

Glucocorticoid-induced TNFR family-related protein (GITR) activation exacerbates murine asthma and collagen-induced arthritis

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Glucocorticoid-induced TNFR family-related protein (GITR) is expressed at low levels on resting T cells, B cells and macrophages but at high levels on regulatory T cells (Treg). Although GITR expression is up-regulated on CD4⁺ effector cells upon activation, the role of GITR in Th1 and Th2 cell development is unclear. We report here that activation of GITR signalling by anti-GITR antibody markedly enhanced the induction of both Th1 and Th2 cytokine production by naive CD4⁺CD25⁻ T cells. Consistent with this observation, anti-GITR antibody significantly enhanced the expression of the key Th1 (T-bet) and Th2 (GATA3) transcription factors *in vitro*. Administration of anti-GITR mAb in a murine model of arthritis significantly exacerbated the severity and onset of joint inflammation with elevated production of TNF- α , IFN- γ , IL-5, and collagen-specific IgG1. Administration of anti-GITR mAb also significantly exacerbated murine allergic airways inflammation with elevated production of OVA-specific IFN- γ , IL-2, IL-4, IL-5, and IgE. Finally, we demonstrated that adoptive transfer of CD4⁺GITR⁺ T cells effectively abolished airway inflammation induced in SCID mice reconstituted with CD4⁺GITR⁻ T cells. Our results therefore provide direct evidence that GITR can modulate both Th1- and Th2-mediated inflammatory diseases, and may be a potential target for therapeutic intervention.

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Introduction

Glucocorticoid-induced TNFR family-related protein (GITR), also known as TNFRSF 18, is constitutively expressed on naive T cells [1–3], and is particularly abundant on CD4⁺CD25⁺ T regulatory (Treg) cells [1,

2]. However, GITR expression is also up-regulated after activation of CD25⁻ effector T cells [1, 2]. Its ligand, GITRL, is expressed on macrophages, DC, and B cells, but not on T cells [4–6]. The interaction of GITR with its ligand provides an early co-stimulatory signal for CD4⁺ T cells, enhancing proliferation and cytokine production of both CD4⁺CD25⁺ and CD4⁺CD25⁻ populations [4, 7, 8]. Activation of CD4⁺CD25⁺ Treg by the agonistic anti-GITR mAb leads to a reversal of the suppressive activity of the Treg cells. Thus, GITR is an important molecule that modulates both regulatory and effector T cell functions. However, the role of GITR in disease, especially the Th1- and Th2- mediated disorders, is still poorly understood. We have investigated the role of GITR in the function of Th1 and Th2 cells *in vitro* and in

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Abbreviations: BAL: bronchoalveolar lavage · CIA: collagen-induced arthritis · GITR: glucocorticoid-induced TNFR family-related protein · Treg: regulatory T cells

murine collagen-induced arthritis (CIA) and allergic airways inflammation *in vivo*.

Asthma is a chronic inflammatory condition of the airways characterised by airway hyper-responsiveness, inflammatory infiltrates in the bronchial wall containing eosinophils, and elevated serum IgE levels. Th2 lymphocytes are thought to play a key role in the initiation and perpetuation of this airway inflammation [9–11]. However, recent evidence suggests that both Th1 and Th2 cells may mediate airway inflammation [12–14]. Rheumatoid arthritis is an autoimmune disease associated with the destruction of cartilage and underlying bone in the joints. There is considerable evidence suggesting that Th1 lymphocytes have an important role in the pathogenesis of this chronic inflammatory process [15–17].

We report here that anti-GITR antibody enhanced both Type 1 (IFN- γ) and Type 2 (IL-5) cytokine production by anti-CD3-activated CD4⁺ and CD4⁺CD25⁻ T cells cultured under neutral, Th1 or Th2 polarising conditions *in vitro*. Consistent with this, anti-GITR antibody treatment also markedly elevated the expression of the key transcription factors T-bet (Th1) and GATA3 (Th2) by CD4⁺CD25⁻ T cells cultured with anti-CD3 antibody *in vitro*. *In vivo*, GITR activation led to disease exacerbation of collagen-induced arthritis and allergic airways inflammation with concomitant elevation of Type1 and Type 2 cytokine synthesis. Thus, our study suggests that GITR is a potential therapeutic target for Th1- and Th2-mediated inflammatory diseases.

Results

Anti-GITR mAb enhances Th1 and Th2 response *in vitro*

Previous work has demonstrated that anti-GITR mAb can abrogate CD4⁺CD25⁺ Treg suppressor function [1, 2], and that GITR is an early costimulatory signal for CD4⁺ T cells enhancing proliferation and cytokine production of CD4⁺CD25⁻ populations [4, 7, 8]. However, the effect of anti-GITR mAb activation on Th1 and Th2 polarisation is unknown. Given the importance of Th1 and Th2 cells in infectious and inflammatory diseases, we therefore investigated the role of GITR in the development of these two key subsets of T cells. CD4⁺ T cells were purified from the lymph nodes of naive BALB/c mice. These CD4⁺ T cells, which have a physiological ratio of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells, were stimulated with anti-CD3 mAb and either anti-GITR or control Ab. Proliferation of CD4⁺ T cells was significantly enhanced in the presence of anti-GITR mAb (Fig. 1A). Both IFN- γ and IL-5

production from CD4⁺ cells was clearly increased after anti-GITR mAb treatment (Fig. 1A). CD4⁺ cells were then further separated into CD4⁺CD25⁺ and CD4⁺CD25⁻ populations. CD4⁺CD25⁻ cells were stimulated with anti-CD3 Ab under neutral, Th1 or Th2 polarising conditions in the presence of anti-GITR or control IgG. CD4⁺CD25⁻ cells cultured under all three conditions showed enhanced IFN- γ and IL-5 production in the presence of anti-GITR mAb compared with control IgG (Fig. 1B,C). FACS analysis was performed to check the GITR expression on the CD4⁺CD25⁻ cells. Consistent with earlier reports [1, 2, 8], GITR expression on naive CD4⁺CD25⁻ T cells was markedly up-regulated within 24 h of CD3 engagement [1, 2]. As expected [1, 2], anti-GITR mAb treatment did not significantly alter the proliferative response or cytokine production by CD4⁺CD25⁺ T cells (data not shown). These results therefore show that cross-linking GITR with an agonist anti-GITR antibody enhances the differentiation of naive T cells into Th1 and Th2 subsets.

Anti-GITR antibody enhances the transcription of T-bet and GATA3

We then explored the mechanism for the anti-GITR antibody-mediated enhancement of Type 1 and Type 2 cytokine synthesis. Since T-bet and GATA3 are the master switches of Th1 and Th2 development, respectively [18, 19], we investigated the effect of anti-GITR antibody on the expression of these two transcription factors following anti-GITR antibody treatment. Real time PCR analysis shows that both T-bet and GATA3 messages were markedly enhanced in CD4⁺CD25⁻ T cells cultured with anti-CD3 antibody under neutral condition in the presence of anti-GITR antibody compared with normal IgG control (Fig. 2A). In addition, anti-GITR mAb enhanced T-bet expression in CD4⁺CD25⁻ T cells cultured under Th1 polarising conditions (Fig. 2B), and GATA3 expression in CD4⁺CD25⁻ T cells stimulated under Th2 polarising conditions (Fig. 2C). Thus, anti-GITR mAb can augment the early Th1 and Th2 responses of CD4⁺CD25⁻ T cells. This enhancing effect is likely through the elevation of the transcription factors T-bet and GATA3. We then proceeded to investigate the relevance of the anti-GITR antibody on a CIA and allergic airways inflammation *in vivo*.

Anti-GITR mAb exacerbates collagen-induced arthritis (CIA)

CIA was induced in DBA/1 mice by i.d. injection of type II collagen (CII) in FCA. The mice were boosted i.p. 21 days later with CII in PBS. Anti-GITR mAb or control IgG was administered i.p. from day 21 (Fig. 3A). Mice

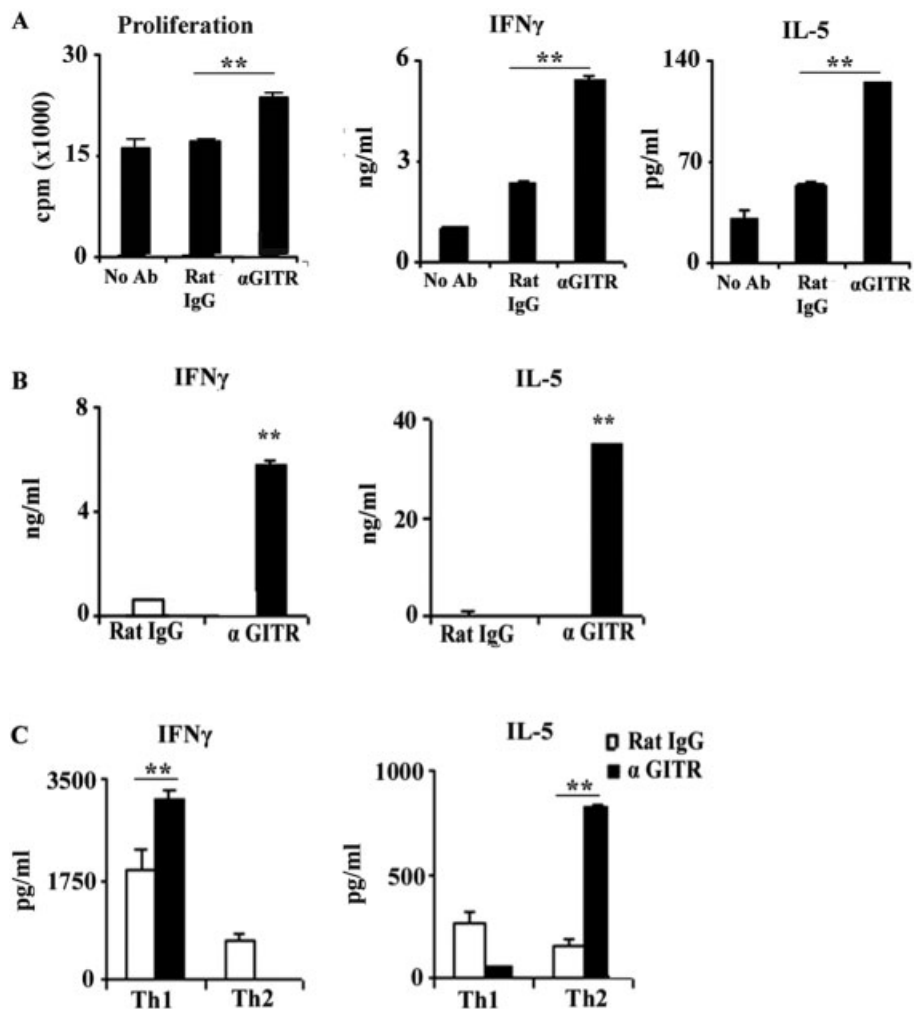


Figure 1. Effect of anti-GITR mAb on Th1 and Th2 cell development *in vitro*. (A) Naive CD4⁺ cells from BALB/c mice were cultured with plate-bound anti-GITR or rat IgG Ab (10 μ g/mL), soluble anti-CD3 Ab (1 μ g/mL), and mitomycin C-treated CD4⁺ cells (as APC). The concentration of anti-GITR used was previously titrated to obtain the optimal concentration for activation. Anti-GITR mAb markedly increased IFN- γ and IL-5 production and proliferation (** p < 0.01 compared with control Ab). (B) CD4⁺CD25⁻ cells from BALB/c mice were purified by MACS as described in Materials and methods section. Naive CD4⁺CD25⁻ cells were then stimulated by anti-CD3 antibody (plate bound 1 μ g/mL) and anti-GITR or control antibody (soluble 10 μ g/mL). After two rounds of stimulation, CD4⁺CD25⁻ showed enhanced Th1 and Th2 cytokine production when cultured in the presence of anti-GITR mAb (** p < 0.01 compared with control Ab). (C) CD4⁺CD25⁻ cells were stimulated with soluble anti-CD3 Ab (1 μ g/mL), and anti-GITR or control Ab (10 μ g/mL), under Th1 or Th2 polarising conditions. IFN- γ and IL-5 production by Th1 and Th2 cells, respectively, was significantly enhanced in the presence of anti-GITR mAb (** p < 0.01 compared with control Ab). Data are representative of three independent experiments, and are shown as mean $\pm$ SEM.

treated with anti-GITR mAb showed a significant increase in articular index (Fig. 3B), number of affected paws (Fig. 3C), and accelerated onset of arthritis (Fig. 3D). Thus, anti-GITR mAb exacerbates the severity and onset of arthritis in CIA.

Immunological analysis of the effect of anti-GITR mAb in CIA

To examine the immunological mechanisms involved in the anti-GITR mAb treatment in CIA, sera were collected from mice at the end of experiment (day 35) and the

cytokine and antibody concentration analysed by ELISA. Mice treated with anti-GITR antibody produced significantly more IFN- γ and TNF- α compared to mice treated with the control normal IgG (Fig. 4A). There was no significant difference in IL-6 production or CII-specific T cell proliferation (Fig. 4A). Interestingly, IL-4 level is also enhanced that is correlated to an enhancement of the collagen-specific IgG1 levels, whereas collagen-specific IgG2a levels were not significantly affected (Fig. 4B). Thus, anti-GITR antibody treatment of CIA resulted in a significant increase in both the Th1 and Th2 response.

Anti-GITR mAb exacerbates OVA-induced airways inflammation

To investigate the potential role of GITR in allergic airways inflammation, we employed an OVA-induced murine asthma model. BALB/c mice were primed and boosted with OVA as described in Materials and methods section. All mice were challenged intranasally (i.n.) on 3 consecutive days beginning on day 25. Anti-GITR mAb or control IgG (1 mg/ mouse) was administered i.p. on

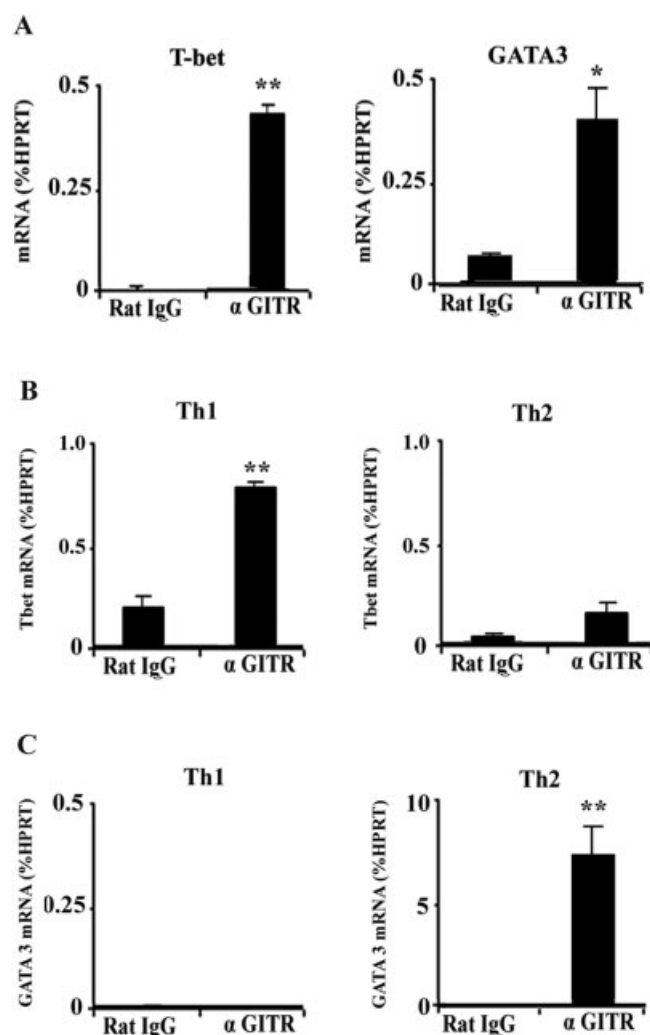


Figure 2. Anti-GITR antibody enhanced the transcription of T-bet and GATA3. (A) Real time PCR analysis shows that both T-bet and GATA3 messages were markedly enhanced in CD4⁺CD25⁻ T cells cultured with anti-CD3 antibody under neutral condition for 48 h in the presence of anti-GITR antibody (** $p < 0.01$ and * $p < 0.05$ compared with control Ab). (B) Anti-GITR mAb enhanced T-bet expression in CD4⁺CD25⁻ T cells cultured with anti-CD3 Ab under Th1 polarising conditions (** $p < 0.01$ compared with control Ab). (C) Anti-GITR mAb enhanced GATA3 expression in CD4⁺CD25⁻ T cells cultured with anti-CD3 Ab under Th2 polarising conditions (** $p < 0.01$ compared with control Ab). Data are representative of two independent experiments, and are shown as mean $\pm$ SEM.

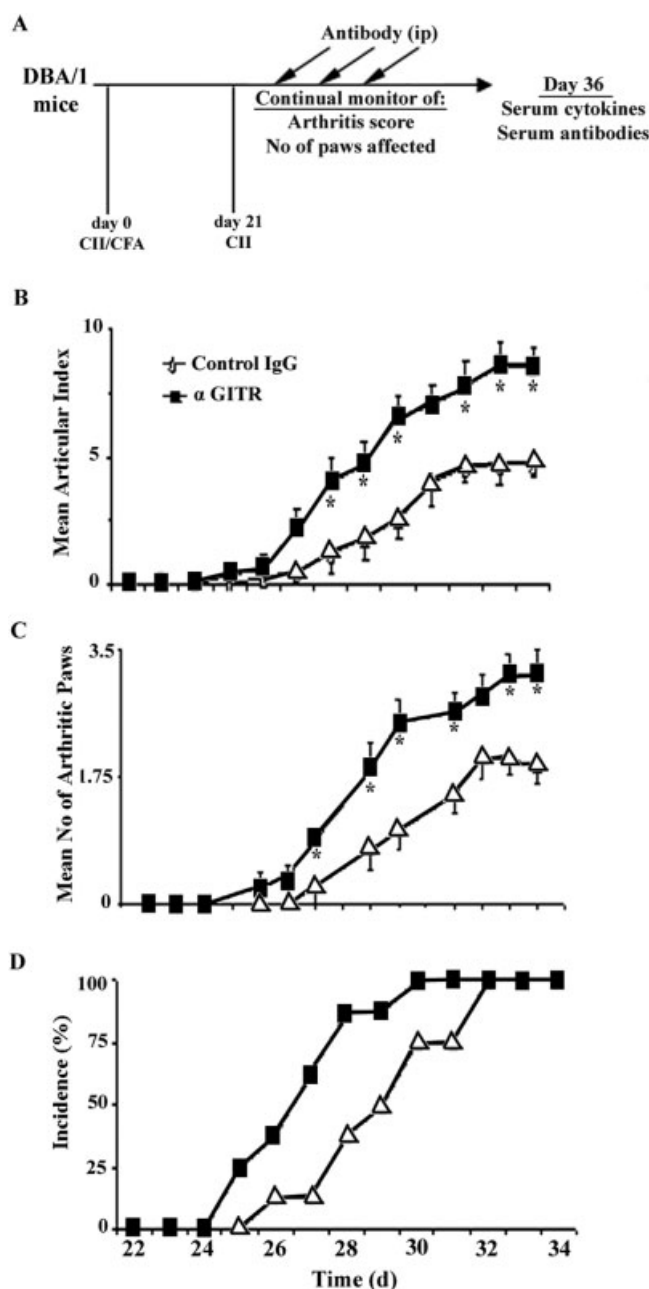


Figure 3. Anti-GITR mAb exacerbated murine collagen-induced arthritis. (A) Experimental protocol. DBA/1 mice were injected i.d. with 200 μ g collagen with CFA on day 0. The mice were boosted i.p. with 200 μ g collagen on day 21. Anti-GITR mAb or control rat IgG (1 mg/mouse) was administered i.p. every 6 days from day 21. The mice were monitored daily for signs of arthritis. Serum was collected on day 35. Cytokines and antibodies concentrations were measured by ELISA. (B) Anti-GITR mAb-treated mice showed a consistently elevated mean articular index after day 28 (* $p < 0.05$ compared to control Ab). (C) The number of affected paws was significantly increased in the mice given anti-GITR mAb (* $p < 0.05$ compared to control Ab). (D) The onset of arthritis was earlier with anti-GITR mAb administration. Data are mean $\pm$ SEM, $n = 8$.

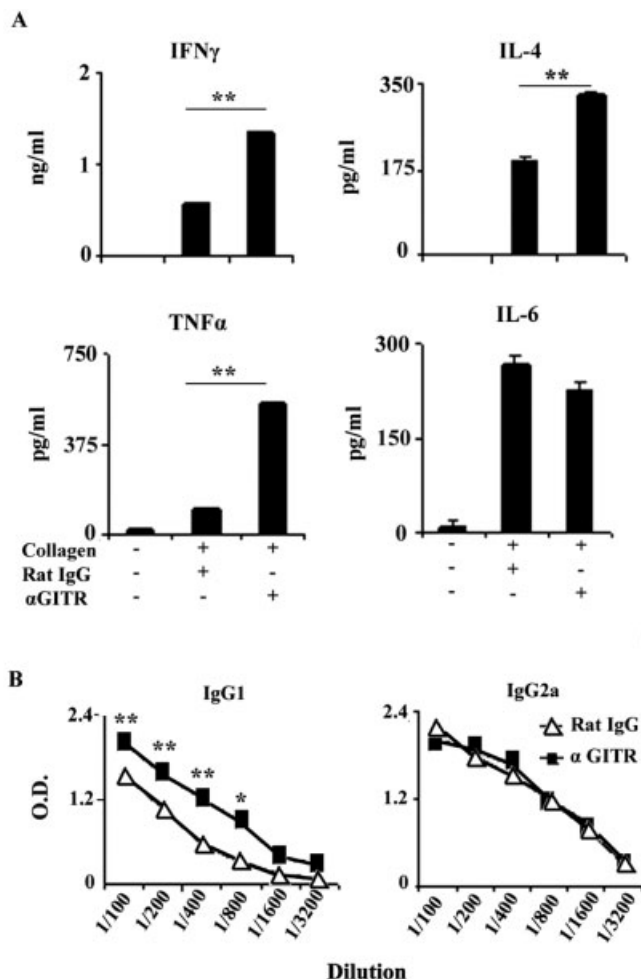


Figure 4. Anti-GITR mAb treatment enhanced Th1 and Th2 responses in murine arthritis. Mice were as described in Fig. 3. (A) Serum IFN- γ , IL-4, and TNF- α levels were increased in mice treated with anti-GITR mAb (** $p < 0.01$ compared with control Ab). IL-6 production was unchanged. (B) Anti-GITR mAb treatment resulted in enhanced IgG1 production (** $p < 0.01$ and * $p < 0.05$ compared with control Ab), with little change in the IgG2a levels. Data are mean $\pm$ SEM of eight mice.

days 24 and 26 (Fig. 5A). The dose of anti-GITR mAb used was determined by preliminary *in vivo* titration. Mice treated with anti-GITR mAb but not control IgG showed a profound increase in the bronchoalveolar lavage (BAL) eosinophilia, without significantly affecting the BAL total cell count (Fig. 5B). Histological analysis demonstrated an amplification of the inflammatory infiltrates seen in the peri-bronchial and perivascular areas of the lungs of mice treated with anti-GITR mAb (Fig. 5C, shown in colour in the supplementary online information). These results demonstrate that anti-GITR mAb could significantly exacerbate the airway inflammation in this model.

Immunological analysis of the effect of anti-GITR mAb in murine allergic airways inflammation

To investigate the immunological mechanism involved in anti-GITR mAb treatment, lymph node cells were harvested from the anti-GITR-treated and rat IgG-treated control mice, and cultured with OVA *in vitro*. Anti-GITR mAb significantly augmented the OVA-specific T cell proliferation compared to those treated with control IgG. The lymphoid cells from anti-GITR-treated OVA sensitised/challenged mice produced significantly more IL-4, IL-5, IL-2, and IFN- γ compared to cells from mice treated with control antibody (Fig. 6A). Serum obtained from mice after anti-GITR treatment also showed an enhanced production of OVA-specific IgE antibody (Fig. 6B), and total IgE (data not shown). The level of serum IgG1 was not significantly increased by anti-GITR mAb treatment (data not shown). Levels of serum IgG2a were low in both groups and unaffected by anti-GITR administration (data not shown). Thus, anti-GITR mAb treatment increased both Th2 and Th1 responses and exacerbated the disease outcome of this model of allergic airways inflammation.

CD4⁺GITR⁺ cells attenuate allergic airways inflammation in SCID mice

Previous studies have demonstrated that CD4⁺GITR⁺ cells that consist mainly of CD4⁺CD25⁺ Treg cells but also contain CD4⁺CD25⁻ Treg cells, suppress Th1-mediated inflammatory bowel disease (IBD) in mice [20]. To investigate whether CD4⁺GITR⁺ T cells could exert an inhibitory effect in a Th2-driven allergic airways disease, we adoptively transferred CD4⁺GITR⁺, CD4⁺GITR⁻, or a combination of CD4⁺GITR⁺ and CD4⁺GITR⁻ cells into SCID mice, which then had airways inflammation induced by the standard protocol described above. Mice transferred with CD4⁺GITR⁻ cells developed marked eosinophilia, whereas mice transferred with CD4⁺GITR⁺ cells failed to show any. Furthermore, co-transfer of CD4⁺GITR⁺ T cells completely suppressed CD4⁺GITR⁻-induced eosinophilia (Fig. 7A). There was no significant difference in the total BAL cell count among the three groups of mice. Draining lymph node cells were harvested and cultured with OVA *in vitro*. Cells from CD4⁺GITR⁻ transferred mice produced significant levels of IL-5 and IFN- γ , while cells from CD4⁺GITR⁺ transferred mice did not. Interestingly, CD4⁺GITR⁺ cells markedly suppressed the production of IL-5 but enhanced the synthesis of IFN- γ by CD4⁺GITR⁻ T cells *in vivo* (Fig. 7A). Histological analysis demonstrated that SCID mice reconstituted with CD4⁺GITR⁻ but not CD4⁺GITR⁺ cells developed airways inflammation. The airway inflammation in mice reconstituted with CD4⁺GITR⁻ T cells

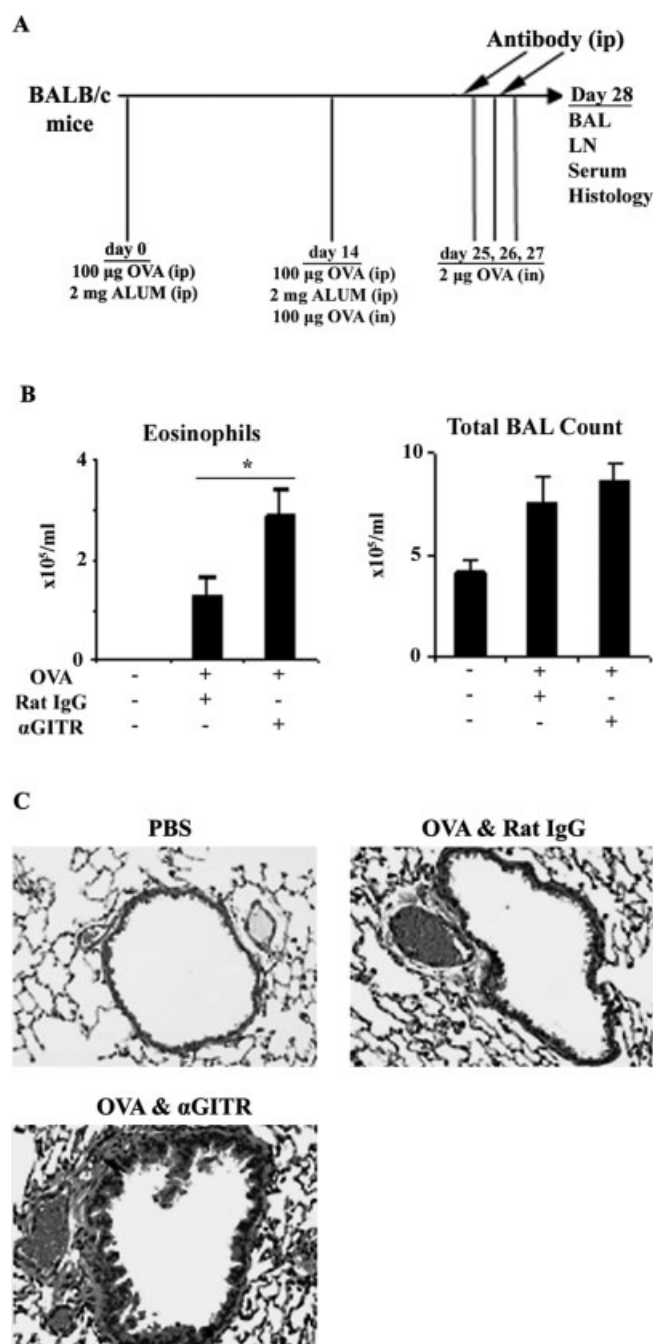


Figure 5. Anti-GITR mAb exacerbated murine allergic airways disease. (A) Experimental protocol. BALB/c mice were injected i.p. with 100 µg OVA and 100 µL 2% Alum on days 0 and 14. The mice were boosted i.n. with 100 µg OVA on day 14. All mice were then challenged i.n. on 3 consecutive days beginning on day 25. Anti-GITR mAb or control Rat IgG (1 mg/mouse) was administered i.p. on days 24 and on 26 when the antibody was given 1 h prior to i.n. challenge. The mice were killed on day 28, and serum, BAL, and lymphoid cells were collected and lung histology studied. (B) BAL total cell concentration remained unchanged, however, the total eosinophil numbers and proportion increased markedly after anti-GITR mAb administration (* $p < 0.05$ compared to control rat Ab). Data are mean $\pm$ SEM, $n = 10$ and are representative of three independent experiments. (C) H&E sections of lungs ($\times 20$ magnification) demonstrate that airways inflammation was increased after anti-GITR administration. Pictures (day 28) are representative of ten mice per group.

and CD4⁺CD25⁻ T cells *in vitro*. It does so via the elevation of the canonical transcription factors, T-bet in Th1 cells and GATA3 in Th2 cell, respectively. Moreover, we provide direct evidence that anti-GITR mAb exacerbates murine models of arthritis and allergic airways inflammation, associated with an increase of both the Th1 and Th2 responses *in vivo*. We also show that passively transferred CD4⁺GITR⁺ cells can attenuate allergic airways inflammation induced in SCID mice reconstituted with CD4⁺GITR⁻ cells. These results therefore demonstrate that activation of GITR not only enhances Th1 and Th2 development *in vitro*, but also has profound effects on both Th1- and Th2-mediated disease *in vivo*.

The enhancing effect of anti-GITR antibody on CD4⁺CD25⁻ cells is likely due to the ability of the antibody to elevate T-bet and GATA3, the master switch of Th1 and Th2 cells respectively. The effect of anti-GITR antibody in this Th1 and Th2 driven system is likely secondary to the TCR activation and the polarisation cytokines. Thus, anti-GITR antibody elevates the expression of these transcription factors following their initial induction by the polarising conditions, suggesting that there is a common pathway by which GITR signalling enhances the expression of these two opposing transcription factors. Given the importance of T-bet and GATA3 in Th cell differentiation, the mechanism by which GITR signalling enhances the expression of these transcription factors merits further investigation.

Extensive work has demonstrated that the addition of anti-GITR mAb to co-cultures of murine responder (CD4⁺CD25⁻) and suppressor (CD4⁺CD25⁺) cells completely abrogates the suppressive effect of these regulatory cells [1, 2]. It is noted that there are several groups of Treg cells that may be important in suppressing inflammation in our animal models, how-

was completely abolished by co-transfer of CD4⁺GITR⁺ T cells (Fig. 7B, shown in colour in the supplementary online information). These results indicate that CD4⁺GITR⁺ T cells are capable of suppressing Th2-mediated allergic inflammation, associated with a reduced Th2 cytokine and increased Th1 cytokine production.

Discussion

Our study demonstrates that an agonist anti-GITR mAb enhances Th1 and Th2 differentiation of naive CD4⁺

ever, our study demonstrates that apart from abrogating the suppressive effect of CD4⁺CD25⁺ Treg cells reported previously, the reversal of suppression by anti-GITR antibody in this culture system could also be due to a direct enhancing effect on the CD4⁺CD25⁻ effector cells. It may be possible that antibody administration in the CIA model may be exacerbating disease by the development of immune complexes. However, the fact that control rat IgG did not significantly enhance CIA at the concentrations used would suggest that this was not the case.

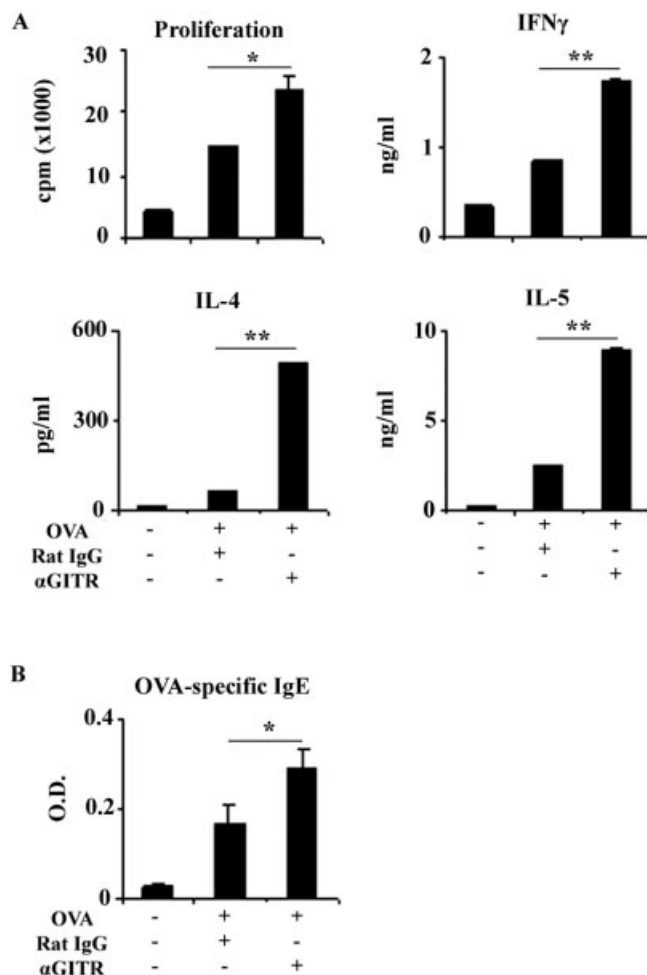


Figure 6. Anti-GITR mAb enhanced Th1 and Th2 responses in murine allergic airways inflammation. Mice were as described in Fig. 5. (A) Lymph node cells were harvested on day 28 and cultured with 1 mg/mL OVA *in vitro*. Culture supernatant was collected at 96 h and cytokine concentrations assayed by ELISA. Cellular proliferation was determined after 8-h culture with [³H]thymidine. Anti-GITR mAb administration resulted in enhanced cellular proliferation, and IFN-γ, IL-4, and IL-5 production (***p* < 0.01 and **p* < 0.05 compared with control Ab). Data are mean ± SEM, *n* = 10 and are representative of three experiments. (B) Sera were collected on day 28. Anti-GITR mAb treatment led to marked increase in OVA-specific IgE (**p* < 0.05 compared to control Ab). Data are mean ± SEM of individual mice, *n* = 6–8.

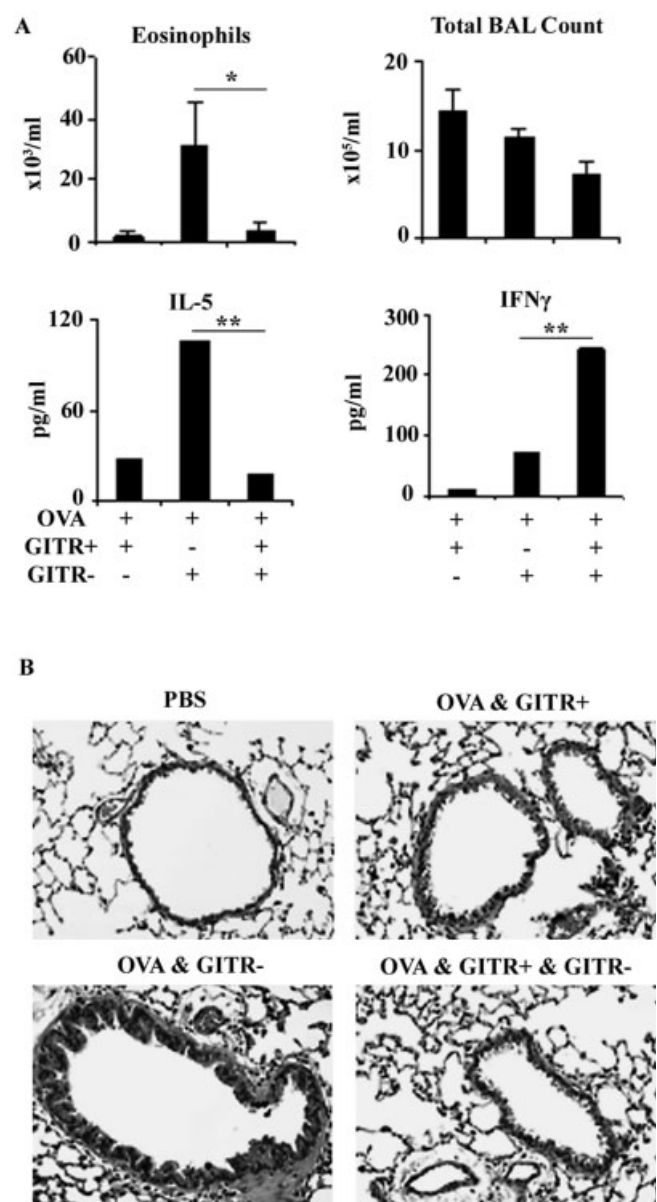


Figure 7. CD4⁺GITR⁺ cells suppress allergic airways inflammation in SCID mice. CD4⁺GITR⁺, CD4⁺GITR⁻, or equal numbers of CD4⁺GITR⁺ and CD4⁺GITR⁻ cells were transferred i.p. into SCID mice, and allergic airways inflammation was induced as described in Materials and methods section. (A) BAL total cell concentration remained unchanged, however, the total number and proportion of eosinophil fell markedly after CD4⁺GITR⁺ cell transfer (**p* < 0.05 compared to CD4⁺GITR⁻ cell transfer alone). Lymph node cell culture supernatant, collected at 96 h, shows that the IL-5 produced by CD4⁺GITR⁻ recipients was significantly suppressed by the presence of CD4⁺GITR⁺ cells. However, IFN-γ production by LN cultures from CD4⁺GITR⁻ treated mice was markedly enhanced when CD4⁺GITR⁺ cells were co-transferred (***p* < 0.01 compared to CD4⁺GITR⁻ cell transfer alone). Data are mean ± SEM, *n* = 5. (B) H&E sections of lungs (×20 magnification) demonstrate that airways inflammation was increased after CD4⁺GITR⁻ cell transfer. This allergic airway inflammation was ameliorated if CD4⁺GITR⁺ cells were co-transferred with the CD4⁺GITR⁻ cells. Pictures (day 28) are representative of five mice per group.

To investigate further the role of CD4⁺GITR⁺ cells *in vivo*, we used an adoptive transfer asthma model. The results demonstrate that CD4⁺GITR⁺ cells suppressed allergic airways inflammation induced in SCID mice reconstituted with CD4⁺CD25⁻ T cells. The disease suppressing effect of CD4⁺GITR⁺ cells was associated with a decrease in the Th2 cytokine and an increase in the Th1 cytokine production. We have not use anti-GITR mAb treatment in this model, as it would not be possible to differentiate the effect of the antibody on CD4⁺CD25⁺ Treg cells and the CD4⁺CD25⁻ effector cells *in vivo*. It has been reported that CD4⁺GITR⁺ cells could adoptively suppress inflammatory bowel disease (IBD), induced in SCID mice reconstituted with CD4⁺CD45RB^{high} cells [20]. In this IBD system, CD4⁺GITR⁺ cells reduced IFN- γ synthesis, and had no effect on the minimal level of IL-4. Thus, although CD4⁺GITR⁺ T cells have a general beneficial effect on a range of inflammatory diseases, the immunological parameters affected by these cells may differ, depending perhaps on the prevailing pro-inflammatory cytokines presence in the disease milieu.

In summary, we demonstrate here that GITR activation results in an exacerbation of both Th1- and Th2-mediated inflammatory diseases through the activation of Th1 as well as Th2 cytokines. This is due to the activation of a pathway common to T-bet and GATA3. Thus our results suggest that GITR may be a potential novel target for a range of Th1 and Th2 mediated inflammatory diseases. Therapeutic potential of reagents such as soluble recombinant GITR receptor is currently being explored.

Materials and methods

Mice

Female BALB/c, and SCID mice (C.B-17) of the BALB/c background (6 to 8 weeks old) were used for the induction of allergic airways inflammation. Collagen-induced arthritis (CIA) was induced in 8–10-week old male DBA/1 mice. All mice were obtained from Harlan-Olac (Bicester, UK) and kept at the Biological Services facilities of University of Glasgow according to the UK Home Office guidelines.

Reagents

OVA (fraction V; Sigma-Aldrich, Poole, UK) and rat IgG (Sigma-Aldrich) were prepared in sterile PBS (Life Technologies, Paisley, UK). Avertin solution was 1,1,1-tribromoethanol (Sigma-Aldrich) dissolved 1/1 w/v in amyl alcohol (Sigma-Aldrich), and then diluted 1/40 in PBS. Alum suspension was 2% Alhydrogel (Brentag Biosector, Fredriksund, Denmark). Bovine type II collagen (CII; Sigma-Aldrich) was prepared in PBS (Life Technologies) and FCA (Difco, Detroit, MI). Anti-mouse GITR mAb was prepared from the DTA-1 hybridoma,

kindly donated by Dr. S. Sagakuchi (Department of Experimental Pathology, Kyoto University, Japan). FITC-conjugated anti-mouse GITR (rat IgG2a) was purchased from R&D Systems (Minneapolis, MN). PE-conjugated anti-mouse CD3 (rat IgG2a), PE-conjugated anti-mouse CD4 (rat IgG2a) and PE-conjugated anti-mouse CD25 (rat IgG1) were purchased from BD PharMingen (San Diego, CA). FITC-conjugated mouse anti-rat IgG F(ab')₂ fragment was purchased from Jackson Immuno Research Laboratories (West Grove, PA). Complete medium consisted of RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 μ g/mL streptomycin, 50 mM 2-mercaptapurine and 10% heat-inactivated FCS (all from Life Technologies).

T cell cultures

CD4⁺ T cells were purified from the lymph nodes of naive BALB/c mice by negative selection using magnetic beads (MACS, Miltenyi Biotec) [21]. CD4⁺ cells were then further separated into CD25⁺ and CD25⁻ populations by MACS using PE-labelled anti-CD25 Ab (BD PharMingen) and anti-PE Ab-conjugated microbeads (Miltenyi Biotec). The purity of cell preparations was determined by FACS analysis and was routinely >95%. Separated cells (1×10^4) and mitomycin C-treated CD4⁻ cells (5×10^4), as APC, were cultured for 96 h in triplicate in U-bottom 96-well plates (Nunc, Roskilde, Denmark). Culture supernatant was collected and cytokine levels determined by ELISA. T cell proliferation was determined on day 3 by incorporation of [³H]thymidine.

In some experiments, CD25⁻ cells purified from naive BALB/c mice were stimulated with graded doses of anti-CD3 mAb (soluble or plate-bound 0.5–5 μ g/mL). Cells were cultured with complete medium for Th0 cell induction; or murine IL-12 (10 ng/mL; Genetics Institute, Cambridge, MA) plus anti-IL-4 antibody (1 μ g/mL) for Th1 cell induction; or murine IL-4 (10 ng/mL) plus anti-IL-12 antibody (1 μ g/mL) for Th2 cell induction (all reagents from R&D Systems, Oxon, UK). Where indicated, 10 μ g/mL control rat IgG Ab or anti-GITR mAb was added to cell culture at the same time as the cytokines and antibody.

Induction of murine allergic airway inflammation

Airway inflammation was induced by a modified protocol [22]. BALB/c mice were immunised by i.p. injection on days 0 and 14 with 100 μ g OVA in an alum suspension made up to a volume of 200 μ L. On day 14, mice were anaesthetised with 250 mL of avertin i.p., and 100 μ g of OVA in 40 μ L of PBS was administered i.n. Mice were again anaesthetised before being challenged i.n. on each of the days 25, 26, and 27 with 10 μ g OVA in 40 μ L of PBS. Control mice were given PBS in place of OVA in both the sensitisation and the challenge stages. Mice were sacrificed on day 28 by administration of a fatal dose of avertin. To test the effect of anti-GITR, 1 mg of mAb was administered i.p. on day 24 and then 1 h prior to the i.n. OVA challenge on day 26.

Induction of CIA

DBA/1 mice were injected i.d. with 200 µg of bovine type II collagen (CII) in CFA and boosted i.p. with 200 µg of CII in PBS on day 21. Mice were monitored daily for signs of arthritis, for which severity scores were derived as follows: 0, normal; 1, erythema; 2, erythema plus swelling; 3, extension/loss of function, and total score, sum of four limbs. Paw thickness was measured with a dial calliper (Kroeplin, Munich, Germany). The quantification of arthritic paws was performed by two treatment-blinded observers. Serum cytokines and Ab production were analysed at the end of the experiment (day 35). To test the effect of anti-GITR mAb, some mice were given 1 mg of antibody i.p. on day 21 and then every six days thereafter, for a total of three injections.

CD4⁺GITR⁺ cell purification and adoptive SCID transfer model

CD4⁺ T cells were purified from the lymph nodes of naive BALB/c mice by negative selection using MACS [21]. CD4⁺ cells were then further separated into GITR⁺ and GITR⁻ populations by MACS using rat anti-GITR mAb (DTA-1 hybridoma) and anti-rat IgG Ab-conjugated microbeads (Miltenyi Biotec). The purity of cell preparations was determined by FACS analysis using anti-GITR mAb and FITC-conjugated mouse anti-rat IgG F(ab')₂ fragment, and was routinely >90%. Cells were washed and re-suspended in PBS. SCID mice were injected i.p. with one or two subpopulations (4 × 10⁵ cells per subpopulation) made to a final volume of 200 µL/mouse. Airway eosinophilia was elicited 24 h later as described for the BALB/c mice.

Bronchoalveolar lavage

Immediately after the administration of a fatal dose of avertin, a blood sample was collected by cardiac puncture. BAL was then harvested using two aliquots of 0.8 mL of PBS. The lavage samples from individual mice were kept on ice until processing. The BAL was centrifuged at 1400 rpm for 5 min, and the supernatant removed. The volume of each supernatant was measured before storage at -70°C until assay for cytokines by ELISA. The BAL cell pellet was then resuspended in 1 mL of PBS, and counted using a haemocytometer (Weber Scientific, Teddington, UK). Cytospin preparations were prepared using a Cytospin (Shandon, Pittsburgh, PA), and stained with Diff-Quick (Triangle Biomedical Sciences, Skelmersdale, UK), by a rapid Romanowsky staining method. Differential cell counts of 400 cells were performed using standard morphological criteria.

Lung histology

After BAL sampling, the lungs were removed and inflated with 1 mL of 10% neutral-buffered formalin and then fixed in 10% neutral-buffered formalin for 72 h. The left lung was then dissected free and embedded in paraffin. Sections (6 µm) were stained with H&E and examined under 20-100X magnifications, and peri-bronchial and peri-vascular inflammation assessed.

Lymphoid cell culture

In the asthma model, thoracic lymph nodes and spleens were harvested and single-cell suspension cultured in complete medium. Cells were cultured at 1 × 10⁶ cells/mL with 1 mg/mL OVA for 96 h in triplicate in U-bottom 96-well plates (Nunc). Supernatants were analysed for cytokine concentrations by ELISA. T cell proliferation was determined by [³H]thymidine incorporation at 72 h.

ELISA

Murine IL-4, IL-5, IL-12, IFN-γ (BD Biosciences, Oxford, UK) and IL-10 (Biosource International, Camarillo, CA) were assayed using paired antibodies. The lower limit of detection for IL-4, IL-5, IL-10 and IL-12 was 10 pg/mL; and for IFN-γ was 40 pg/mL. OVA-specific IgE was measured using a modification of the OptiEIA ELISA kit (BD Biosciences), in that the plate coating Ab was substituted for 10 µg/mL OVA. Serum anti-collagen II Ab was measured as described previously [17]. Briefly, plates were coated with 2 µg/mL CII, and samples were added the following day. Bound IgG1/2a was detected with biotin-conjugated anti-mouse IgG1 or IgG2a respectively (BD PharMingen).

Quantitative PCR

This was carried out as described previously [23]. The primers and probes were as follows: for T-bet, GCCAGGGAACCGCT-TATATG (forward), AACCTCCTGGCGCATCCA (reverse), and CCCAGACTCCCCAACACCGGA (probe); for GATA3, TCCTCCTCTACGCTCCTTGCTA (forward), AACTGATTC-CTTGGCGCTC (reverse), and TCGTGATCGGAAGAGCAACCGTCTC (probe); and for HPRT, GCAGTACAGCCCCAAATGG (forward), AACAAAGTCTGGCCTGTATCCAA (reverse), and TAAGTTGCAAGCTTGCTGGTGAAAAGGA (probe).

Statistical analysis

Analysis of variance (ANOVA) was used to compare differences between multiple groups. Statistical significance between individual groups was then examined by Student's *t*-tests. **p* < 0.05 was considered significant, and ***p* < 0.01 was deemed highly significant.

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