Therapeutic Glucocorticoid-Induced TNF Receptor-Mediated Amplification of CD4+ T Cell Responses Enhances Antiparasitic Immunity

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Therapeutic Glucocorticoid-Induced TNF Receptor-Mediated Amplification of CD4+ T Cell Responses Enhances Antiparasitic Immunity

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Chronic infectious diseases and cancers are often associated with suboptimal effector T cell responses. Enhancement of T cell costimulatory signals has been extensively studied for cancer immunotherapy but not so for the treatment of infectious disease. The few previous attempts at this strategy using infection models have lacked cellular specificity, with major immunoregulatory mechanisms or innate immune cells also being targeted. In this study, we examined the potential of promoting T cell responses via the glucocorticoid-induced TNF receptor (GITR) family-related protein in a murine model of visceral leishmaniasis. GITR stimulation during established infection markedly improved antiparasitic immunity. This required CD4+ T cells, TNF, and IFN-γ, but crucially, was independent of regulatory T (Treg) cells. GITR stimulation enhanced CD4+ T cell expansion without modulating Treg cell function or protecting conventional CD4+ T cells from Treg cell suppression. GITR stimulation substantially improved the efficacy of a first-line visceral leishmaniasis drug against both acute hepatic infection and chronic infection in the spleen, demonstrating its potential to improve clinical outcomes. This study identifies a novel strategy to therapeutically enhance CD4+ T cell-mediated antiparasitic immunity and, importantly, achieves this goal without impairment of Treg cell function. 

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*Immunology and Infection Laboratory, Queensland Institute of Medical Research and The Australian Center for Vaccine Development; †Bone Marrow Transplantation Laboratory, Queensland Institute of Medical Research, Herston, Queensland, Australia; ‡London School of Hygiene and Tropical Medicine, London, United Kingdom; and §Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan.

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Address correspondence and reprint requests to Dr. Christian R. Engwerda, Queensland Institute of Medical Research, 300 Herston Road, Herston, Queensland 4006, Australia. E-mail address: christian.engwerda@qimr.edu.au

Abbreviations used in this paper: GITR, glucocorticoid-induced TNF receptor; IG, immature granuloma; iKC, infected Kupffer cell; MFI, mean fluorescence intensity; MG, mature granuloma; MNC, mononuclear cell; p.i., postinfection; Sb(V), sodium stibogluconate; Treg, regulatory T; VL, visceral leishmaniasis.

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during infection using a strategy that avoids broader leukocyte modulation or Treg cell suppression. In this study, we demonstrate for the first time the therapeutic potential of agonistic signaling via GITR during infection to enhance CD4+ T cell responses, without compromising Treg cell function, to improve parasite clearance by TNF- and IFN-γ-dependent mechanisms.

### Materials and Methods

#### Mice

Inbred female C57BL/6 were purchased from the Australian Resource Centre (Canning Vale, Western Australia, Australia), and maintained under conventional conditions. Furosamide mice (43) were backcrossed six to seven times onto the C57BL/6 background, bred, and maintained at the Queensland Institute of Medical Research Animal Facility along with IFN-γR−/−deficient C57BL/6 mice. All mice used were age matched (6–12 wk) and were housed under specific-pathogen free conditions. All animal procedures were approved and monitored by the Queensland Institute of Medical Research Animal Ethics Committee.

#### Parasites and infection of mice

*L. donovani* (LV9) was maintained by passage in B6.RAG-1−/− mice, and amastigotes were isolated from the spleens of chronically infected animals, as described previously (44, 45). Mice were infected by injecting 2 × 10^5 amastigotes i.v. via the lateral tail vein, killed at the times indicated in the text by CO2 asphyxiation, and bled via cardiac puncture. Spleens and perfused livers were removed, and parasite burdens were determined from Diff-Quik–stained impression smears (Lab Ads, Narrabeen, New South Wales, Australia) and expressed in Leishman-Donovan units (the number of amastigotes per 1000 host nuclei multiplied by organ weight in grams) (46). Liver and spleen tissue were also preserved in Tissue-Tek OCT Compound (Sakura, Torrance, CA). Hepatic mononuclear cells (MNCs) were isolated immediately following death as described previously (44, 45).

#### Abs and drugs for in vivo administration

All hybridomas (DTA-1 [anti-GITR], PC61 [anti-CD25], 145-2C11 [anti-CD3ε], YTS191.1 [anti-CD4], and 53-5.8 [anti-CD8b]) were grown in 5% CO2 asphyxiation, and bled via cardiac puncture. Spleens and perfused livers were removed, and parasite burdens were determined from Diff-Quik–stained impression smears (Lab Ads, Narrabeen, New South Wales, Australia) and expressed in Leishman-Donovan units (the number of amastigotes per 1000 host nuclei multiplied by organ weight in grams) (46). Liver and spleen tissue were also preserved in Tissue-Tek OCT Compound (Sakura, Torrance, CA). Hepatic mononuclear cells (MNCs) were isolated immediately following death as described previously (44, 45).

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#### Flow cytometry

**For the staining of cell surface Ags, liver MNCs or splenocytes were harvested and first preincubated with CD16/32 mAb (2.4G2; grown-in-house) to avoid nonspecific binding of Abs to FcγRs. Cells were then incubated with fluorochrome-conjugated Abs on ice for 20 min. T cells, NKT cells, and NK cells were enumerated with Pacific Blue or FITC-conjugated anti-TCRβ chain (H57-597), PE-Cy5–conjugated anti-CD4 (GK1.5), PE-conjugated anti-CD8a (H1.2F3), and allophyocyanin-conjugated anti-CD8a (NK1.1) (PK136). B cells were enumerated using FITC-conjugated anti-CD19 (6D5) and Pacific Blue-conjugated anti-B220 (RA3-6B2). NKT cells were stained using PE-conjugated α-galactosylceramide–loaded CD1d tetramers (a gift from Prof. D. Godfrey, Department of Microbiology and Immunology, University of Melbourne, Melbourne, Victoria, Australia). All mAbs were purchased from Biolegend (San Diego, CA) or BD Biosciences. Intracellular staining for Foxp3 was performed using Alexa 647–conjugated anti-Foxp3 mAb (150D) (Biolegend) in conjunction with a Foxp3–staining buffer kit (eBioscience) used according to the manufacturer’s instructions. For intracellular cytokine staining, splenocytes were first incubated for 3 h at 37°C to 5% CO2 in 5% (v/v) FCS and RPMI 1640 containing 10 μg/ml brefeldin A (Sigma-Aldrich) prior to FcγR blockade and cell surface labeling as detailed above. For intracellular Ab labeling of cytokines or Bcl-2, surface-labeled cells were washed, fixed, and permeabilized using BD cytofix/cytoperm fixation/ permeabilization kits according to the manufacturer’s instructions (BD Biosciences). Cells were incubated with PE-conjugated mAbs against IFN-γ or Bcl-2 (or appropriate isotype control Abs) for 40 min on ice, extensively washed, and fixed in 1–2% (v/v) formaldehyde in PBS. Assessment of BrdU incorporation was performed using a FITC-based BrdU detection kit (BD Biosciences) according to the manufacturer’s instructions. Flow cytometric analysis was performed on a FACS Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo Software (Tree Star, Ashland, OR).

#### In vitro Treg cell suppression assay

CD4+ TCRβFoxp3+ Treg cells, CD4+ TCRβFoxp3+ CD44hi naive T cells, and CD4+ TCRβFoxp3+ CD44− effector T cells from C57BL/6 mice were sorted using a FACS Aria cell sorter (BD Biosciences). Cell purity after sorting was confirmed to exceed 95%. Between 25,000 and 45,000 naïve or activated CD4+ T cells were incubated at 37°C to 5% CO2 in U-bottomed 96-well plates with CD4+ TCRβFoxp3+ Treg cells at ratios between 1:1 and 128:1. Cells were stimulated with 1 μg/ml anti-CD3ε (145-2C11; made in-house) in the presence of 10,000 irradiated CD11c+ dendritic cells from naïve C57BL/6 mice. Briefly, CD11c+ dendritic cells were positively selected from spleen mononuclear cells by magnetic- conjugated anti-mouse CD11c Abs (N418) and positive selection columns, according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). After 72 h of stimulation, wells were pulsed for 20 h with 1 μCi of [3H] thymidine. Thymidine incorporation was then measured using a 1450 Microbeta plate reader (PerkinElmer, Boston, MA).

#### Measurement of serum cytokine levels

Serum harvested from blood was assessed for the presence of cytokines using flow cytometry during experimental VL, we treated *L. donovani*-infected C57BL/6 mice with the agonistic anti-GITR mAb DTA-1, either on the day of infection (day 0), or 5 d.p.i. We chose these two time points to model two scenarios; firstly, one in which parasite-specific T cells were naive (day 0), and secondly, where parasite-specific T cells had been activated through interaction with APCs displaying cognate Ag. Liver parasite burdens at day 14 p.i. were significantly reduced in mice.
administered DTA-1 at 5 d p.i., compared with control rat IgG-treated mice, but not in mice administered DTA-1 at the time of infection (Fig. 1A). Thus the positive effect of DTA-1 on parasite control was dependent on the timing of administration. We further assessed the effect of DTA-1 treatment on day 5 p.i. on parasite burdens in the liver and spleen 14 and 28 d p.i. (Fig. 1B). We noted that DTA-1 treatment reduced the peak level of infection in the liver, although it is interesting to note that the rate of parasite clearance from days 14–28 p.i. was similar for both DTA-1- and control treated mice. DTA-1 also exerted positive effects in the spleen, by significantly delaying, although not preventing, establishment of infection in this organ. These data demonstrate that DTA-1 treatment after the establishment of infection enhanced parasite control both in the liver and spleen.

**GITR stimulation enhances liver granuloma assembly and function**

We next examined the effect of DTA-1 treatment 5 d p.i. on liver leukocyte numbers and granuloma formation after *L. donovani* infection. The formation of granulomas around infected Kupffer cells is a critical event in resolution of hepatic infection in this disease model (35). We observed a substantial increase by day 14 p.i. in the number of leukocytes in the livers of DTA-1-treated mice, compared with control rat IgG-treated mice (Fig. 2A). However, by day 28 p.i. similar granulomatous responses were evident in both DTA-1 and control treated mice (Fig. 2A). These data suggested that DTA-1 treatment had accelerated the process of granuloma formation over the first 14 d of infection. A detailed assessment of granuloma formation and maturation at day 14 p.i. (Fig. 2B) indicated that DTA-1 treatment reduced the total number of infected foci, compared with control infected mice (Fig. 2C). The foci present in the livers of control rat IgG treated mice were predominantly infected Kupffer cells, with relatively few immature or mature granulomas present. In contrast, the majority of infected foci in the livers of DTA-1–treated mice were immature and mature granulomas. These data indicate that DTA-1 treatment at day 5 p.i. reduced parasite burden by increasing hepatic leukocyte numbers and enhancing granuloma formation in the liver.

**Increased GITR expression by conventional CD4+ T cells, CD8+ T cells, and Treg cells in the liver following infection**

We hypothesized that the importance of DTA-1 treatment timing could be explained by changes in GITR expression on specific cell types over the first 5 d of infection. GITR expression by all cell types analyzed in the spleen changed very little over this time (Fig. 3A). However, GITR expression significantly increased on Foxp3+ CD4+ Treg cells, conventional CD4+ T cells and CD8+ T cells in the liver (Fig. 3B). In contrast, GITR expression by liver NKT cells, NK cells, and B cells was unchanged over this time-frame, suggesting that despite being targets for DTA-1 from the day of infection, GITR stimulation of these cells was unlikely to contribute to DTA-1–mediated enhanced immunity (Fig. 3B). Instead, these data suggested that increased targeting of DTA-1 to liver CD4+ Treg cells, conventional CD4+ T cells, and CD8+ T cells at day 5 p.i. might account for the enhanced immunity generated by GITR stimulation.

**DTA-1 enhances CD4+ T and CD8+ T cell responses**

We next examined the effect of DTA-1 treatment on hepatic T cell responses, because of their importance in controlling parasite growth, and in the case of CD4+ T cells, their critical role in granuloma formation (34, 35). We found a significant increase (*p < 0.01*) at day 14 p.i. in the number of conventional CD4+ and CD8+ T cells in the livers of mice treated with DTA-1 on day 5 p.i. compared with those treated with control rat IgG (Fig. 4A). Importantly, however, DTA-1 treatment did not induce changes in the small number of Treg cells found in the liver (Fig. 4A), as previously reported in other experimental systems (8). The number of IFN-γ–producing CD4+ T cells was dramatically increased by DTA-1 treatment (Fig. 4B, 4C). In contrast, the number of IFN-γ+CD8+ T cells was only slightly enhanced by DTA-1 treatment (Fig. 4C). Relatively few NK or NKT cells produced IFN-γ at this time, either in control or DTA-1–treated infected mice (Fig. 4C). These data demonstrate that DTA-1 treatment had a dramatic effect on CD4+ T cell responses, a moderate effect upon CD8+ T cell responses, and no effect on the numbers of CD4+ Treg cells in the liver.

**DTA-1–mediated immunity is dependent on CD4+ T cells but not CD8+ T cells**

To determine the relative roles of CD4+ and CD8+ T cells in DTA-1–mediated enhanced antiparasitic immunity, both cell types were independently depleted from the time of DTA-1 administration on day 5 p.i. Depletion of CD4+ T cells completely abrogated DTA-1–mediated enhanced immunity, whereas depletion of CD8+ T cells had no effect (Fig. 5). Importantly, CD4+ or CD8+ T cell depletion in the absence of GITR activation had little impact on parasite burdens at day 14 p.i. (Fig. 5), because the effects of T cell absence are not readily apparent until later in infection (34). Therefore, although DTA-1 treatment moderately enhanced CD8+ T cell responses, GITR stimulation improved parasite control via a mechanism requiring enhanced CD4+ T cell activation but not CD8+ T cell activation.
GITR stimulation boosts CD4+ T cell proliferation but does not enhance protection against apoptosis

To determine whether the increased number of activated CD4+ T cells in the livers of GITR-stimulated mice was due to enhanced proliferation, we assessed the incorporation of the synthetic nucleotide BrdU into the DNA of CD4+ T cells. By day 14 p.i., the number of BrdU+CD4+ T cells in the livers of infected mice was significantly greater in mice administered DTA-1 on day 5 p.i., compared with mice administered control rat IgG (p < 0.05) (Fig. 6A). In contrast, the number of BrdU+CD8+ T cells in the liver was not significantly increased by the administration of DTA-1 (Fig. 6A). In addition to boosting proliferation, it was also possible that GITR stimulation could have been enhancing protection of CD4+ T cells from apoptosis. To examine this, we measured expression of Bcl-2, an important antiapoptotic protein, in both CD4+ T cells and CD8+ T cells. We observed that ~90% of CD4+ T cells in the livers of naive mice expressed detectable levels of Bcl-2 (Fig. 6B). This percentage dropped to ~45% of CD4+ T cells harvested from the livers of infected mice treated with control rat IgG, suggesting that during L. donovani infection, CD4+ T cells in the liver become more susceptible to apoptosis by day 14 p.i. (Fig. 6B). Furthermore, the proportion of Bcl-2+CD4+ T cells in the liver of day 5 p.i., DTA-1-treated mice was significantly lower than that of control infected mice (Fig. 6B). Taken together, the BrdU incorporation and intracellular Bcl-2 expression data demonstrate that GITR stimulation specifically stimulated CD4+ T cell proliferation but did not provide them with enhanced protection from apoptosis.

DTA-1-mediated immunity during L. donovani infection is independent of modulation of Treg cell function

GITR stimulation has been reported to reduce the suppressive capacity of Treg cells. To determine whether DTA-1 treatment was also acting directly on these cells to reduce suppression and enhance
immunity, we depleted CD25hi Treg cells from naive mice 14 d preinfection with *L. donovani*. We have previously shown that this regimen depletes CD25hiFoxp3+CD4+ Treg cells but does not deplete conventional CD4+ T cells generated during infection (49, 50). Control and CD25hi Treg cell-depleted mice were infected and treated with control rat IgG or DTA-1 on the day of infection, or alternatively, with DTA-1 or control rat IgG on day 5 p.i. As shown above (Fig. 1), DTA-1 treatment enhanced parasite control in the liver when administered at day 5 p.i. but had no effect if given on the day of infection (Fig. 7A). This pattern was also observed in mice depleted of CD25hi Treg cells prior to infection (Fig. 7A). These data indicate that GITR stimulation enhances immunity to *L. donovani* via a mechanism that is independent of CD25hi Treg cells.

To further analyze any potential effects of GITR stimulation on Treg cell function, we adopted a routine in vitro suppression assay, in which titrated numbers of CD4+ Treg cells are able to block proliferation of anti–CD3ε-stimulated naive CD4+ T cells. To sort viable Foxp3+CD4+ Treg cells, we used C57BL/6.foxp3gfp/gfp mice, which express eGFP under the control of the *foxp3* promoter (43). The eGFP+ (Foxp3+) CD4+ Treg cells were sorted from naive mice and infected mice 4 d after administration of either control rat IgG or DTA-1 (administered on day 5 p.i.). These cells were then assessed in vitro for their ability to suppress proliferation of Foxp3+CD4+CD44hi naive T cells (Fig. 7B). We observed that CD4+ Foxp3+ Treg cell function was not altered by DTA-1 treatment. GITR stimulation of Ag-specific CD4+ T cells has also been reported to render these cells refractory to Treg cell-mediated suppression (9, 20). To explore this possibility during *L. donovani* infection, we assessed the ability of sorted CD4+CD44hiFoxp3–activated T cells from infected control mice, and infected DTA-1–treated mice, to proliferate in the presence of naive CD4+Foxp3hi Treg cells. We observed no difference between activated CD4+ T cells from control infected mice or DTA-1–treated mice in their ability to resist Treg cell-mediated suppression (Fig. 7C). Taken together, these data demonstrate that during *L. donovani* infection,
GITR stimulation does not modulate either Treg cell function, or the susceptibility of conventional CD4+ T cells to Treg cell-mediated suppression. Hence, GITR stimulation enhances immunity via a mechanism that is independent of CD25hi Treg cells. DTA-1–mediated immunity is dependent upon TNF and IFN-γ

We next examined which cytokines are important in DTA-1–mediated, CD4+ T cell-dependent antiparasitic immunity. We noted significantly enhanced levels of both TNF and IFN-γ at day 14 p.i. in the serum of infected mice treated with DTA-1, compared with control infected mice (Fig. 8A). Both these cytokines are crucial for controlling parasite numbers in the liver (32, 33, 36). To determine the requirement for IFN-γ in DTA-1–mediated enhanced immunity, IFN-γR–deficient C57BL/6 mice were infected and treated with control rat IgG or DTA-1 on day 5 p.i. Wild-type C57BL/6 mice were also infected and treated in the same manner. Although DTA-1 treatment reduced parasite burden in wild-type mice as expected, this reduction in parasite burden was not observed in IFN-γR–deficient mice (Fig. 8B), indicating a critical role for IFN-γ in DTA-1–mediated antiparasitic immunity. To determine the role of TNF in DTA-1–mediated enhanced immunity, infected mice were given control rat IgG or DTA-1 on day 5 p.i. and treated with control human IgG or Enbrel etanercept, a human TNF blocking molecule that also blocks TNF in mice (47, 48). The enhanced protection elicited by DTA-1 treatment of control human IgG-treated wild-type mice was completely abrogated in mice treated with Enbrel etanercept (Fig. 8C), indicating an important role for TNF in DTA-1–mediated immunity against L. donovani. Taken together, these data show that enhanced production of both IFN-γ and TNF are required for the antiparasitic immunity elicited by GITR stimulation.

**Therapeutic GITR stimulation enhances drug-mediated parasite clearance**

A pentavalent antimonial drug, Sb(V) (also known as Pentostam), is one of the most widely used treatments for VL in humans, despite its toxicity, requirement for several weeks of daily dosing, and emerging parasite drug resistance (25, 29, 30). Therefore, an important clinical goal is to clear parasites using lower, less frequent doses of Sb(V). Because Sb(V) relies on the host immune response for its efficacy (51, 52), we used a well-validated system for testing adjunctive therapies in VL (38, 39, 51–53) to investigate whether DTA-1 could improve the efficacy of a supoptimal dose of Sb(V) during an established L. donovani infection.

To assess the potential for DTA-1 to target conventional CD4+ T cells at later time points during infection, we first analyzed GITR expression on conventional CD4+ and CD8+ T cells, as well as Treg cells in mice infected for 14 d with L. donovani, a time when liver parasite burdens are maximal, and infection has become established in the spleen (Fig. 9A) (24, 31). We observed moderate upregulation of GITR on Foxp3+CD4+ Treg cells and CD8+ T cells in the liver and spleen at this time point, and similarly, minimal upregulation of GITR on splenic conventional CD4+ T cells. However, substantial upregulation of GITR was observed on conventional CD4+ T cells in the liver, indicating that activated, conventional CD4+ T cells were potential targets for DTA-1 treatment during established infection.

We administered DTA-1 at day 14 p.i. either alone or with a suboptimal dose of Sb(V). One week after administration of Ab and/or drug (on day 21 p.i.), liver and spleen parasite burdens were determined (Fig. 9B, 9C). DTA-1 treatment alone enhanced parasite clearance in the liver, although this was not statistically significant, but did not affect splenic parasite burdens. A suboptimal dose of Sb(V) (50 mg/kg), partially reduced liver and spleen parasite burdens, relative to mice administered a full therapeutic dose of Sb(V) (500 mg/kg). Importantly, DTA-1 substantially improved liver and spleen parasite burdens in infected mice treated with either control rat IgG or DTA-1 (Fig. 9B, 9C) (24, 31, 32). We observed a marked enhancement of liver parasite burdens in mice treated with either control rat IgG or DTA-1 (n = 5 mice/group) on day 21 p.i. with Sb(V) (50 mg/kg). This confirms that DTA-1–mediated immunity may have the potential to enhance the efficacy of Sb(V) treatment.

**Figure 5.** GITR stimulation enhances immunity via a CD4+ T cell-dependent, CD8+ T cell-independent mechanism. Infected C57BL/6 mice were treated on day 5 p.i. with control rat IgG or DTA-1. Some groups of mice (n = 5 mice/group) were also treated with either anti-CD8 Abs or anti-CD4 Abs from day 5 onward, as indicated, and liver parasite burdens were determined at 14 d p.i. **p < 0.01. Data presented are representative of two independent experiments.

**Figure 6.** GITR stimulation enhances CD4+ T cell proliferation but does not increase protection against apoptosis. A, Naive mice and infected mice (n = 5 mice/group) treated with either control rat IgG or DTA-1 were administered BrDU from day 6 p.i. At day 14 p.i., liver CD4+ T cells and CD8+ T cells were assessed for incorporation of BrDU by flow cytometry. B, Naive mice and infected mice treated with either control rat IgG or DTA-1 (n = 5 mice/group) were assessed at day 14 p.i. for intracellular expression of the antiapoptotic protein Bcl-2 by liver CD4+ T cells and CD8+ T cells using flow cytometry. Data presented are representative of two independent experiments. "**"p < 0.01.
parasite clearance in mice treated with the suboptimal dose of Sb(V) in both the liver and spleen. In fact, DTA-1 combined with supoptimal drug treatment was as successful at clearing parasites from the liver and spleen, as the full therapeutic dose of Sb(V). These data indicate that GITR stimulation has potential as an adjunct to conventional chemotherapy for the treatment of VL by clearing parasites from the liver and spleen, the two main infected organs in mice and humans.

Discussion

Manipulation of T cell activation via T cell costimulatory receptors has received relatively little attention in the context of infectious disease, despite its reported efficacy in improving antitumor immune responses (5–9, 18, 54, 55). We employed a murine model of VL to assess the effect of GITR stimulation during infection. We discovered that GITR stimulation enhanced parasite clearance via mechanisms requiring CD4+ T cells, IFN-γ, and TNF but not CD8+ T cells or Treg cells. GITR stimulation specifically enhanced CD4+ T cell proliferation, IFN-γ and TNF production but did not render CD4+ T cells refractory to CD4+ Treg cell-mediated suppression or increase levels of the antiapoptotic molecule Bcl-2. In fact, given that GITR stimulation appeared to further activate CD4+ T cells and reduce their Bcl-2 levels, it is possible that activation induced cell death may have been accelerated among this cell population. Most important, however, is the fact that GITR stimulation did not alter the number or suppressive capacity of Treg cells. Thus, activation of GITR during experimental VL specifically enhanced a suboptimal

![Image](http://www.jimmunol.org/Downloaded_from/FIGURE_7.jpg)

**FIGURE 7.** GITR stimulation does not modulate CD4+ Treg cell function during *L. donovani* infection. A, CD25^hi^CD4^+^Foxp3^+^ T cell-depleted and control mice were infected and treated with DTA-1 on the day of infection or 5 d.p.i. Control mice received rat IgG. Liver parasite burdens were determined 14 d.p.i. ***p < 0.001; **p < 0.01. These data are representative of two independent experiments. B, CD4^+^Foxp3^+^ Treg cells were sorted 9 d.p.i. from pooled spleens of infected C57BL/6^foxp3^<sup>3<sup>6<sup>6</sup>6</sup>^ mice treated with DTA-1 or control rat IgG on day 5 p.i. Treg cells were titrated into wells containing naive CD4^+^CD44^lo^Foxp3<sup>2</sup> T cells from C57BL/6^foxp3^<sup>3<sup>6<sup>6</sup>6</sup>^ mice and stimulated with anti-CD3ε Ab and irradiated CD11c<sup>+</sup>APCs. CD4^+^ T cell proliferation was measured by [^3H^] thymidine incorporation. Data are representative of two independent experiments. C, CD4<sup>+</sup> Treg cells sorted from uninfected C57BL/6^foxp3^<sup>3<sup>6<sup>6</sup>6</sup>^ mice were titrated against effector CD4<sup>+</sup>CD44^hi^ Foxp3<sup>2</sup> T cells from pooled spleens of infected C57BL/6^foxp3^<sup>3<sup>6<sup>6</sup>6</sup>^ mice treated either with DTA-1 or control rat IgG on day 5 p.i. Effector cells were stimulated and proliferation assessed as in B. Data presented here are representative of three independent experiments.

![Image](http://www.jimmunol.org/Downloaded_from/FIGURE_8.jpg)

**FIGURE 8.** GITR stimulation enhances immunity via IFN-γ- and TNF-dependent mechanisms. A, Analysis of serum IFN-γ and TNF levels in day 14-infected mice treated 5 d.p.i with DTA-1 or control rat IgG (n = 5 mice/group). Horizontal bars indicate median values per group. Data presented are pooled from three independent experiments. B, Wild-type and IFN-γR-deficient C57BL/6 mice were infected and treated with DTA-1 or control rat IgG on day 5 p.i. (n = 5 mice/group). Liver parasite burdens were determined at day 14 p.i. Data presented are representative of two independent experiments. C, C57BL/6 mice were infected and then treated with DTA-1 or control rat IgG on day 5 p.i. Also from day 5 p.i. onward, mice were treated with control human IgG or the TNF blocking protein, Enbrel etanercept (n = 5 mice/group). Liver parasite burdens were determined at day 14 p.i. Data presented are representative of two independent experiments. ***p < 0.001; **p < 0.01.
antiparasitic CD4+ T cell response, without impairing Treg cell function. Furthermore, therapeutic administration of GITR during an established infection significantly enhanced the efficacy of suboptimal drug treatment. This last observation highlights the potential for GITR stimulation to shorten the long, toxic, chemotherapy regimens currently used, and to improve parasite clearance in VL patients.

Three recent studies demonstrated that GITR stimulation could enhance antitumor immune responses in mice (8, 9, 18). These studies are consistent with our data in that CD4+ T cells mediated enhanced protection. Others have reported that GITR activation stimulates proliferation of Treg cells (8, 9). We did not observe this phenomenon during *L. donovani* infection. The reasons for this are unclear at present but could be related to the immune cell and cytokine environment that exists during this infection. We did not observe any change in the suppressive capacity of Treg cells in vitro after GITR stimulation in vivo, which is consistent with the observations of others (9). Critically, our in vivo Treg cell depletion studies clearly demonstrate that GITR stimulation does not enhance immunity via direct modulation of their function.

Previous studies suggested that GITR stimulation protected CD4+ T cells from suppression by Treg cells (9, 20). We did not find this to be the case during *L. donovani* infection. Again, the reasons for this are unclear but could be due to differences in experimental design in at least one case (9). We specifically purified Foxp3+CD4+CD44hi T cells as the responder CD4+ T cell population, whereas the previous study classified responders as being CD4+CD25+ cells. CD4+CD25+ cells would have contained naive CD4+ T cells,

**FIGURE 9.** GITR stimulation enhances the efficacy of suboptimal drug therapy. *A*, Analysis of GITR expression by indicated cell types from livers and spleens of mice infected 14 d previously with *L. donovani*. Lines indicate GITR expression by individual mice from a group of five infected mice compared with a representative naive mouse (solid gray histogram). Liver (*B*) and splenic (*C*) parasite burdens in mice 7 d after being given various indicated treatments on day 14 p.i. Data presented are representative of two independent experiments. **p < 0.01.
activated CD4+ T cells, and CD25- Foxp3+CD4+ T cells, the relative proportions of which may have varied between treatment groups. Therefore, this previous study did not directly compare responder CD4+ T cells from control and DTA-1 treated mice but instead studied mixed populations of CD4+ T cells.

In the current study, increases in CD8+ T cell responses were modest in comparison with that of CD4+ T cell responses. This pattern was similar to observations made using murine antitumor models (8, 9). The reasons for this pattern are unclear, given that both CD8+ and CD4+ T cells express intermediate levels of GITR, and both upregulate its expression during infection. The signaling pathways triggered by GITR stimulation of CD4+ T cells in vivo remain to be fully elucidated but could provide an explanation for the preferential effects of GITR activation on CD4+ T cells and not CD8+ T cells.

Finally, we observed that the enhanced antiparasitic immunity mediated by GITR stimulation was more effective if administered p.i., rather than at the time of infection. This observation is consistent with others, who observed that antitumor immunity was boosted by GITR stimulation if administered the day after tumors were implanted, but not the day before or on the day of implantation. The reasons for this are not linked to Treg cells in our system, because we observed this phenomenon in the presence or absence of CD25hiCD4+ Treg cells in vivo. Instead, we hypothesize that GITR signaling pathways are inefficiently triggered in CD4+ T cells during initial encounters with parasite Ag but that increased GITR expression on CD4+ T cells following activation makes them amenable to enhanced GITR-mediated costimulation.

In conclusion, the data presented in this study demonstrate that CD4+ T cell responses during L. donovani infection are suboptimal and can be enhanced by GITR stimulation to improve antiparasitic immunity. Importantly, GITR stimulation did not affect the capacity of Treg cells to suppress immune responses, suggesting this major regulatory mechanism for preventing autoimmunity was unaffected. Therefore, GITR stimulation is a viable strategy, particularly in the context of adjunctive immunotherapy, for specifically boosting endogenous T cell responses during infection.

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Disclosures

The authors have no financial conflicts of interest.

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