INFLAMMATORY BOWEL DISEASE

GITR modulates innate and adaptive mucosal immunity during the development of experimental colitis in mice

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Background: Uncontrolled T cell activation and abnormal function of the innate immune system against normal enteric bacterial flora play a critical part in the pathogenesis of inflammatory bowel disease (IBD). Therefore, pharmacological strategies directed to restore the normal responsiveness of the immune system could be efficacious in the treatment of these pathological conditions. Glucocorticoid-induced tumour necrosis factor receptor (GITR)-related gene is a member of the tumour necrosis factor receptor superfamily that is constitutively expressed at high levels on regulatory T cells and at low levels on unstimulated T cells, B cells and macrophages. GITR triggering leads to activation of T effectors and reversal of suppressive function of regulatory T cells.

Aim: To investigate the role of GITR in the development of experimental colitis in mice.

Results: Using GITR^{-/-} mice, GITR deletion protected against 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis by reducing innate immune responses and effector T cell activity. Effector T cells isolated from GITR^{-/-} mice were less effective than T cells isolated from GITR^{+/+} mice to transfer colitis in immunodeficient mice. Blocking the GITR/ligand for GITR (GITRL) signal by giving soluble GITR prevented TNBS-induced colitis in normal GITR^{+/+} and also in lymphocyte-deficient SCID mice.

Conclusions: Collectively, these data suggest that GITR plays a critical part in regulating both acquired and innate mucosal immune responses during the development of experimental colitis in mice. Therefore, targeting the GITR/GITRL system signalling may represent a potential pharmacological tool for the treatment of IBD.

Mouse glucocorticoid-induced tumour necrosis factor (TNF) receptor-related gene (GITR) is a 22-kDa protein that belongs to the TNF receptor superfamily.^{1–3} GITR is expressed in different cells, including T lymphocytes and macrophages.^{1–2} Like the other members of the TNF receptor superfamily, GITR is overexpressed on T lymphocyte activation, suggesting a role in the development of an immune response.^{4–5} Moreover, high levels of GITR are constitutively expressed on CD4CD25 regulatory T (Treg) cells, suggesting a role for GITR in the activity of this T cell subset.^{6–8} This possibility was supported by functional studies in which the addition of an agonistic anti-GITR monoclonal antibody (mAb) to cocultures of suppressor and effector T cells led to the reversal of the suppressive function of Treg cells.^{7–8} Moreover, in vivo administration of agonistic anti-GITR mAbs results in autoimmune gastritis in mice and increases disease activity in experimental models of Th1-mediated chronic diseases, suggesting that GITR signalling causes a break in immunological self-tolerance.^{8–13} The ligand for GITR (GITRL) has recently been cloned and has been identified in antigen presenting cells, such as B lymphocytes, dendritic cells and macrophages.^{14–15} Confirming the results with agonistic anti-GITR mAbs, the interaction between GITR and GITRL coactivates effector T lymphocytes, renders them less susceptible to Treg-mediated suppression and inhibits the suppressor activity of Treg cells.^{4–15–18}

Crohn's disease and ulcerative colitis, collectively referred to as inflammatory bowel diseases (IBD), are relatively common inflammatory diseases of the gastrointestinal tract, characterised by dysfunction of mucosal immune responses, abnormal cytokine production and cellular inflammation that ultimately lead to mucosal damage.^{19–20} Although the aetiology of IBD remains unknown, circumstantial evidence supports a central role for dysregulated CD4 Th1 effector cell responses to the

normal enteric bacterial flora as a common disease mechanism.^{20–24} Interestingly, lamina propria CD4 GITR T cells with regulatory activity were considerably increased in patients with active IBD, suggesting that GITR may contribute to the intestinal immune homeostasis.²⁵ In addition, a defective function of the innate immune system, a complex network including sensors for invading pathogens, antibacterial proteins and cells, such as macrophages, neutrophils and polymorphonuclear cells (PMNs), has been recently shown to play a crucial part in the development of IBD.^{26–29}

In recent years, several animal models of IBD have been developed. Among these, the hapten-induced model of colonic inflammation in which 2,4,6-trinitrobenzene sulphonic acid (TNBS) is delivered intrarectally to normal mice and the T cell transfer model of colitis in immunodeficient mice display human Crohn's disease-like features, notably a predominant Th1 activation of the mucosal CD4 T cell population.²⁰ We and others have recently shown that intrarectal instillation of TNBS can also induce colitis in SCID and RAG-1^{-/-} mice, confirming the importance of cells and factors of the innate immune system in regulating mucosal inflammatory responses.^{30–31}

In this study, we have investigated whether GITR has a role in the development of TNBS-induced mucosal inflammation in mice and whether inhibition of GITR signalling could exert therapeutic activity in this model.

Abbreviations: FITC, fluorescence isothiocyanate; GITR, glucocorticoid-induced tumour necrosis factor receptor; GITRL, ligand for GITR; IBD, inflammatory bowel disease; IFN, interferon; LPMC, lamina propria mononuclear cell; mAb, monoclonal antibody; MPO, myeloperoxidase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMN, polymorphonuclear cell; TNF, tumour necrosis factor; TNBS, 2,4,6-trinitrobenzene sulphonic acid; Treg, regulatory T cells

METHODS

Animals

129/SvEv GTR-deficient (GTR^{-/-}) mice were generated as previously described.⁵ 129/SvEv RAG-2^{-/-} mice were obtained from Taconic Farms (Germantown, New York, USA). Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of the University of Perugia, Perugia, Italy. Mice were used at the age of 8–12 weeks. Protocols were approved by the animal study committees of the University of Perugia according to governmental guidelines for animal care.

Induction of colitis and study design

TNBS-induced colitis model

Colitis was induced in 8–12-week-old male 129/SvEv GTR^{+/+} and GTR^{-/-} mice as previously described.³² To induce colitis, 2 mg of the hapten reagent TNBS (Sigma Chemical, Milan, Italy) in 50% ethanol was slowly administered into the colonic lumen using a 3.5 F catheter fitted on to a 1-ml syringe. Mice were killed at day 7 and their colons analysed. To assess whether blockade of GTR signalling could protect against the development of colitis, 129/SvEv GTR^{+/+} mice receiving 2.0 mg TNBS were randomised to receive no treatment (TNBS alone) or a fusion protein between the cysteine-rich region of mouse GTR and the Fc portion of human immunoglobulin (Ig)G1 (GTR:Fc; Alexis Corporation, San Diego, USA). A total of 0.5 mg/kg GTR:Fc was administered intraperitoneally for 6 days, starting on the same day as TNBS intrarectal instillation. Mice were killed at day 7 and their colons analysed. Finally, to investigate the role of innate immunity on protection exerted by GTR:Fc, SCID mice were treated by intracolonic injection of TNBS (2 mg/mouse), alone or in combination with GTR:Fc. The fusion protein was given intraperitoneally after injection of TNBS and daily thereafter for 6 days at a dose of 0.5 mg/kg. Mice were killed at day 7 and their colons analysed.

T cell transfer model of colitis

Colitis was induced in 129/SvEv RAG-2^{-/-} mice as previously described.³³ RAG-2^{-/-} mice were injected intraperitoneally with 100 µl of phosphate-buffered saline (PBS) containing one or two subpopulations of sorted spleen CD4 T cells: (1) control (PBS alone); (2) 4 × 10⁵ CD4CD45RB^{high} alone isolated from GTR^{+/+} mice; (3) 4 × 10⁵ CD4CD45RB^{high} alone isolated from GTR^{-/-} mice; (4) 4 × 10⁵ CD4CD45RB^{high} isolated from GTR^{+/+} mice and 2 × 10⁵ CD4CD25 Treg cells isolated from GTR^{+/+} mice; (5) 4 × 10⁵ CD4CD45RB^{high} isolated from GTR^{+/+} mice and 2 × 10⁵ CD4CD25 Treg cells isolated from GTR^{-/-} mice. Cells were sorted by Epics Altra Cell Sorter (Beckman Coulter, Barcelona, Spain). Before cell sorting, CD4 cells were enriched by the CD4 isolation kit (Miltenyi, Biotec, Bergisch Gladbach, Germany). Purity of T cell subpopulations was >98%, as evaluated by flow cytometry analysis (FACScan, Beckton Dickinson). T cells were further characterised using anti-GTR mAbs (goat IgG AF524, R&D Systems, Minneapolis, Minnesota, USA) and anti-CD25 mAbs. Mice were killed 18 weeks after the T cell injection was given and their colons analysed and used to isolate LP T cells. To investigate whether the CD4CD45RB^{high} population represent an interferon (IFN)γ responsive effector cell population on GTR activation, CD4CD45RB^{high} were stimulated for 72 h with 0.5 µg/ml soluble anti-CD3 agonistic mAbs (BD PharMingen, San Diego, CA) with or without 2.0 µg/ml soluble agonistic anti-GTR mAb (goat IgG AF524, R&D Systems). IFNγ concentration was then measured using a specific ELISA kit (R&D Systems, Abingdon, UK).

Histological examination

For histological examination, tissues were removed, fixed in 10% buffered formalin phosphate, embedded in paraffin,

sectioned and stained with haematoxylin and eosin. The degree of inflammation on microscopic cross-sections of the colon was graded semiquantitatively from 0 to 4 (0, no signs of inflammation; 1, very low level; 2, low level of leucocyte infiltration; 3, high level of leucocyte infiltration, high vascular density, thickening of the colon wall; 4, transmural infiltration, loss of goblet cells, high vascular density and thickening of the colon wall). Grading was done in a blinded fashion, by two experienced pathologists using an Olympus BX60 microscope (Olympus, Tokyo, Japan).

Myeloperoxidase assay

Neutrophil infiltration in the colon was monitored by measuring tissue myeloperoxidase (MPO) activity using a spectrophotometric assay with tetramethylbenzidine as substrate, according to a previously published method.³⁴

Reverse transcriptase-polymerase chain reaction and real-time polymerase chain reaction

Mice were killed and the colons were removed and immediately snap-frozen in liquid nitrogen and stored at -80°C until used. Small colon pieces (50 mg) were homogenised in 1 ml TRIzol Reagent (Invitrogen, Milan, Italy), and total RNA was isolated according to the manufacturer's instructions. Reverse transcription of total RNA (1 µg) was performed with Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen) during 50 min at 42°C. Polymerase chain reaction (PCR) experiments were performed using specific primers. The amplification of cDNA (2 µl) was achieved in 20 µl mixture containing 200 µM dNTP, 1.5 mM MgCl₂, 1 µM of gene specific sense and antisense primers and 1 U of platinum Taq DNA polymerase (Invitrogen) using iCycler (Bio Rad, Milan, Italy). Gene-specific oligonucleotide primers for murine cytokines were designed from published mouse sequences. Initial experiments were conducted to determine the optimal annealing temperature for each set of gene-specific primers and also the linear phase of the product amplification curve (data not shown). PCR was conducted as follows: after an initial denaturation at 94°C for 2 min, 35 cycles of amplification (94°C for 30 s, 58°C for 15 s, 72°C for 30 s) were performed, followed by 5 min final extension at 72°C. The quality of RNA samples was evaluated using β-actin-specific primers. PCR products were separated by electrophoresis on 2% agarose gel stained with 0.5 µg/ml ethidium bromide. Each assay was carried out in quadruplicate. The level of gene expression of each transcript was normalised to that of the housekeeping gene β-actin.

For real-time PCR, the sense primer for GTR was AAGGTTCCAGAACGGAAGTG and the antisense primer was GGGTCTCCACAGTGGTACT; for GTR1, the sense primer was CGAGTCCTGCATGGTTAA and the antisense primer was TCAGCTTCCCATCAGATGTC; for HPRT the sense primer was TCATTATGCCGAGGATTGG and the antisense primer was CCCTTGAGCACACAGAG. PCR was carried out in the CHROMO 4 (MJ Research Bio Rad) using DyNAmo HS SYBR GREEN qPCR kit (Finnzymes, Celbio Milano, Italy). Gene expression was quantitated relative to the expression of HPRT.

Isolation and purification of lamina propria T cells, macrophages and polymorphonuclear cells

T cells from the lamina propria of the colon were prepared as previously described.³² The population was >98% pure, as evaluated by flow cytometry analysis (FACScan, Beckton Dickinson). Isolation of T cells from the lamina propria, macrophages and PMNs was carried out using an Epics Altra Cell sorter (Beckman Coulter). CD4 T cells from the lamina propria were isolated by negative selection using CD8, CD11, Mac-1 and B220-coated magnetic beads (Miltenyi, Biotec,

Bergisch Gladbach, Germany). Macrophages were sorted as CD11b and negative for other markers. PMNs were sorted as GR-1⁺ and GR-1⁻ for other markers. Fluorescence isothiocyanate (FITC) anti-mouse CD3ε (CD3 ε chain, clone 145-2C11), FITC anti-mouse CD11b (clone M1/70) and FITC anti-mouse Ly-6G (GR-1) antibodies were obtained from BD PharMingen (San Diego, California, USA). The purity of all cell populations was >98%, as evaluated by flow cytometry analysis.

Culture of total lamina propria mononuclear cells, macrophages and CD4 T cells for assay of cytokine production

Total lamina propria mononuclear cells (LPMC), lamina propria macrophages and CD4 T cells from lamina propria from different treatment regimens were suspended in complete medium (RPMI 1640, 10% heat-inactivated fetal calf serum, 3 mM L-glutamine, 10 mM Hepes buffer, 10 µg/ml penicillin, 100 U/ml streptomycin and 0.05 mM 2-mercaptoethanol) and cultured at a concentration of 10⁶ cells/ml for 48 h. CD4 T cells from the lamina propria were placed in uncoated culture wells (to measure production by unstimulated cells) or in wells containing immobilised murine anti-CD3ε mAbs (PharMingen) and 1 µg/ml soluble anti-CD28 antibody (PharMingen; to measure production by stimulated cells). At the end of the incubation period, culture supernatants were harvested and assayed for cytokine concentration using specific ELISA kits (R&D Systems, Abingdon).

Statistical analysis

All values are expressed as mean (standard deviation (SE)) of the number mice per group. Comparisons of more than two groups were made by a one-way analysis of variance with a retrospective Tukey's test. Comparison of two groups was made using Student's t test for unpaired data when appropriate. Differences were considered significant if *p* was <0.05.

RESULTS

Role of GITR expression in the development of TNBS-induced colitis in immunocompetent mice

When given to wild-type mice, TNBS resulted in a severe illness characterised by a significant mortality and loss of body weight (fig 1A–C). On microscopic examination, the colons of TNBS-treated GITR^{+/+} mice showed a marked mononuclear cell infiltration associated with loss of goblet cells (fig 1E). The histological grading of colon sections increased from 0.5 (SE 0.2) in ethanol-treated mice to 3.5 (SE 0.2) in TNBS-treated mice (*p*<0.01; fig 1D). Consistent with these histological changes, intrarectal instillation of TNBS resulted in a significant increase in colonic MPO activity (fig 1D) and colon mucosal wall thickness (data not shown). In contrast with wild-type mice, GITR^{-/-} mice were almost completely resistant to TNBS-induced colitis, as shown by the low mortality (fig 1A), the absence of wasting disease (fig 1B,C), the reduction in histological score (fig 1D,F), colonic MPO content (fig 1D) and colon mucosal wall thickness (data not shown).

As shown in fig 2A, GITR mRNA was constitutively expressed by T cells from the lamina propria, PMNs and macrophages of wild-type mice and was significantly up regulated in all cell subtypes during the development of TNBS-induced colitis. GITRL mRNA was also constitutively expressed by macrophages from the lamina propria and, to a lesser extent, by PMNs from the lamina propria, but was not modulated by intrarectal instillation of TNBS (fig 2B).

Having shown that cells of the innate and adaptive mucosal immune systems express GITR, we then investigated whether deletion of the GITR gene resulted in the production of a

different cytokine profile in response to intracolonic administration of TNBS. As shown in fig 3A, LPMCs isolated from TNBS-injected GITR^{-/-} mice released considerably less amounts of IL12 than that harvested from GITR^{+/+} colitic mice. Similarly, GITR^{-/-} macrophages from the lamina propria isolated from TNBS-treated mice was found to release considerably less TNFα and IL6 than GITR^{+/+} macrophages from the lamina propria isolated from colitic wild-type mice (fig 3B). Moreover, although the percentages of T cell subsets from the lamina propria were similar in GITR^{+/+} and GITR^{-/-} mice, and were not influenced by TNBS administration (table 1), the T cell-derived cytokine profile production was markedly different between TNBS-treated GITR^{+/+} and GITR^{-/-} mice. Indeed, CD4 lymphocytes from the lamina propria isolated from GITR^{+/+} mice released higher amounts (*p*<0.01) of Th1-derived cytokines, such as IL2 (data not shown), IFNγ and TNFα with respect to CD4 T cells from the lamina propria isolated from TNBS-treated GITR^{-/-} mice (fig 3C). On the other hand, release of the regulatory cytokines transforming growth factor β and IL10 by CD4 T cells from the lamina propria isolated from TNBS-treated GITR^{-/-} mice was significantly (*p*<0.01) increased as compared with CD4 T cells from the lamina propria isolated from TNBS-treated GITR^{+/+} mice (fig 3D).

Role of GITR expression in the development of colitis induced by transfer of CD4CD45RB^{high} T cells in immunodeficient RAG-2^{-/-} mice

When spleen CD4CD45RB^{high} T cells from GITR^{+/+} mice were transferred into syngenic RAG-2^{-/-} mice, the recipients developed severe colitis and a wasting disease characterised by diarrhoea and a body weight persistently lower than controls (fig 4A–D). On the other hand, colitis induced in RAG-2^{-/-} mice reconstituted with CD4CD45RB^{high} T cells isolated from GITR^{-/-} mice was markedly attenuated, as shown by the clinical, histological and immunological analyses (fig 4A–D). Cotransfer of spleen CD4CD45RB^{high} T cells from GITR^{+/+} mice together with spleen CD4CD25 Treg cells isolated from GITR^{-/-} or GITR^{+/+} mice prevented the development of colitis (fig 4A–D). GITR^{-/-} Treg cells were more potent than GITR^{+/+} Treg cells, although the difference was not statistically significant owing to the already high efficacy of GITR^{+/+} Treg cells (fig 4A–D).

As shown in fig 5A,B, we found that the spleen CD4CD45RB^{high} T cells used to transfer colitis constitutively express GITR, but not the CD25 receptor, proliferate at high levels (data not shown) and produce high amounts of IFNγ when stimulated with anti-CD3. Agonistic anti-GITR mAbs further increased the proliferation rate (data not shown) and IFNγ production of CD3-stimulated T cells (fig 5B), indicating that the GITR^{+/+} donor CD4CD45RB^{high} T cells represent an IFNγ responsive effector population on GITR stimulation.

Administration of soluble GITR protects against development of TNBS-induced colitis in wild-type mice

GITR^{+/+} mice were randomised to receive TNBS alone or TNBS and GITR:Fc for 6 days after induction of colitis. As shown in fig 6, we found that TNBS-induced colitis was almost completely prevented by GITR:Fc administration. Indeed, a striking improvement of the wasting disease became apparent (fig 6A,B) and macroscopic and histological grading of colons on day 7 showed a significant (*p*<0.01) reduction in inflammatory activity in animals receiving GITR:Fc (fig 6C,F,G). GITR:Fc administration also reduced the amount of infiltrating PMNs, as detected by MPO activity and reduced IFNγ mucosal content (fig 6D,E).

Figure 2 (A) REAL-time polymerase chain reaction (PCR) analysis on glucocorticoid-induced tumour necrosis factor receptor (GTR) mRNA expression on lamina propria infiltrating T lymphocytes (T cell), polymorphonuclear cells (PMNs) and macrophages (MØ) from control and 2,4,6-trinitrobenzene sulphonic acid (TNBS)-treated GTR^{+/+} mice. (B) REAL-time PCR analysis on GTRL mRNA expression on LP infiltrating T lymphocytes (T cell) from the lamina propria, polymorphonuclear cells (PMNs) and macrophages (MØ) from control (ethanol-treated) and TNBS-treated wild-type mice. *p<0.05 versus TNBS-treated mice.

Figure 3 Glucocorticoid-induced tumour necrosis factor receptor (GITR) $^{-/-}$ mice are resistant to 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis. (A–D) Cytokine profile production by isolated lamina propria mononuclear cell (LPMC), macrophages from the lamina propria (MØ) and CD4 $^{+}$ T cells from the lamina propria. LPMC and MØ from the lamina propria were isolated from mice killed 7 days after intrarectal administration of ethanol or TNBS, suspended in complete medium and cultured for 48 h at a concentration of 10^6 cells/ml. CD4 T cells lamina propria were suspended in complete medium and cultured for 48 h at a concentration of 10^6 cells/ml in wells containing immobilised murine anti-CD3 ϵ mAbs and 1 μ g/ml soluble anti-CD28 antibody. At the end of the incubation period, culture supernatants were harvested and assayed for cytokine concentration using specific ELISA kits. * $p < 0.05$ versus GITR $^{-/-}$ mice.

TNBS-colitis can be induced in immunodeficient SCID mice and is still prevented by GITR:Fc administration

As shown in fig 7A, SCID mice developed colitis in response to intrarectal instillation of TNBS that was roughly comparable to that seen in wild-type mice. Histological analysis of colons from

these mice showed a significant degree of inflammation (fig 7B). Likewise, TNBS-induced increase in MPO activity and mucosal IFN γ levels was seen regardless of the presence of lymphocytes (fig 7C,D). Administration of GITR:Fc to SCID mice abrogated inflammation induced by TNBS, resulting in a

Table 1 Percentages of spleen and T cell subpopulations from the lamina propria in GITR $^{+/+}$ and GITR $^{-/-}$ mice

	CD4	CD8	CD4CD25	CD8CD25
Spleen				
Control GITR $^{+/+}$	49.9 (0.2)	19.1 (0.7)	5.3 (0.1)	2.0 (0.5)
Control GITR $^{-/-}$	42.0 (7.1)	15.9 (4.3)	5.8 (0.1)	1.5 (1.0)
TNBS GITR $^{+/+}$	45.9 (4.0)	23.1 (4.8)	4.4 (0.8)	2.0 (1.0)
TNBS GITR $^{-/-}$	44.0 (4.5)	18.1 (3.1)	4.0 (0.7)	1.5 (1.0)
Lamina propria				
Control GITR $^{+/+}$	50.0 (2.0)	20.0 (1.0)	5.0 (1.0)	1.0 (1.0)
Control GITR $^{-/-}$	43.0 (6.0)	19.0 (3.0)	4.5 (1.0)	2.0 (1.0)
TNBS GITR $^{+/+}$	46.0 (2.0)	22.5 (1.0)	5.0 (1.0)	1.0 (0.5)
TNBS GITR $^{-/-}$	44.4 (4.0)	19.0 (3.0)	4.5 (1.0)	0.8 (1.0)

GITR, glucocorticoid-induced tumour necrosis factor receptor; TNBS, 2,4,6-trinitrobenzene sulphonic acid.

Figure 4 (A–D) T cell transfer model of colitis in immunodeficient mice. RAG-2^{-/-} mice were injected intraperitoneally with 100 μ l of phosphate-buffered saline (PBS) containing one or two subpopulations of sorted spleen CD4 T cells: group 1, PBS alone; group 2, 4×10^5 CD4CD45RB^{high} alone isolated from glucocorticoid-induced tumour necrosis factor receptor (GITR)^{+/+} mice; group 3, 4×10^5 CD4CD45RB^{high} alone isolated from GITR^{-/-} mice; group 4, 4×10^5 CD4CD45RB^{high} isolated from GITR^{+/+} mice and 2×10^5 CD4CD25 Treg cells isolated from GITR^{+/+} mice; group 5, 4×10^5 CD4CD45RB^{high} isolated from GITR^{+/+} mice and 2×10^5 CD4CD25 Treg cells isolated from GITR^{-/-} mice. * $p < 0.05$ versus all groups; ** $p < 0.05$ versus group 3.

complete protection against the wasting syndrome, histological changes and recruitment of PMNs (fig 7A–C). Furthermore, GITR:Fc administration decreased IFN γ production in the colon of TNBS-treated SCID mice (fig 7D); data were also confirmed by RT-PCR (data not shown).

Discussion

Intrarectal administration of TNBS in mice induces a transmural inflammation of the colon that is due to Th1 cell-mediated response to TNBS-haptenated colonic proteins or

cross-reactive luminal antigens.²⁰ On this basis, TNBS-induced colitis is considered to be an experimental model of Crohn's disease, which is similarly characterised by transmural inflammation and production of Th1-derived cytokines, such as IL12, IL2, IFN γ and TNF α . Moreover, we and others^{30–31} have shown that colitis can also be induced in mice in the absence of lymphocytes, suggesting that, although T cells are important in the development of colitis, the disease can also develop through interaction with other cell types, such as that of the innate immune system.

Figure 5 (A) Glucocorticoid-induced tumour necrosis factor receptor (GITR) and CD25 expression in CD4CD45RB^{high} cells. Splenic GITR^{+/+} CD4 T cells were isolated (see Methods) and were stained with anti-CD45RB, anti-GITR and anti-CD25 mAbs. Unshaded histogram represents the control (goat immunoglobulin G) staining. (B) Increased interferon (IFN) γ production upon GITR costimulation. CD4⁺CD45RB^{high} cells, sorted from both GITR^{+/+} and GITR^{-/-} T cells (see Methods), were stimulated by the indicated mAbs, on irradiated splenocytes, for 72 h. Supernatants were harvested and used for IFN γ ELISA assay. The results represent the mean (standard error (SE)) of three independent experiments. * $p < 0.05$ versus GITR^{-/-} mice.

Figure 6 Early administration of glucocorticoid-induced tumour necrosis factor receptor (GITR:Fc) prevents the wasting syndrome and colitis induced in $GITR^{+/-}$ mice by intrarectal instillation of 2,4,6-trinitrobenzene sulphonic acid (TNBS). $GITR^{+/-}$ SvEv/129 mice were given 2 mg TNBS/mouse intracolonic and followed for 7 days. GITR:Fc, or saline, was injected intraperitoneally at a dose of 0.5 mg/kg starting on the same day of TNBS administration. (A–C) GITR:Fc reduces the clinical and histological signs of TNBS-induced colitis. (D,E) Early administration of GITR:Fc reduces colonic MPO activity and IFN γ mucosal content. * $p < 0.05$ versus mice treated with TNBS alone. (F,G) Histological analysis (H&E staining) of the colon from mice killed 7 days after intrarectal instillation of TNBS without (F) and with (G) GITR:Fc treatment.

In this study, we have discussed the role of GITR in the TNBS-model of colitis, showing that elimination of GITR signalling fully protects mice against TNBS-induced mucosal inflammation by down regulating both the innate and adaptive mucosal immune responses elicited by TNBS. We have shown that GITR and its ligand are constitutively expressed not only by T cells from the lamina propria, but also by the cells of the mucosal innate immune system, such as macrophages from the lamina propria and PMNs. Moreover, we found that TNBS administration markedly increased GITR mRNA expression on all cell subtypes, suggesting a functional role for this receptor during the development of colonic mucosal inflammation. The fact that GITR plays an important part in the development of the innate mucosal immune responses was further confirmed by showing that LPMCs isolated from GITR-deficient mice released low levels of IL12 in response to TNBS injection. IL12 is essential in maintaining TNBS-induced colitis and a persistent local Th1 cytokine response,^{35, 36} and plays a critical part in

Crohn's disease.^{37, 38} Therefore, deletion of the GITR gene may protect mice against TNBS-induced colitis by down regulating the mucosal Th1 response dependent on locally released IL12. Moreover, in accordance with previous work showing a role for GITR signalling in activation of macrophages,^{39, 40} macrophages from the lamina propria isolated from TNBS-treated $GITR^{-/-}$ mice produced low levels of IL6 and TNF α in comparison with those isolated from colitic $GITR^{+/-}$ mice. As macrophages play a critical part in the development of experimental colitis^{30, 31} and in human IBD,^{41, 42} the protection afforded by deletion of the GITR gene against TNBS-induced colitis may result, at least partly, from the down regulation of the response of macrophages from the lamina propria against TNBS.

GITR triggering induces both anti-apoptotic and pro-apoptotic effects on T cells,^{1, 4, 43} abrogates the suppressive activity of Treg cells⁸ and costimulates effector T cells.^{4, 5} In this study, we showed that the percentages of T cell subpopulations from the lamina propria were similar in $GITR^{+/-}$ and $GITR^{-/-}$ mice, and

Figure 7 (A–D) Glucocorticoid-induced tumour necrosis factor receptor (GITR:Fc) reduces the clinical, histological and immunological signs of 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis in SCID mice. SCID mice were given 2 mg TNBS/mouse intracolically and followed for 7 days. GITR:Fc, or saline, was injected intraperitoneally at a dose of 0.5 mg/kg starting on the same day of TNBS administration. * $p < 0.05$ versus mice treated with TNBS alone.

were not modified by TNBS administration, suggesting that the survival of peripheral and T cells from the lamina propria does not differ between GITR^{+/+} and GITR^{-/-} mice. Moreover, we found that although T cells from the lamina propria isolated from TNBS-treated GITR^{+/+} mice were strongly activated and showed the typical Th1-like cytokine profile, those T cells isolated from TNBS-treated GITR^{-/-} mice were only slightly activated and released amounts of Th1-derived cytokines similar to lamina propria T cells isolated from control mice. The reduced Th1 response observed in GITR^{-/-} mice may be dependent, at least partly, on the low levels of IL12 released from mucosal innate immune cells, such as macrophages and dendritic cells. However, we speculate that deletion of the GITR gene directly modulates functions of T cells from the lamina propria, as GITR^{-/-} CD4CD45RB^{high} T cells were markedly less able to transfer colitis in immunodeficient mice than GITR^{+/+} CD4CD45RB^{high} T cells. Moreover, in accordance with the fact that GITR signalling abrogates T-regulatory cell functions both in vitro and in vivo,² T cells from the lamina propria isolated from TNBS-treated GITR-deficient mice produced higher levels of the regulatory cytokines IL10 and transforming growth factor β , suggesting that in the TNBS model of colitis, GITR exerts the opposite effect on Th1 and T-regulatory cells.

Perhaps one of the most striking aspects of our data is that the same protective effect afforded by deletion of the GITR gene against TNBS-induced colitis can also be obtained by the administration of a soluble form of GITR. Indeed, GITR:Fc administration fully prevented the clinical, histological and immunological signs of TNBS-induced colonic inflammation in wild-type mice. Moreover, confirming the role of GITR in the

development of innate mucosal immune responses against TNBS, we have shown that administration of GITR:Fc was also able to prevent the development of colitis in SCID mice. As it has been shown that GITRL is expressed on antigen-presenting cells,^{14,15} and that GITR/GITRL binding enhances effector T cell activity,¹⁶ but inhibits the function of T-regulatory cells,¹⁸ we speculate that the soluble form of GITR exerts its therapeutic anti-inflammatory activity by interfering with the GITR/GITRL pathway.

In conclusion, we have shown that the GITR/GITRL system plays a critical part in the regulation of mucosal immune responses in two rodent models of colitis, and that modulation of GITR signalling exerts therapeutic activity on the development of TNBS-induced colitis in both normal and immunodeficient mice. These results suggest that the GITR/GITRL system represents a target for therapeutic intervention in autoimmune or inflammatory disorders such as IBD.

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