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

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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 regulatory T (Treg) cells and activated $CD4^+$ T cells. We investigated the roles of GITR in the development of colitis in mice. **METHODS:** Chronic enterocolitis was induced by the transfer of wild-type or $GITR^{-/-}$ $CD4^+$ T cells to $GITR^{-/-} \times Rag^{-/-}$ or $Rag^{-/-}$ mice. We determined the severity of colitis by 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 nonfractionated $CD4^+$ cells from wild-type or $GITR^{-/-}$ donors induced colitis in $GITR^{-/-} \times Rag^{-/-}$ 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 $GITR^{-/-} \times Rag^{-/-}$ 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 $GITR^{-/-} \times Rag^{-/-}$ mice following transfer of nonfractionated $CD4^+$ 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 induction of colitis. 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.

BASIC AND TRANSLATIONAL AT

Inflammatory bowel diseases, which are divided into Crohn's disease and ulcerative colitis, are idiopathic diseases. Some phenotypes of Crohn's disease 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 $CD4^+CD25^+$ T regulatory (Treg) cells.

As a tumor necrosis factor receptor superfamily member, glucocorticoid-induced tumor necrosis factor receptor-related gene (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 $CD4^+CD25^-$ effector T (Teff) cells.[1] However, it is not conclusive yet whether signaling through GITR will break or enhance immunologic tolerance.[1–4]

GITR is also expressed in antigen-presenting cells (APCs), including monocytes, macrophages, neutrophils, and dendritic cells (DCs).[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 trinitrobenzene sulfonic acid (TNBS)-induced colitis,[7] suggesting an involvement of GITR signaling in regulating the function of APCs and the development of colitis.

GITR ligand (GITR-L), the nonpromiscuous ligand of GITR, is highly expressed in plasmacytoid dendritic cells (pDCs),[8] macrophages, and Langerhans DCs.[9,10] Engagement of GITR-L (or TNFSF18) by GITR in pDCs activates an inhibitory enzyme, indoleamine 2,3-dioxygenase (IDO), which protects mice against allergic bronchopulmonary aspergillosis.[8] 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 DCs to the draining lymph node.[9] 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 pheno-

Abbreviations used in this paper: APC, antigen-presenting cell; DAI, disease activity index; DC, dendritic cell; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GITR, glucocorticoid-induced tumor necrosis factor receptor-related gene; GITR-L, glucocorticoid-induced tumor necrosis factor receptor-related gene ligand; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; MLN, mesenteric lymph node; OVA, ovalbumin; pDC, plasmacytoid dendritic cell; Teff, effector T cell; Th, T-helper; Treg cell, regulatory T cell; wt, wild-type.

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type reminiscent of another tumor necrosis factor receptor family member (HEVM).[13] This may reflect the balance among 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 APCs acts in tolerance induction in vivo, we used a well-known model of chronic enterocolitis. To this end, we evaluated the pathogenesis of colitis on the transfer of unfractionated $CD4^+$ T cells or combinations of GITR-deficient, GITR-L–deficient, and GITR-L–sufficient $CD4^+$ T-cell subsets into *Rag*$^{-/-}$ or *GITR*$^{-/-}$ × *Rag*$^{-/-}$ recipients. Unexpectedly, the outcomes of these studies show that the presence of GITR on the surface of DCs and macrophages is requisite for controlling colitis. On transfer of nonfractionated $CD4^+$ cells into *GITR*$^{-/-}$ × *Rag*$^{-/-}$ mice, disease develops because of an imbalance between Treg and T-helper (Th) 1 cell proliferation in the lamina propria and mesenteric lymph node (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[TCRαTCRβ]425Cbn/J) were purchased from The Jackson Laboratory (Bar Harbor, ME). *GITR*$^{-/-}$ mice were provided by Dr C. Riccardi and Dr P. P. Pandolfi.[14] FoxP3-IRES-EGFP knockin C57BL/6 mice were generously provided by Dr V. Kuchroo.[15] *GITR*$^{-/-}$ mice were crossed with *Rag*$^{-/-}$ mice to generate *GITR*$^{-/-}$ × *Rag*$^{-/-}$ double knockout mice. F_2 mice were interbred and used for experiments. *GITR-L*$^{-/-}$ C57BL/6 mice were generated as described in Supplementary Figure 1. 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 at Beth Israel Deaconess Medical Center.

Antibodies

Anti–CD3ϵ-biotin, CD11b-Pacific Blue, CD4-PE (fluorescein isothiocyanate [FITC]), CCR7-PE, GITR-PE, IDO, and streptavidin-PerCP were from Biolegend (San Diego, CA). Anti–FoxP3-FITC, interleukin (IL)-17A-PE, and GITR-L-PE were products of eBioscience (San Jose, CA). Anti–CCR9-allophycocyanin was from R&D Systems (Minneapolis, MN). Anti–CD25-PE, CD115-PE, CD11c-PE, CD45RB-FITC, Ly6C-PerCP, CD103-APC, TCR$v\beta$5-biotin, and interferon (IFN)-γ-PE were from BD Biosciences (San Jose, CA). Anti–PDCA1-FITC was purchased from Miltenyi Biotec (Auburn, CA).

Induction and Assessment of Colitis

Briefly, $CD4^+CD45RB^{hi}$, $CD4^+CD25^+$, or $CD4^+$ T cells were sorted by fluorescence-activated cell sorting (FACS) and intraperitoneally injected into *GITR*$^{-/-}$ × *Rag*$^{-/-}$ or *Rag*$^{-/-}$ recipients. Recipient mice were analyzed for disease activity index (DAI) on the first observance of diarrhea as previously described.[16] 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 the colon for analyzing 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 described.[17] Briefly, after disruption of epithelial cells from the mucosa in Hank's balanced salt solution/EDTA buffer, collagenase D and deoxyribonuclease 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 wild-type (*wt*) or *GITR*$^{-/-}$ spleens or MLNs using a $CD11c^+$ DC isolation kit (Miltenyi Biotec). DCs primed with 2 mg/mL chicken egg ovalbumin (OVA) were irradiated with an X-ray irradiator (3000 rad) and used to activate OTII-Tg $CD4^+$ T cells labeled with carboxyfluorescein diacetate succinimidyl ester (at 5:1 ratio) for 72 hours. TCR$v\beta5^+$ 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*$^{-/-}$ × *Rag*$^{-/-}$ and *Rag*$^{-/-}$ mouse was mashed in phosphate-buffered saline supplemented with phenylmethylsulfonyl fluoride and protease inhibitors as described previously.[18] IDO protein levels were measured by immunoblotting with an αIDO antibody. IDO activity was quantified by the production of L-kynurenine.[19]

Cytokine Production

Cytokines in cell or colon cultures were analyzed using cytometric bead array mouse inflammatory kits (#552364; BD Biosciences).

Statistical Analysis

All data were analyzed with Prism 4.0c software (GraphPad, San Diego, CA) and presented as the mean values ± SD. Statistical comparisons were performed by 2-tailed Student *t* test. $P < .05$ was considered statistically significant.

Results

Transfer of Nonfractionated $CD4^+$ T Cells Into GITR$^{-/-}$ × Rag$^{-/-}$ 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*$^{-/-}$ × *Rag*$^{-/-}$ or *Rag*$^{-/-}$ recipients. Surprisingly, when nonfractionated *wt* $CD4^+$ T cells were transferred into *GITR*$^{-/-}$ × *Rag*$^{-/-}$ recipients, an aggressive chronic enterocolitis developed. In contrast, weight loss, DAI, and histology scores were low in *Rag*$^{-/-}$ mice kept in the same cage (Figure 1*A–D*). Proinflammatory cytokines produced by ex vivo colon cultures (Figure 1*E*) or in vitro cultures of MLN $CD4^+$ T cells demonstrated a preference for a Th1-type inflammation, whereas the number of IL-17A–producing $CD4^+$ T cells was reduced (Figure 1*F*).