nodes between CD4⁺ cells and bacterial antigens presented by specialized dendritic cells and macrophages. Migration of aggressor macrophages or tolerogenic dendritic cells determines the outcome of the inflammatory responses, which are completed by neutrophil infiltration. Because expression of GITR on the surface of these cell types is poorly understood^{5, 7}, we first employed anti-GITR to detect expression on dendritic cells and macrophages in colitic *Rag*^{-/-} recipients upon transfer of CD45RB^{hi} CD4⁺ T cells. As shown in Figure 6A, GITR is expressed on the surface of dendritic cells and macrophages in the lamina propria of colitic mice. In contrast, without the transfer of CD45RB^{hi} CD4⁺ T cells, lamina propria cells isolated from the colon of *Rag*^{-/-} mice do not express GITR (Supplement Figure S10). Because LPS-activation also induces the expression of GITR on the surface of splenic DCs and macrophages (Figure 6B-C), we conclude that GITR is an activation marker for monocyte-derived cells.

To examine whether the absence of GITR in *Rag*^{-/-} recipients altered the induction of colitis, we used an agonistic anti-CD40 monoclonal antibody, which causes colitis in *Rag*^{-/-} mice²⁴. Because the response in *GITR*^{-/-}*xRag*^{-/-} mice was similar to that in *Rag*^{-/-} mice, the effect of GITR deficiency on colitis was not simply due to a gross abnormality of DC or macrophage functions (Supplement Figure S11).

Furthermore, soluble GITR is thought to initiate a signaling pathway in GITR-L expressing pDCs, which results in activation of the enzyme IDO. We found that the levels of IDO activity in the colon of *GITR*^{-/-}*xRag*^{-/-} mice in which colitis had been induced by the transfer of non-fractionated CD4⁺ cells was comparable to that of *Rag*^{-/-} mice (Figure 6D-E). Thus, this DC-intrinsic pathway was unlikely to be involved in the disease of *GITR*^{-/-}*xRag*^{-/-} recipients.

Collectively, our data demonstrate that the absence of GITR on the surface of dendritic cells and macrophages does not cause colitis by itself, but rather indicates that the inflammation is caused by the aberrant interaction of APCs with the donor CD4⁺ T cells.

DCs from MLN of *GITR*^{-/-} mice preferentially drive Th1 responses instead of Treg expansion

Due to the influence of GITR on the nonlymphocyte immune system and the inducible expression of GITR on DCs, we assessed whether *GITR*^{-/-} DCs differ functionally from their *wt* counterparts *in vitro*. Interestingly, OVA-loaded CD11c⁺ DCs from *GITR*^{-/-} MLN aggressively drove a Th1 response of OTII CD4⁺ cells (Figure 7A and Supplement Figure S12A-B), which was consistent with the Th1-colitis in *GITR*^{-/-}*xRag*^{-/-} recipients after transfer of CD4⁺ T cells (Figure 1). In contrast, OTII CD4⁺ cells secreted equal amounts of IFN γ when activated by *wt* or *GITR*^{-/-} splenic DCs (Figure 7B).

To address why the absence of GITR on the surface of DCs causes an enhanced induction of Th1 cells, the frequency of tolerogenic PDCA1⁺ CD11c⁺ pDCs and CD103⁺ DCs was assessed. As shown in Figure 7C-D and Supplement Figure S12C-D, the numbers of both CD103⁺ DCs in the MLN and PDCA1⁺CCR9⁺ pDCs in the spleen were reduced in *GITR*^{-/-} mice compared to that of *wt* mice under steady state. The number of PDCA1⁺ pDCs in *GITR*^{-/-}*xRag*^{-/-} mice, with or without inflammation induced by the transfer of CD4⁺ T cells, was significantly lower than in their *wt* counterparts (Figure 7E-F).

We conclude that the composition of tolerogenic DC subsets is altered by GITR deficiency, which causes a Th1-colitis after transfer of CD4⁺ T cells into the *GITR*^{-/-}*xRag*^{-/-} recipients.

Discussion

GITR is highly expressed on the surface of naturally occurring CD4⁺CD25⁺ Treg cells and on the surface of effector CD4⁺ T cells upon activation of naïve cells¹. However, naïve *GITR*^{-/-} CD4⁺ CD45RB^{hi} T cells are as equally efficient as their *wt* counterparts in the induction of colitis in *Rag*^{-/-} recipients. CD45RB^{hi} CD4⁺ T cell-induced colitis is preventable by the co-transfer of *GITR*^{-/-} Treg cells. We conclude that GITR on the surface of CD4⁺ T cells is dispensable for the induction and suppression of colitis. The results suggest that in general Treg / Teff interactions that are known to operate *in vivo* are not dependent on the presence of GITR.

Unexpectedly, upon transfer of non-fractionated CD4⁺ T cells or naïve CD4⁺ T cells alone into *GITR*^{-/-}*xRag*^{-/-} recipients an accelerated Th1 type inflammation develops in the colon as compared to *Rag*^{-/-} recipients, which manifests as early as 3-4 weeks. Non-fractionated CD4⁺ T cell-induced colitis in *GITR*^{-/-}*xRag*^{-/-} recipients does not depend on Treg cells, while naïve CD4⁺ T cells somehow are refractory to Treg-mediated suppression *in vivo* and induce inflammation in the colon of *GITR*^{-/-}*xRag*^{-/-} mice. Abrogation of Treg-mediated suppression *in vivo* is not caused by an aberrant interaction between GITR on the surface of a cell in the recipient *Rag*^{-/-} mouse (and not in the *GITR*^{-/-}*xRag*^{-/-} animal) could interact with GITR-L on the surface of either a Treg or a Teff cells. Because CD4⁺ T cells do not express GITR-L in contrast with a previous report²³ as judged by FACS analysis with α GITR-L monoclonal antibodies and by RT-PCR both *ex vivo* and *in vitro*, nor does a GITR-L deficiency affect the colitis-inducing function of naïve T cells as well as the colitis-preventing function of regulatory T cells.

We do detect *Helicobacter bilis* in our *GITR*^{-/-}*xRag*^{-/-} and *Rag*^{-/-} mice by a PCR-based method. However, *H. bilis* may not play a key role in the development of colitis in *GITR*^{-/-}*xRag*^{-/-} recipients after transfer of non-fractionated CD4⁺ T cells. Because the

Rag^{-/-} recipients, which are kept in the same cage with *GITR*^{-/-}*xRag*^{-/-} recipients and are positive for *H. bilis*, develop milder colitis after the transfer of CD4⁺CD45RB^{hi} naïve T cells as well as the transfer of non-fractionated CD4⁺ T cells (Figure 3E-F and Figure 4). However, we cannot rule out the possibility that *GITR*-deficient *Rag*^{-/-} mice are more prone to the infection by other pathogenic bacteria. *GITR*^{-/-} APCs exposed to the pathogenic bacterial antigens drive a strong Th1 type inflammation.

GITR^{-/-}*xRag*^{-/-} mice develop an accelerated colitis by the transfer of naïve CD4⁺ T cells in recipients reminiscent that of the *HVEM*^{-/-}*xRag*^{-/-} mice¹³, in which a radio-resistant cell but not DCs is proposed to cause the accelerated disease. In contrast to *HVEM*^{-/-} mice, in which the function of DCs is not greatly affected, *GITR*-deficiency causes a significant reduction of tolerogenic CD103⁺ DCs and PDCA1⁺ pDCs. *GITR*^{-/-} MLN DCs induced CD4⁺ T cells to secrete significantly higher IFN γ than their *wt* counterparts *in vitro*, consistent with the previous reports that CD103⁻ DCs are more efficient at driving Th1 immune responses than CD103⁺ DCs²⁵⁻²⁸. IFN γ alone may not induce the devastating disease in *GITR*^{-/-}*xRag*^{-/-} mice, it may initiate the escalating Th1 inflammation.

Treg-mediated suppression is abrogated in *GITR*^{-/-}*xRag*^{-/-} mice, which cannot be mimicked by a traditional *in vitro* suppression assay, suggesting a migratory factor may be involved in the abrogation of Treg-mediated suppression *in vivo*. As the precursor of the pro-inflammatory macrophages and DCs^{29, 30}, monocytes are the strong candidates with migratory ability. Increased Th1-cytokines produced by an abnormal DC-CD4⁺ T cell interaction inevitably affect the migration of monocytes to the inflamed sites and the subsequent differentiation into aggressor macrophages or dendritic cells, which determines the outcome of the inflammatory responses^{5, 7}. The effect of *GITR* deficiency on the function of monocytes is still under active investigation.

There is also significantly increased IL-6 in *ex vivo* colon cultures of *GITR*^{-/-}*xRag*^{-/-} recipients after transfer of CD4⁺ T cells. T cells secrete trace amount of IL-6; therefore, it must be produced by an APC (macrophages and/or DCs) accumulated in the colon of *GITR*^{-/-}*xRag*^{-/-} mice during the inflammation. IL-6 trans-signaling is known to inhibit the generation of inducible Treg cells from naïve T cells and to render CD4⁺CD25⁻ Teff cells resistant to Treg-mediated suppression^{31, 32}. Increased IL-6 in inflamed *GITR*^{-/-}*xRag*^{-/-} mice by the transfer of CD4⁺ cells may explain the reduction of FoxP3⁺ Treg cells, which is caused both by the loss of FoxP3 expression in Treg cells and by the *de novo* generation of inducible FoxP3⁺ Treg cells from naïve T cells. The percentage of Treg cells lose FoxP3 expression in *GITR*^{-/-}*xRag*^{-/-} recipients, in which no colitis is induced, is comparable to that in *Rag*^{-/-} recipients after the transfer of CD4⁺FoxP3⁺ Treg cells alone. This suggests the reduction of CD4⁺FoxP3⁺ Treg cells in *GITR*^{-/-}*xRag*^{-/-} recipients after transfer of non-fractionated CD4⁺ T cells is secondary to the inflammation.

Taken together, by using a CD4⁺ T cell transfer model of chronic enterocolitis we establish that the expression of *GITR* on the surface of regulatory and effector CD4⁺ T cells is dispensable for development of disease. In contrast, the abnormal development and function of DC subsets in recipient *GITR*^{-/-}*xRag*^{-/-} mice results in a Th1-inflammatory phenotype and reduced activity of Treg cells when unfractionated CD4⁺ T cells are transferred.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

APC	antigen-presenting cell
CBA	cytometric bead array
CFSE	carboxyfluorescein diacetate, succinimidyl ester
DAI	Disease Activity Index
DC	dendritic cell
GITR	Glucocorticoid-Induced TNF Receptor-related gene
GITR-L	GITR ligand
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
MLN	mesenteric lymph node
OTII-Tg	C57BL/6-Tg(TCRaTCRb)425Cbn/J transgenic
SD	Standard Deviation
Treg	CD4+CD25+ regulatory T cells
Teff	effector T cells
WT	wild type

Fig. 1. CD4⁺ T cells induce colitis in $GITR^{-/-} \times Rag^{-/-}$ but not in $Rag^{-/-}$ recipients
CD4⁺ T cells obtained from wt spleens by FACS sorting were *i.p.* injected into $Rag^{-/-}$ (n=17) or $GITR^{-/-} \times Rag^{-/-}$ (n=14) hosts [1×10^6 cells/mouse]. Mice were euthanized when signs of diarrhea, hunching, and wasting disease manifested. Statistical significance was determined by two-tailed Student's *t*-test.

1A Weight loss as a percentage of the initial weight. Values represent the mean weight $\pm$ SD.

1B & 1C DAI and Histology score. Mean and individual values of each group are indicated.

1D Representative Histology. Original magnification 10×.

1E Cytokine production by short-term colon cultures. Colon tissue was isolated from *GITR*^{-/-}*xRag*^{-/-} (n=7) and *Rag*^{-/-} (n=6) mice with *wt* CD4⁺ T cells transfer and cultured for 24 hours. Supernatants were collected after centrifugation and analyzed for inflammatory cytokines with the multiplex CBA assay.

1F MLN CD4⁺ T cells differentiate into Th1 cells upon transfer into *GITR*^{-/-} *x Rag*^{-/-} mice. MLN cells isolated from *GITR*^{-/-}*xRag*^{-/-} (n=5) and *Rag*^{-/-} (n=5) mice into which *wt* CD4⁺ T cells had been transferred were stimulated with 10 µg/ml of plate bound αCD3ε for 24 hours. Cells were stained with monoclonal antibodies against CD4, IFN-γ and IL-17A. Representative staining of each group is shown.

Fig. 2. $GITR^{-/-}$ $CD4^{+}$ T cells induce colitis upon transfer into $GITR^{-/-} \times Rag^{-/-}$ recipients. $CD4^{+}$ T cells obtained from $GITR^{-/-}$ spleens by FACS sorting were *i.p.* injected into $GITR^{-/-} \times Rag^{-/-}$ or $Rag^{-/-}$ hosts [1×10^6 cells/mouse]. Disease parameters were determined as described in methods and Figure 1.

2A DAI.

2B Histology score.

2C Cytokine production by short-term colon cultures.

Ex vivo colon cultures from $GITR^{-/-} \times Rag^{-/-}$ (n=8) and $Rag^{-/-}$ (n=8) mice into which $GITR^{-/-}$ $CD4^{+}$ T cells had been transferred were prepared for determining the inflammatory cytokines as in Figure 1.

Fig. 3. Tregs do not suppress colitis after their co-transfer with CD45RB^{hi} cells into $GITR^{-/-} \times Rag^{-/-}$ recipients

$GITR^{+/+}$ or $GITR^{-/-}$ CD4⁺CD45RB^{hi} cells alone (5×10^5 cells/mouse) or together with CD4⁺CD25⁺ cells (5×10^4 cells/mouse) were transferred to $Rag^{-/-}$ or $GITR^{-/-} \times Rag^{-/-}$ recipients. Mice were euthanized at week 4 after cell transfer. DAI and histology score were assigned as described in Figure 1. Each filled or open circle represents one mouse. Statistical significance was determined by the two-tailed Student's *t*-test. Mean and individual values of each group are indicated.

3A & 3B DAI and histology score upon cell transfer into $GITR^{-/-} \times Rag^{-/-}$ recipients.

3C & 3D DAI and histology score upon cell transfer into $Rag^{-/-}$ recipients.

3E Weight loss as a percentage of the initial weight after transfer of CD4⁺CD45RB^{hi} cells into *Rag*^{-/-} and *GITR*^{-/-}*xRag*^{-/-} recipients.

Values represent the mean weight $\pm$ SD.

3F DAI upon transfer of CD4⁺CD45RB^{hi} cells into *Rag*^{-/-} and *GITR*^{-/-}*xRag*^{-/-} recipients.

Fig. 4. GITR deficiency in *Rag*^{-/-} recipients caused an over expansion of naïve T cells as well as a loss of FoxP3 expression on Treg cells

CD4⁺CD45RB^{hi} naïve T cells sorted from Thy1.1⁺ B6 congenic mice were mixed with CD4⁺FoxP3⁺ Treg cells from Thy1.2⁺ FoxP3-IRES-GFP reporter mice at 9:1 ratio and adoptively transferred to *GITR*^{-/-}x*Rag*^{-/-} or *Rag*^{-/-} hosts (5 mice for each group). Mice were weighed weekly and euthanized for colitis activity as described in Figure 1. Spleen, MLN and lamina propria cells from *GITR*^{-/-}x*Rag*^{-/-} or *Rag*^{-/-} recipients were isolated and evaluated for FoxP3⁺, Thy1.1⁺ and Thy1.2⁺ cells.

4A Weight loss as a percentage of the initial weight. Values represent the mean weight ± SD.

4B DAI. Mean and individual values of each group are indicated.

4C Ratio of Thy1.1⁺ and Thy1.2⁺ cells after cell transfer. Thy1.1⁺ cells represent CD4⁺CD45RB^{hi} naïve T cells. Thy1.2⁺ cells represent FoxP3⁺(GFP⁺) cells.

4D Percentage of FoxP3(GFP)⁺/Thy1.2⁺ cells after cell transfer.

4E Percentage of FoxP3⁺ cells expressing Thy1.1⁺ and Thy1.2⁺. Splenocytes from *GITR*^{-/-}*xRag*^{-/-} or *Rag*^{-/-} recipients were stained with CD4, Thy1.1 and FoxP3. Thy1.1 and FoxP3 expressing cells were compared among gated CD4⁺ T cells.

A **B**

Fig. 5. GITR expression by non-T hematopoietic cells, but not GITR-L by CD4⁺ T cells, abrogated the Treg-mediated suppression
5A DAI.
5B Histology score.

GITR-L^{-/-} CD4⁺CD45RB^{hi} (5×10^5 cells/mouse) and CD4⁺CD45RB^{lo} (5×10^5 cells/mouse) T cells were co-injected into *GITR*^{-/-} *x Rag*^{-/-} or *Rag*^{-/-} hosts. DAI and Histology scores were determined as in Figure 1. Mean and individual values of each group are indicated.

Fig. 6. GITR expression on the surface of lamina propria DCs and macrophages of colitic mice and on splenic monocyte-derived cells upon activation

6A Dendritic cells and macrophages isolated from the lamina propria of colitic Rag^{-/-} mice express GITR. CD4⁺CD45RB^{hi} wt T cells were transferred to Rag^{-/-} recipients (5×10⁵ cells/mouse). Once diarrhea started, monocyte derived cells were isolated from the colonic lamina propria and stained with αCD11c, αCD11b, αLy6C and αGITR. Different cell subsets were gated to compare the GITR expression levels. Bold black line represents αGITR; gray shaded area represents IgG isotype control.

6B & 6C LPS induces GITR expression on splenic DCs and macrophages. Freshly isolated wt or GITR^{-/-} splenocytes (upper panel) or those stimulated with LPS (lower panel)

for 24 hours were stained with monoclonal antibodies directed against CD11c, Ly6C and GITR. The expression levels of GITR on different cell subpopulations (gated as in **6B**) were compared. Bold black line represents GITR^{+/+} cells; gray shaded area represents GITR^{-/-} cells.

6D & 6E IDO protein levels and enzymatic activity in the colon of GITR^{-/-} x Rag^{-/-} mice are identical to that of Rag^{-/-} mice. IDO protein levels and enzymatic activity in the colon extracts of GITR^{-/-}xRag^{-/-} and Rag^{-/-} mouse were measured by immunoblot with an α IDO antibody and by the production of L-kynurenine respectively.

Fig. 7. DCs from MLN of colitic *GITR*^{-/-} mice preferentially drive Th1 responses instead of Treg expansion

7A *GITR*^{-/-} DCs from MLN preferentially stimulate Th1 cells. CD11c⁺ DCs from MLN of *wt* and *GITR*^{-/-} mice were primed with 2 mg/ml of chicken ovalbumin for 18 hours. OVA-loaded DCs were irradiated [3000 Rad] and cocultured with OTII CD4⁺ T cells at 1 : 5 ratio for 3 days. Supernatants were used for assessing the cytokines by CBA. Data represent mean $\pm$ SD of triplicate. Results are representative of 3 individual experiments.

7B Cytokines secreted by CD4⁺ T cells in the presence of splenic DCs. CD11c⁺ DCs from spleen of *wt* and *GITR*^{-/-} mice were loaded with OVA protein and used to stimulate

OTII CD4⁺ T cells as described in 7A. Supernatants were used for assessing the cytokines by CBA. Data represent mean $\pm$ SD of triplicate.

7C-D Percentage of MLN CD103⁺ DCs and splenic PDCA1⁺CCR9⁺ pDCs of *GITR*^{-/-} mice was reduced under steady state. MLN (7C) and spleen (7D) cells from *wt* and *GITR*^{-/-} were stained for comparing the percentage of different DC subsets. Each filled or open circle represents one mouse. Mean and individual values of each group are indicated.

7E Reduced number of pDCs in spleen of *GITR*^{-/-}*xRag*^{-/-} mice without transfer of CD4⁺ T cells. Splenocytes from *Rag*^{-/-} and *GITR*^{-/-}*xRag*^{-/-} mice (5 for each group) were stained for MHCII, CD11c and PDCA1.

7F Reduced number of pDCs in *GITR*^{-/-}*xRag*^{-/-} mice during inflammation induced by CD4⁺ T cells. CD4⁺ T cells from *wt* mice were adoptively transferred into *GITR*^{-/-}*xRag*^{-/-} and *Rag*^{-/-} recipients (5 for each group). Mice were euthanized at week 4. Spleen, MLN or colon LP cells were pooled for CD11c, PDCA1 staining. Result represents two separate experiments.