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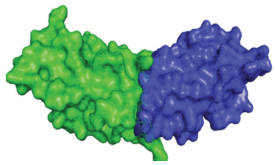

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Th2 Responses to Helminth Parasites Can Be Therapeutically Enhanced by, but Are Not Dependent upon, GITR–GITR Ligand Costimulation In Vivo

Nienke van der Werf,*,† Stephen A. Redpath,*,† Alexander T. Phythian-Adams,*,† Miyuki Azuma,‡ Judith E. Allen,*,† Rick M. Maizels,*,† Andrew S. MacDonald,*,† and Matthew D. Taylor*,†

The immune suppression that characterizes human helminth infections can hinder the development of protective immunity or help to reduce pathogenic inflammation. Signaling through the T cell costimulator glucocorticoid-induced TNFR-related protein (GITR) counteracts immune downregulation by augmenting effector T cell responses and abrogating suppression by Foxp3$^+$ regulatory T cells. Thus, superphysiological Ab-mediated GITR costimulation represents a novel therapy for promoting protective immunity toward parasitic helminths, whereas blocking physiological GITR–GITR ligand (GITRL) interactions may provide a mechanism for dampening pathogenic Th2 inflammation. We investigated the superphysiological and physiological roles of the GITR–GITRL pathway in the development of protective and pathogenic Th2 responses in murine infection models of filariasis (*Litomosoides sigmodontis*) and schistosomiasis (*Schistosoma mansoni*). Providing superphysiological GITR costimulation using an agonistic anti-GITR mAb over the first 12 d of *L. sigmodontis* infection initially increased the quantity of Th2 cells, as well as their ability to produce Th2 cytokines. However, as infection progressed, the Th2 responses reverted to normal infection levels, and parasite killing remained unaffected. Despite the Th2-promoting role of superphysiological GITR costimulation, Ab-mediated blockade of the GITR–GITRL pathway did not affect Th2 cell priming or maintenance during *L. sigmodontis* infection. Blockade of GITR–GITRL interactions during the acute egg phase of *S. mansoni* infection resulted in reduced Th2 responses, but this effect was confined to the spleen and did not lead to changes in liver pathology. Thus, although superphysiological GITR costimulation can therapeutically enhance Th2 responses, physiological GITR–GITRL interactions are not required for the development of Th2-mediated resistance or pathology in murine models of filariasis and schistosomiasis. ***The Journal of Immunology*****, 2011, 187: 1411–1420.**

Human helminth infections are characterized by immune suppression resulting in parasite survival and chronic infection (1–3). Chronically infected filarial and schistosome patients present impaired immune responses, as shown by reduced production of IL-5 and IFN-γ, and occasionally IL-4, in response to parasite Ags (4–7). Although some elements of immune suppression are reversible upon drug-mediated clearance (6, 8), drug treatments do not induce immune protection against parasite challenge, and individuals rapidly become reinfected. Therefore, strategies to induce long-term protective immunity are needed that can counteract infection-induced immune regulation.

The glucocorticoid-induced TNFR-related protein (GITR) is a costimulatory receptor and a member of the TNF receptor superfamily (9–11). It is constitutively expressed at high levels on Foxp3$^+$ regulatory T cells (Tregs) and at low levels on naive CD4$^+$ T cells; expression increases in both cell populations upon activation (12). The GITR ligand (GITRL) is highly expressed on activated plasmacytoid dendritic cells (13, 14) and macrophages (15). Costimulation through GITR augments CD4$^+$ effector T cell (Teff) responses (12, 16) and, by enhancing CD4$^+$ Teff activation can abrogate Foxp3$^+$ Treg-mediated suppression (14, 17). Therefore, GITR costimulation provides a potential strategy for counteracting immune regulation and boosting immune responses.

We and other investigators demonstrated that agonistic anti-GITR mAb treatment can enhance immunity to helminth parasites (18, 19), and similar results were found with other infections (20–22) and tumors (23, 24). As such, there is growing interest in using GITR costimulation as a novel vaccine adjuvant (25–28). In contrast, GITR ligation can exacerbate pathology during bacterial or protozoan infections (29, 30), suggesting that blocking GITR–GITRL interactions could be used to alleviate pathology. Most studies have focused on the effects of agonistic anti-GITR costimulation, yet these superphysiological effects of GITR ligation might be quite different from natural ligation through GITRL. Few studies have shown a physiologic role for GITR–GITRL interactions during immune challenge, all of which are Th1 related (13, 31–33). Thus, the physiological roles of GITR are not well defined, particularly in regard to Th2 responses.

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Abbreviations used in this article: GITR, glucocorticoid-induced TNFR-related protein; GITRL, GITR ligand; LsAg, *Litomosoides sigmodontis* Ag; Mf, microfilariae; MLN, mesenteric lymph node; p.i., postinfection; Teff, effector T cell; tLN, thoracic lymph node; Treg, regulatory T cell.

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Infection of resistant (C57BL/6) and susceptible (BALB/c) mice with the filarial nematode *Litomosoides sigmodontis* provides a unique model to dissect out the mechanisms of immune regulation in Th2-mediated resistance to helminths (34). In a complementary system, infection of mice with the trematode *Schistosoma mansoni* allows the study of pathogenic Th2 responses toward the highly immunogenic Th2-driving eggs that become trapped in tissue microvasculature, such as the liver, resulting in granuloma formation (35). We demonstrated that *L. sigmodontis* infection induces an early Foxp3$^+$ Treg response that, in susceptible mice, inhibits protective immunity and promotes parasite survival (19, 36, 37). Resistant C57BL/6 mice show equivalent early Foxp3$^+$ Treg responses; however, in contrast, they simultaneously present a more active CD4$^+$Foxp3$^-$ Teff response with upregulation of GITR. This raises an interesting question about what role GITR plays in priming and maintaining Th2 effector responses and/or in their resistance to Foxp3$^+$ Treg-mediated suppression, the balance of which may influence protective immunity during filarial infection.

Although susceptible BALB/c mice develop a downregulated immune phenotype leading to a fully developed patent infection 55–60 d postinfection (p.i.) (19, 36, 37), defined by the presence of transmission stage microfilariae (Mf) circulating within the blood (34), they eventually overcome immune regulation, resulting in late-phase killing and clearance of *L. sigmodontis* after 70 d (34, 38, 39). GITR expression on BALB/c CD4$^+$Foxp3$^-$ T cells increases during the later stages of infection (19, 36), and superphysiological GITR costimulation can be used therapeutically alongside depleting CD4$^+$CD25$^+$Foxp3$^+$ Tregs to enhance parasite killing (19). Thus, GITR costimulation may also be involved in the reversal of immune regulation and late-phase parasite killing following patency.

We tested the superphysiological and physiological roles of the GITR–GITRL pathway in the development and maintenance of protective Th2 responses during filarial infection and pathogenic Th2 responses during *S. mansoni* infection. Providing superphysiological GITR costimulation during immune priming using an agonistic anti-GITR mAb increased Th2 responses toward *L. sigmodontis* in susceptible BALB/c mice. However, these augmented Th2 responses were short-lived and did not result in a long-term enhancement of protective immunity. Blocking physiological GITR–GITRL interactions with a neutralizing anti-GITRL mAb during *L. sigmodontis* infection did not inhibit Th2 priming in resistant C57BL/6 mice or the late-phase parasite clearance in susceptible BALB/c mice. Although GITRL blockade during the egg-deposition stage of *S. mansoni* infection had a partial effect on the expansion of splenic IL-4–producing Th2 cells, it had no significant impact on liver granuloma formation. Thus, although superphysiological GITR costimulation enhanced Th2 responses during filarial infection, physiological GITR–GITRL interactions were neither a prerequisite for resistance nor a requirement for the induction or maintenance of Th2 responses. Similarly, GITR–GITRL interactions were not required for the development of Th2-mediated immunopathology during *S. mansoni* infection.

Materials and Methods

Animals, infections, Ag, and immunizations

Female BALB/c, C57BL/6, and BALB/c 4get IL-4gfp reporter mice (courtesy of Markus Mohrs, Trudeau Institute, Saranac Lake, NY) (40) were bred in-house and maintained under specific pathogen-free conditions at the University of Edinburgh. Mice were used at 6–12 wk of age, and all animal work was conducted in accordance with the Animals (Scientific Procedures) Act 1986. The *L. sigmodontis* life cycle was maintained in gerbils using the mite vector *Ornithonyssus bacoti* (41). Mice were infected s.c. on the upper back with 25 *L. sigmodontis* L3 larvae. Adult parasites were recovered by lavage. *L. sigmodontis* Ag (LsAg) was prepared by collecting the PBS-soluble fraction of homogenized adult male and female worms. *Biomphalaria glabrata* snails infected with *S. mansoni* were obtained from F. Lewis (Biomedical Research Institute, Rockville, MD). Experimental mice were infected percutaneously with ~30 *S. mansoni* cercariae. *S. mansoni* eggs were quantified following liver digestion in 4% potassium hydroxide buffer/g liver tissue at 37°C. To measure liver granulomas, paraffin-embedded liver sections were stained with Masson's trichrome, and the area of each granuloma containing a single egg was calculated using a compound microscope (LEICA) and LEICA analysis software. From this, the average granuloma size for each individual mouse was calculated. *S. mansoni* eggs were isolated from C57BL/6 mouse livers and stored at −80°C. *S. mansoni* soluble egg Ag was prepared in-house, as previously described (42). Mice were immunized s.c. in the footpad with 1500 *S. mansoni* eggs.

In vivo Ab and BrdU treatments

Mice received i.p. injections of 1 mg agonistic anti-GITR mAb (DTA-1; in-house) (17) on days 0 and 7 of *L. sigmodontis* infection. To block GITRL, mice were given i.p. injections with 200 μg anti-GITRL mAb (MIH44; in-house) (43) every 3 d. Treatment started on day 0 or 59 of *L. sigmodontis* infection, day 0 of *S. mansoni* egg injection, and day 36 following *S. mansoni* infection. An equivalent dose of rat IgG (Sigma-Aldrich) was used as control. For BrdU treatment, mice received an i.p. injection of 1 mg BrdU (Sigma-Aldrich) 24 h prior to autopsy.

Cell purifications and in vitro restimulations

The parathymic, posterior, mediastinal, and paravertebral lymph nodes were taken as a source of thoracic lymph nodes (tLNs) draining the pleural cavity. Pleural cavity cells were recovered by lavage. Mesenteric lymph nodes (MLNs), tLNs, and spleen cells were dissociated and washed in RPMI 1640 (Invitrogen) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and either 0.5% mouse sera (Caltag-Medsystems) for *L. sigmodontis* studies or 5% FCS for *S. mansoni* studies. To purify GFP$^-$CD4$^+$ T cells from IL-4gfp mice, tLN cells were enriched for CD4$^+$ T cells by negative selection using anti-CD8 (53-6.72), anti-B220 (RAB632), anti-MHC class II (M5/114.15.2), anti-Gr1 (RB6-8C5), and anti-F4/80 (A3-1), followed by sheep anti-rat IgG Dynal Beads (Invitrogen). Cells were stained with allophycocyanin-conjugated anti-CD4 (RM4-5), and GFP$^-$CD4$^+$ T cells were purified using a FACSAria flow sorter (Becton-Dickinson). To isolate liver mononuclear cells, the liver was diced and digested at 37°C with 250 μg/ml collagenase D and 10 μg/ml DNase in Hanks' medium supplemented with 50 U/ml penicillin-50 μg/ml streptomycin. To stop the enzymatic digestion, 100 μl 0.1 M EDTA (pH 7.3) was added. The digest was passed through a 70-μm cell strainer (BD Biosciences) to obtain a single-cell suspension. Leukocytes were isolated by resuspension in 33% isotonic Percoll (GE Healthcare) and centrifuged at 700 × *g*. For restimulation with LsAg (10 μg/ml) or *S. mansoni* soluble egg Ag (15 μg/ml), whole tLN cells were cultured at 5×10^5 cells/well, and spleen cells were cultured at 1×10^6 cells/well. Purified GFP$^-$CD4$^+$ T cells were cultured at 5×10^4 cells/well with 1×10^6 irradiated (30 Gy) naive splenocytes. Supernatants were harvested at 72 h. To measure intracellular cytokines, cells were stimulated for 4 h with 0.5 μg/ml PMA and 1 μg/ml ionomycin, with 10 μg/ml brefeldin A added for the final 2 h (all from Sigma-Aldrich).

Flow cytometry

The following Abs were used: Alexa Fluor 700-conjugated anti-CD4 (RM4-5), polyclonal anti-GFP (eBioscience), Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen), PE-conjugated anti–IL-4 (11B11), Alexa Fluor 647-conjugated anti–IL-13 (ebio13A; eBioscience), allophycocyanin-conjugated anti–IL-5 (TRFK5), E450-conjugated anti–IFN-γ (XMG1.2; eBioscience), FITC-conjugated anti-GITR (DTA-1; in-house), FITC-conjugated anti-BrdU with DNase (B44), and allophycocyanin-conjugated anti-Foxp3 (FJK-16s; eBioscience). Nonspecific binding was blocked with 4 μg rat IgG/1×10^6 cells. Intracellular staining for Foxp3 was performed with a Foxp3-staining buffer kit (eBioscience). For intracellular cytokine staining, dead cells were excluded using Aqua Dead Cell Stain Kit (Molecular Probes), and the cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit. Flow cytometric acquisition was performed on a FACSCanto II or LSR II (BD Biosciences), and data were analyzed using FlowJo Software (Tree Star). Reagents were obtained from BD Biosciences unless otherwise stated.

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Cytokine and Ab ELISA

Ab pairs used for cytokine ELISA were IL-4 (11B11/BVD6-24G2), IL-5 (TRFK5/TRFK4), IL-10 (JES5-2A5/SXC-1), IFN-γ (R4-6A2/XMG1.2), capture anti–IL-13 (38213; R&D Systems), and biotinylated polyclonal anti–IL-13 (PeproTech). Recombinant murine IL-4, IL-5, IL-10, IFN-γ (Sigma-Aldrich), and IL-13 (R&D Systems) were used as standards. Biotin-detection Abs were used with ExtrAvidin-alkaline phosphatase conjugate and SIGMA*FAST p*-nitrophenyl phosphate substrate (both from Sigma-Aldrich). To measure *L. sigmodontis*-specific Abs, ELISA plates (NUNC) were coated with 5 μg/ml LsAg diluted in 0.45 M $NaHCO_3$/0.18 M Na_2CO_3 (Sigma-Aldrich). Plates were incubated with serial dilutions of serum, and a representative dilution from the linear section of the dilution curve was selected for each isotype. Detection of Ab isotypes was performed using HRP-conjugated anti-mouse IgG1, IgG2a, or IgM (Southern Biotechnology Associates) and an 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) peroxidase substrate system (KPL).

Statistics

Statistical analysis was performed using JMP version 8. The data were first checked for homogeneity of variance and normality. If the raw data failed to meet these requirements for parametric analysis, log10 transformations were applied. Parametric analysis of combined data from multiple repeat experiments or experiments containing more than two groups was performed using ANOVA, followed by the Tukey post hoc test. When using two-way ANOVA to combine data from multiple experiments, it was verified that there were no significant qualitative interactions between experimental and treatment effects. For nonparametric data, the unpaired Mann–Whitney *U* test was used. Figures depict means when parametric tests were used and medians when nonparametric tests were used.

Results

Th2 responses are retarded during the initial stages of L. sigmodontis *infection*

Th2 responses are critical for resistance toward *L. sigmodontis* (44, 45). However, it has been difficult to accurately quantify Th2 responses in the early stages of infection. To track Th2 cell development during *L. sigmodontis* infection, susceptible BALB/c 4get IL-4gfp reporter mice (46) were infected, and the percentage of $CD4^+$IL-4gfp$^+$ Th2 cells in the pleural cavity (infection site) and tLN was quantified over the course of infection. The development of Th2 responses during the early infection stages was slow, with no initial increase over naives in the proportion of IL-4gfp$^+$ Th2 cells in the pleural cavity at day 6 p.i. and only a weak, albeit significant, 2-fold increase in their proportion at day 12 p.i. (Fig. 1*A*). The weak nature of the day-12 increase was notable because it did not reach significance within every individual experiment, although it was significant when meta-analysis was used to combine data from multiple experiments. Not until the later stages of infection did the proportion of IL-4gfp$^+$ Th2 cells gradually increase, with a 6-fold expansion by day 40 p.i., when adult parasites have developed, and a 17-fold increase after the onset of patency at day 60 p.i. (Fig. 1*A*). A similar pattern was found within the tLN, with a modest initial expansion followed by a gradual increase to reach a 4-fold upregulation at day 60 p.i. (Fig. 1*B*). Thus, the initial Th2 response to *L. sigmodontis* is remarkably slow to emerge, becoming robust only during the later infection stages.

Therapeutic administration of anti-GITR during immune priming enhances the magnitude of the Th2 response

We previously showed that resistance to *L. sigmodontis* infection in C57BL/6 mice is associated with increased expression of GITR on $CD4^+$Foxp3$^-$ Teffs during the early infection stages, whereas susceptible BALB/c mice fail to upregulate GITR and show reduced $CD4^+$Foxp3$^-$ Teff proliferation (36). To test the hypothesis that providing superphysiological GITR costimulation could be used to boost the initial Th2 responses and reverse susceptibility,

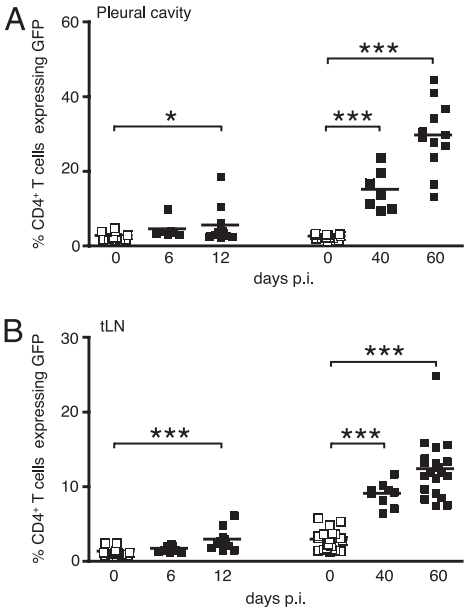

FIGURE 1. Kinetics of Th2 cell induction following *L. sigmodontis* infection. IL-4gfp reporter mice were infected with *L. sigmodontis* (filled symbols) or left uninfected (open symbols), and the proportion of IL-4gfp$^+$ Th2 cells in the pleural cavity (*A*) and tLN (*B*) was quantified. Symbols represent individual mice. Lines represent means, and data are from pooled experiments. $*p < 0.05$, $***p < 0.001$, one-way ANOVA, followed by Tukey's post hoc test based on combined data from at least two experiments per time point.

we infected BALB/c IL-4gfp reporter mice with *L. sigmodontis* and treated them with agonistic anti-GITR mAb at days 0 and 7 p.i. Treatment resulted in a 60% increase in the frequency of IL-4gfp$^+$ Th2 cells in the pleural cavity at 12 d p.i. (Fig. 2*A*). This increment was restricted to the infection site; although anti-GITR treatment increased the percentage of IL-4gfp$^+$ Th2 cells in the tLN, this was independent of infection (Fig. 2*B*). Despite the augmented Th2 differentiation, anti-GITR did not increase the numbers of total cells or $CD4^+$ T cells in the tLN or pleural cavity (data not shown).

In 4get reporter mice, GFP$^+$ cells are those that express IL-4 mRNA and are competent to produce IL-4, but the GFP label does not necessarily mark the production of IL-4 protein (40). To confirm that anti-GITR treatment increases the production of Th2 cytokines, we assessed the ability of IL-4gfp$^+$ Th2 cells in the pleural cavity to produce IL-4, IL-5, and IL-13 by intracellular staining for protein. Although infection alone at this early time point only caused a minor nonsignificant increase in the proportion of IL-4gfp$^+$ Th2 cells producing IL-5 and IL-13 (Fig. 2*D*, 2*E*), anti-GITR treatment of infected mice significantly increased the percentage of IL-4gfp$^+$ Th2 cells producing IL-4, IL-5, and IL-13 (Fig. 2*C*–*E*). Although anti-GITR treatment did not increase the percentage of IL-4gfp$^+$ Th2 cells in the tLN in response to infection, it resulted in increased LsAg-specific IL-5 production following in vitro restimulation of tLN cells (Fig. 2*F*). IL-4 and IL-13 were not consistently detectable following in vitro restimulation, emphasizing the weak nature of the early Th2 response (data not shown). In line with Th2 induction, *L. sigmodontis* infection tends to reduce the proportion of $CD4^+$ T cells producing IFN-γ; however, in addition to its effects on Th2 immunity, anti-GITR treatment increased the proportion of pleural cavity $CD4^+$ T cells producing IFN-γ at 12 d p.i. (Fig. 2*G*).

To investigate whether anti-GITR treatment also boosts humoral immunity, we determined the levels of LsAg-specific Abs in the

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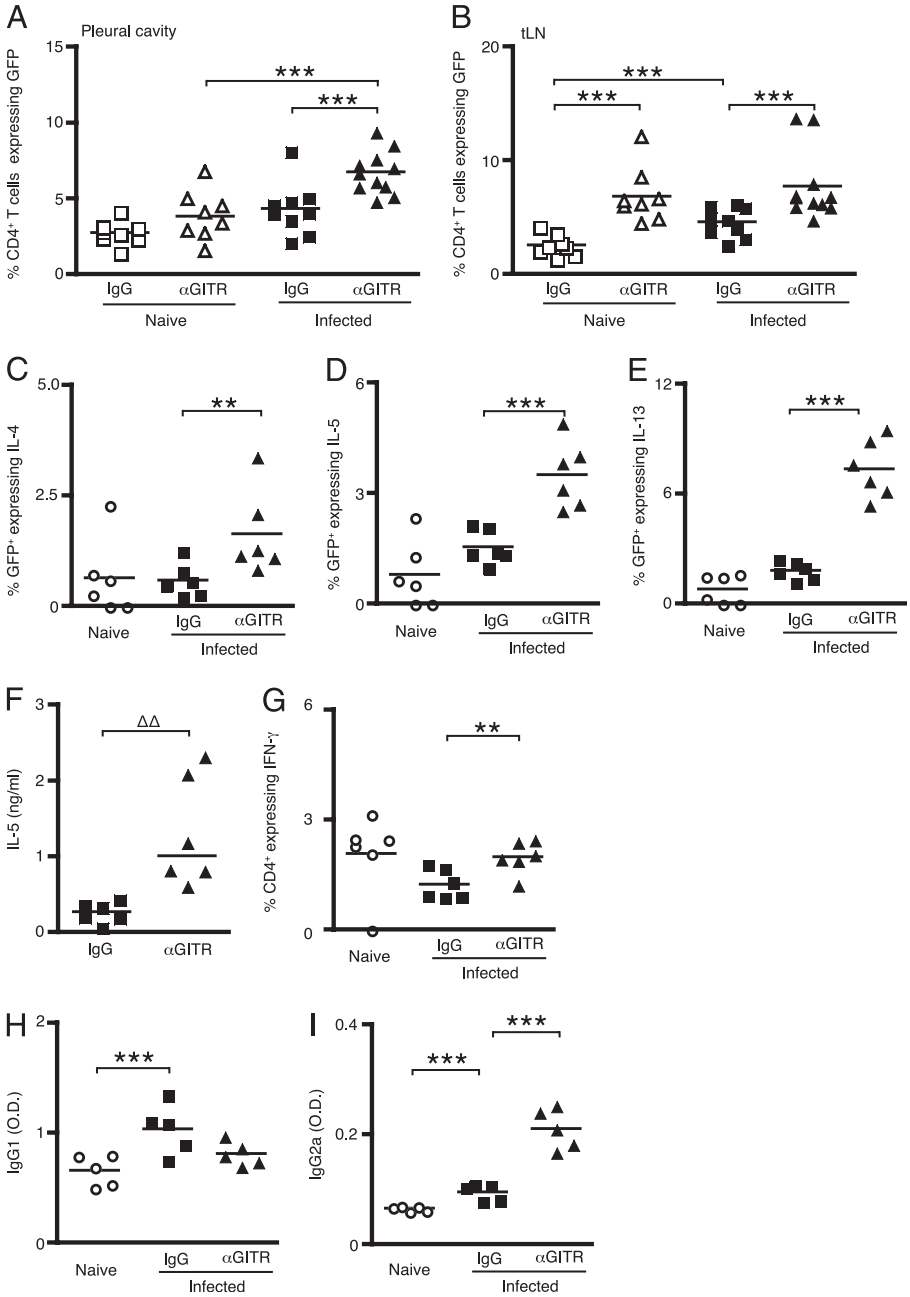

FIGURE 2. Therapeutic administration of agonistic anti-GITR mAb enhances the magnitude of the Th2 response to *L. sigmodontis*. *L. sigmodontis*-infected (filled symbols) BALB/c IL-4gfp reporter mice (*A–E*) or BALB/c mice (*F–I*) were treated with agonistic anti-GITR mAb (triangles) or rat IgG (squares) and autopsied 12 d p.i. Open circles indicate naive untreated mice. Lines show mean values unless otherwise stated, and symbols represent individual mice. The proportion of $CD4^+$ T cells expressing IL-4gfp within the pleural cavity (*A*) and tLN (*B*). Panels show combined data from two independent experiments. Proportion of pleural cavity $CD4^+GFP^+$ Th2 cells producing intracellular IL-4 (*C*), IL-5 (*D*), and IL-13 (*E*). One representative experiment out of two is shown. *F*, In vitro production of IL-5 by tLN cells following restimulation with LsAg. One of four independent experiments is shown. Lines show median values. *G*, Proportion of $CD4^+$ T cells producing IFN-γ. Serum levels of *L. sigmodontis*-specific IgG1 (*H*) and IgG2a (*I*). One representative experiment of three is shown. $^{**}p < 0.01$, $^{***}p < 0.001$, two-way ANOVA, followed by Tukey's post hoc test based on pooled data from denoted experiments; $^{\Delta\Delta}p < 0.01$, Mann–Whitney test.

serum at 12 d p.i. Despite enhancing the cellular Th2 response, anti-GITR treatment did not result in increased production of the Th2 Ab isotype IgG1 (Fig. 2*H*). However, the increased production of IFN-γ was associated with elevated levels of serum IgG2a (Fig. 2*I*). Serum IgM, IgG2b, and IgG3 were unaffected by anti-GITR treatment (data not shown).

Thus, superphysiological GITR costimulation during immune priming enhances both Th1 and Th2 responses to *L. sigmodontis*, with the increased Th2 immunity at the infection site due to both elevated numbers of IL-4gfp$^+$ Th2 cells and increased functional quality of the Th2 population, as measured by their production of Th2 cytokines.

Therapeutic administration of anti-GITR during immune priming fails to increase parasite killing and results in a long-term enhancement of Th1, but not Th2, immunity

For anti-GITR treatment to be effective as an adjuvant or therapeutic agent, it would need to induce a long-term change in protective immunity. Therefore, we asked whether agonistic anti-GITR treatment results in a permanent or temporary enhancement of Th2 responses. Susceptible BALB/c IL-4gfp$^+$ reporter mice were treated with anti-GITR on days 0 and 7 p.i. and autopsied at day 60 when Mf are circulating in the bloodstream.

By day 60 p.i., *L. sigmodontis*-infected mice have established a stronger Th2 response, with 34 ± 9% of $CD4^+$ T cells in the pleural cavity expressing IL-4gfp (Fig. 3*A*) compared with 5 ± 5% at day 12 (Fig. 1*A*). Despite the early boosting effect of anti-GITR treatment on Th2 responses, there were no differences in the proportion (Fig. 3*A*, 3*B*) or number (data not shown) of IL-4gfp$^+$ Th2 cells between anti-GITR–treated and IgG-infected mice by 60 d p.i. There were also no differences in the LsAg-specific production of IL-5 (Fig. 3*C*), IL-4, or IL-13 (data not shown) by tLN cells following in vitro restimulation. Consistent with the failure to induce a long-term increase in Th2 immunity, adult parasite burdens and Mf counts in peripheral blood remained unaffected following anti-GITR treatment (Fig. 3*D*, 3*E*).

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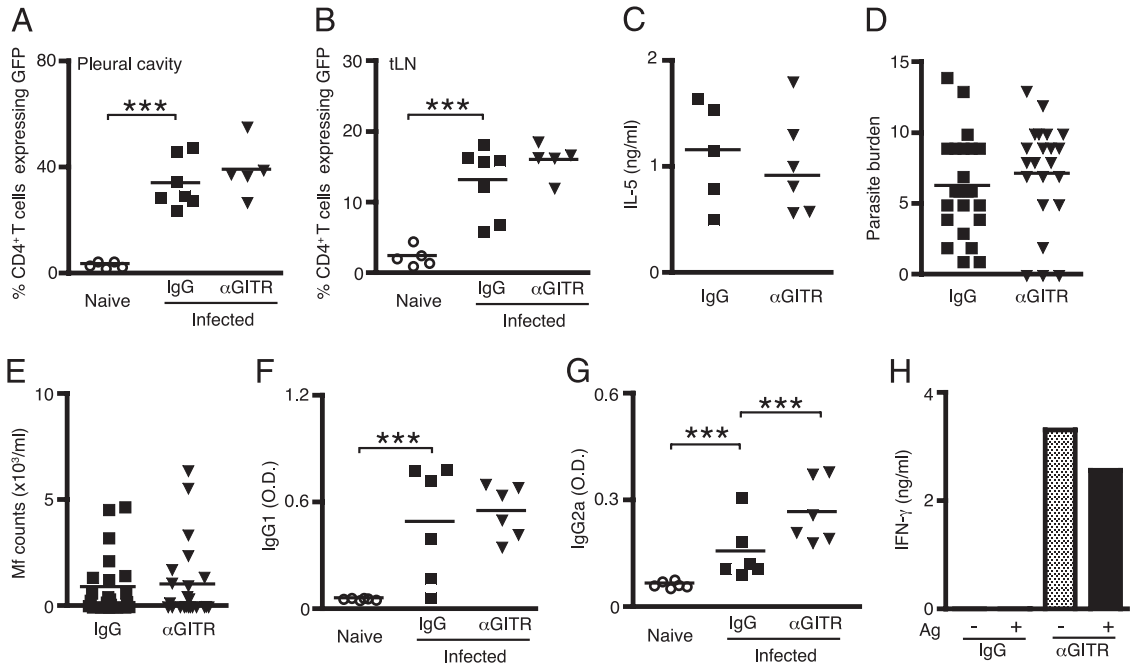

FIGURE 3. Therapeutic administration of agonistic anti-GITR during immune priming does not enhance long-term protective Th2 immunity. *L. sigmodontis*-infected BALB/c IL-4gfp reporter mice were treated with an agonistic anti-GITR mAb (▼) or rat IgG (■) on days 0 and 7 p.i. and autopsied at day 60. Open circles represent naive untreated mice. Symbols represent individual animals, and lines represent mean values, unless otherwise stated. Proportion of $CD4^+$ T cells expressing GFP in the pleural cavity (*A*) and tLN (*B*). One representative experiment of two is shown. *C*, In vitro production of IL-5 by tLN cells following restimulation with LsAg. One representative experiment of three is shown; lines represent median values. Adult parasite burden in the pleural cavity (*D*) and number of Mf/ml within the peripheral blood (*E*). Pooled data from four independent experiments are shown. Serum levels of *L. sigmodontis*-specific IgG1 (*F*) and IgG2a (*G*). One representative experiment of two is shown. *H*, In vitro IFN-γ production in response to LsAg by pooled GFP^-CD4^+ non-Th2 cells purified from the tLN of *L. sigmodontis*-infected mice treated with IgG or anti-GITR. One representative experiment of two is shown. ***$p < 0.001$, two-way ANOVA, followed by Tukey's post hoc test based on combined data from denoted experiments.

In agreement with the transitory nature of the enhanced cellular Th2 response, we did not find any long-term effect of anti-GITR treatment on *L. sigmodontis*-specific serum levels of IgG1 (Fig. 3*F*). However, *L. sigmodontis*-specific IgG2a was still elevated in the sera at day 60 p.i. (Fig. 3*G*), and IFN-γ production by purified tLN IL-4gfp$^-$ non-Th2 cells was enhanced, albeit in an Ag-nonspecific manner (Fig. 3*H*). Thus, the augmenting effects of anti-GITR treatment on Th2 priming seem to be temporary and do not result in increased parasite killing. In contrast, although *L. sigmodontis* infection mainly induced a Th2 response, anti-GITR treatment resulted in a minor, but long-term, enhancement of Th1 immunity.

GITR–GITRL interactions are not required for priming of Th2 responses to L. sigmodontis

Resistant C57BL/6 mice upregulate GITR on $CD4^+Foxp3^-$ Teffs during the early stages of *L. sigmodontis* infection, suggesting that their stronger initial Teff response and resistance may be related to GITR costimulation (36). To determine whether GITR–GITRL interactions are required for the development of Th2 responses and resistance to filarial infections, we treated *L. sigmodontis*-infected C57BL/6 mice over the first 12 d of infection with an anti-GITRL mAb that was shown to block GITRL activity in vivo (13, 24, 43).

GITRL blockade failed to reduce the proportion of $CD4^+Foxp3^-$ Teffs proliferating in vivo in response to infection, thus GITR–GITRL interactions could not explain the enhanced Teff response of resistant C57BL/6 mice (Fig. 4*A*). Similarly, GITR–GITRL interactions were not required for the induction of Th2 responses, because the production of LsAg-specific IL-5 and IL-13 by tLN cells was unaffected (Fig. 4*B*, 4*C*). GITRL blockade also failed to impact on the resistant phenotype, with both control and anti-GITRL–treated mice showing similar parasite recoveries at day 47 p.i. (data not shown). These data suggest that although super-physiological GITR costimulation can enhance Th2 responses during priming, physiological GITR–GITRL interactions are not required for Th2 priming following *L. sigmodontis* infection.

GITR–GITRL interactions are not required for late-phase killing of L. sigmodontis

Although patency is associated with immune downregulation (19, 36, 37), BALB/c mice show a late-phase killing response and clear infection after 70 d (34, 38, 39). We found that expression of GITR on Foxp3$^-$ Teffs at the infection site increases as infection progresses and that treating infected mice with agonistic anti-GITR Ab during established infection can promote protective immunity (19, 36). These results raise the hypothesis that late-phase killing is due to increasing levels of GITR costimulation, naturally boosting Th2 cell responses and leading to a reversal in immune regulation.

To investigate this possibility, *L. sigmodontis*-infected susceptible BALB/c mice, either wild-type or IL-4gfp reporter mice, were treated with a blocking anti-GITRL mAb from days 60–80 p.i. Despite high levels of GITR expression (19, 37), blockade of GITRL was not found to impact on parasite burden or Mf levels at 82 d p.i. (Fig. 5*A*, 5*B*). Accordingly, no differences were seen in the total number of IL-4gfp$^+$ Th2 cells in the pleural cavity (Fig. 5*C*) or the tLN (Fig. 5*D*) or in the proportion of pleural cavity IL-4gfp$^+$ Th2 cells producing IL-4 and IL-5 protein (data not shown). In vitro LsAg-specific production of IL-5 (Fig. 5*E*) and IL-4 (data not shown) by tLN cells also remained unaltered. Thus, GITR–

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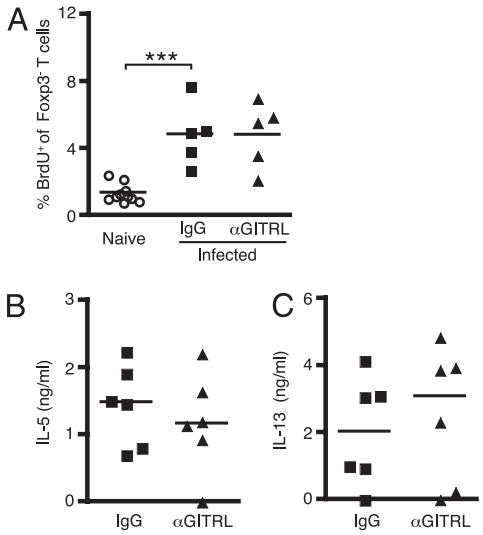

FIGURE 4. GITRL blockade during immune priming does not affect Th2 development. *L. sigmodontis*-infected C57BL/6 mice were treated during immune priming with a blocking anti-GITRL mAb (▲) or rat IgG (■) over the first 12 d of infection. Open circles represent naive untreated controls. *A*, Proportion of Foxp3$^-$CD4$^+$ T cells that incorporated BrdU. One representative experiment of three is shown. In vitro production of IL-5 (*B*) and IL-13 (*C*) by tLN cells following restimulation with LsAg. One representative experiment of three is shown. Symbols denote individual mice, and lines show mean (*A*) or median (*B*, *C*) values. ***$p < 0.001$, two-way ANOVA, followed by Tukey's post hoc tests based on combined data from three experiments.

GITRL interactions are not required for Th2 maintenance or late-phase killing during *L. sigmodontis* infection.

GITR–GITRL interactions are partially required for Th1 and Th2 responses during S. mansoni *infection*

Infection with *S. mansoni* results in immunopathology when eggs become trapped in the liver microvasculature, leading to immune inflammation and granuloma formation (35). We found that expression of GITR was strongly upregulated on CD4$^+$Foxp3$^-$ Teffs in the spleen and liver at the peak of egg-induced inflammation (week 8 p.i.) (Fig. 6*A*, 6*B*), suggesting that GITR signaling is potentiating the inflammatory Th2 response. Therefore, we tested whether blocking GITR–GITRL interactions can be used to moderate Th2-mediated inflammation and granuloma formation during *S. mansoni* infection.

Because early infection with *L. sigmodontis* larvae induces a relatively weak Th2 response, and increases in IL-4 protein are difficult to detect, we injected *S. mansoni* eggs s.c. to confirm the role of GITR–GITRL interactions in the context of a stronger Th2 stimulus. In this setting, 11% of CD4$^+$ T cells expressed IL-4gfp 12 d p.i.; however, GITRL blockade still failed to affect Th2 cell priming because the total number of IL-4gfp$^+$ Th2 cells in the draining popliteal lymph node (Fig. 6*C*), as well as the total number of IL-4gfp$^+$ Th2 cells producing IL-4 (data not shown), remained unaltered.

To investigate whether GITR–GITRL interactions promote egg-induced Th2 responses during *S. mansoni* infection, BALB/c IL-4gfp reporter mice were treated with blocking anti-GITRL mAb from the stage of initial egg deposition (week 5 p.i.) until the time of peak inflammation (week 8 p.i.). Blockade of GITRL resulted in a significant 43% reduction in the number of IL-4gfp$^+$ Th2 cells expressing IL-4 protein in the spleen (Fig. 6*D*). Despite the reduced number of IL-4gfp$^+$ Th2 cells secreting IL-4 protein in the spleen, there was no effect on *S. mansoni* egg Ag-specific splenic production of IL-4, IL-5, IL-10, or IL-13 following in vitro restimulation (data not shown). Consistent with a partial reduction in Th2 responses, GITRL blockade had no effect on the proportion of IL-4gfp$^+$ Th2 cells secreting IL-4 protein in the liver (Fig. 6*E*) and no effect on the size of egg-induced liver granulomas (Fig. 6*F*). In addition to a diminished Th2 response, there was a small, but consistently significant, reduction in the proportion of IFN-γ–producing CD4$^+$ T cells in the spleen (Fig. 6*G*) and liver (Fig. 6*H*). Thus, in contrast to *L. sigmodontis* infection, blockade of GITR–GITRL interactions partially reduced both Th2 and Th1 responses toward *S. mansoni*.

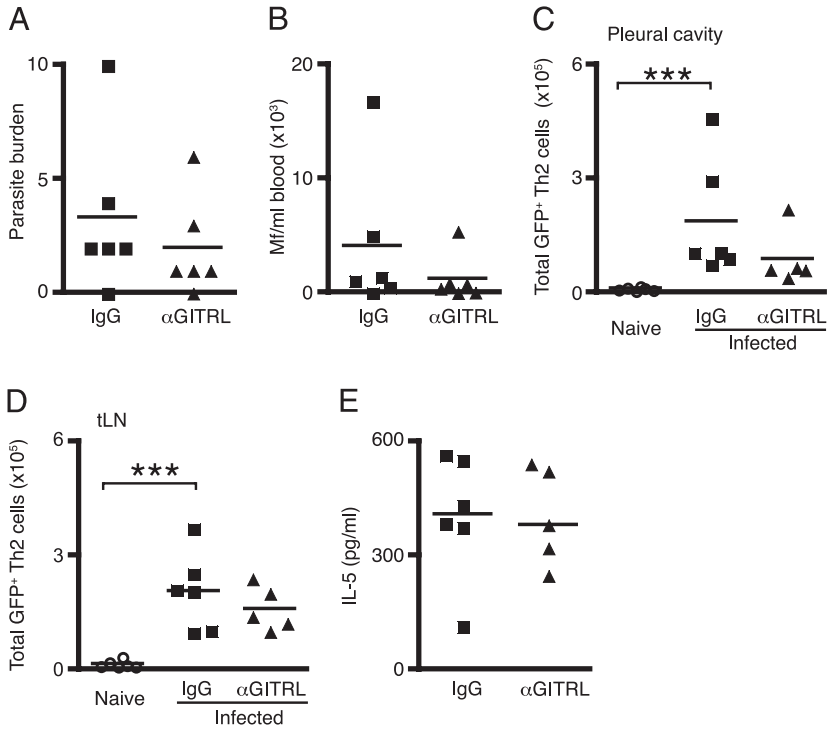

FIGURE 5. GITR–GITRL interactions are not required for late-phase killing in susceptible BALB/c mice. *L. sigmodontis*-infected BALB/c WT or BALB/c IL-4gfp reporter mice were treated with a blocking anti-GITRL mAb (▲) or rat IgG (■) from days 60–80 p.i., and mice were autopsied on day 82. Circles represent naive untreated mice, and symbols represent individual mice. Adult parasite burden in the pleural cavity (*A*) and Mf/ml in peripheral blood (*B*). One representative experiment of three is shown. Lines show mean values. The number of CD4$^+$GFP$^+$ Th2 cells in the pleural cavity (*C*) and tLN (*D*). One representative experiment of two is shown; lines denote mean. *E*, In vitro production of IL-5 by tLN cells following restimulation with LsAg. One representative experiment of three is show; lines represent median values. ***$p < 0.0001$, two-way ANOVA, followed by Tukey's post hoc test based on combined data from three experiments.

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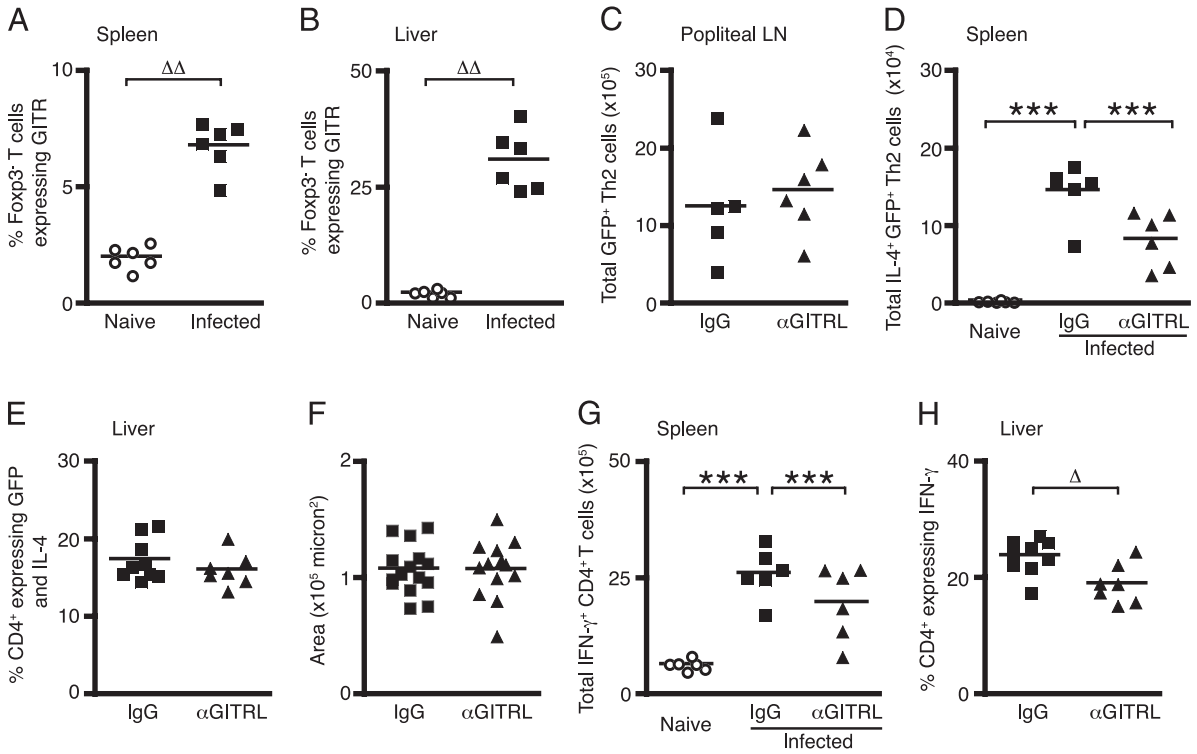

FIGURE 6. GITRL blockade impairs Th2 and Th1 responses toward *S. mansoni* eggs. Percentage of CD4$^+$Foxp3$^-$ T cells expressing GITR in the spleen (*A*) and liver (*B*) of *S. mansoni*-infected (filled symbols) or naive (open symbols) BALB/c mice 8 wk p.i. One representative experiment of two is shown. *C*, BALB/c IL-4gfp mice were injected s.c. in the footpad with *S. mansoni* eggs and treated with anti-GITRL mAb (▲) or rat IgG (■) for 12 d. The numbers of IL-4gfp$^+$ Th2 cells in the popliteal lymph node were quantified at day 12. One representative experiment of two is shown. *D–H*, *S. mansoni*-infected IL-4gfp mice were treated with anti-GITRL mAb (▲) or rat IgG (■) from wk 5 to autopsy at wk 8. Open symbols represent naive untreated controls. One representative experiment of two is shown. *D*, Total number of splenic CD4$^+$IL-4gfp$^+$ Th2 cells producing IL-4 protein. *E*, Percentage of liver CD4$^+$ T cells producing IL-4 protein. *F*, Mean area of liver granulomas. *G*, Total number of splenic CD4$^+$ T cells producing IFN-γ. *H*, Percentage of liver CD4$^+$ T cells producing IFN-γ. Symbols represent individual animals, and lines represent means. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, Mann–Whitney test; $^{***}p < 0.001$, two-way ANOVA, followed by Tukey's post hoc test based on combined data from two experiments.

GITR–GITRL interactions are not required for Foxp3$^+$ Treg expansion following helminth infection

L. sigmodontis infection results in the rapid expansion of GITR$^+$ Foxp3$^+$ Tregs at the infection site in both susceptible and resistant mice (36). There are conflicting reports that GITR costimulation promotes Foxp3$^+$ Treg expansion (13, 18, 32, 43, 47); therefore, to test whether GITR–GITRL interactions are responsible for the initial Foxp3$^+$ Treg expansion, GITRL was blocked over the first 12 d of *L. sigmodontis* infection in resistant C57BL/6 mice. As previously shown, there was an increased percentage of CD4$^+$ T cells expressing Foxp3 in the pleural cavity of *L. sigmodontis*-infected mice, but this was unaffected by blockade of GITRL (Fig. 7*A*). In the converse experiment, treating susceptible BALB/c mice with agonistic anti-GITR mAb on days 0 and 7 p.i. also failed to affect the percentage of CD4$^+$ T cells expressing Foxp3 (Fig. 7*B*). Similarly, blockade of GITRL during the acute egg phase of *S. mansoni* infection (weeks 5–8 p.i.) had no effect on Foxp3$^+$ Treg expansion in the spleen (Fig. 7*C*) or liver (Fig. 7*D*). Thus, GITR–GITRL interactions do not play a major role in the expansion of Foxp3$^+$ Tregs during *L. sigmodontis* or *S. mansoni* infections.

Discussion

Costimulation of GITR through agonistic mAb is known to augment Teff responses (10, 16), overcome Foxp3$^+$ Treg–mediated suppression (14, 17), and boost resistance to infection with helminths, protozoa, and viruses (18–21, 48), demonstrating the potential for targeting GITR as a therapeutic adjuvant. In contrast to this superphysiological GITR costimulation, understanding of the physiological role of GITR is limited, particularly in Th2 settings. In the current study, we investigated both the superphysiological and physiological role of the GITR–GITRL pathway in the development and maintenance of protective and pathogenic Th2 and Foxp3$^+$CD4$^+$ Treg responses in murine models of filariasis and schistosomiasis. Providing superphysiological GITR costimulation using agonistic anti-GITR mAb during immune priming initially augmented Th2 responses toward the filarial nematode *L. sigmodontis*. However, this effect was temporary, with the Th2 response reverting to normal infection levels by the onset of patency (day 60 p.i.), and it failed to enhance parasite killing. Despite the Th2-enhancing effect of agonistic anti-GITR treatment, GITRL blockade failed to affect Th2 cell priming and only showed a partial effect on Th2 cell maintenance during *S. mansoni* infection. Thus, although superphysiological GITR costimulation can enhance Th2 responses, GITR–GITRL interactions are not essential for the priming or maintenance of Th2 responses under physiological conditions.

Filarial parasites have been shown to rapidly suppress host immunity (36, 49); in agreement with this, we found that the expansion of IL-4gfp$^+$ Th2 cells was relatively weak in the pleural cavity (infection site) and draining lymph nodes of susceptible BALB/c mice over the first 12 d of infection. This contrasts with the rapid expansion of CD4$^+$Foxp3$^+$ Tregs seen by day 7 p.i. in filarial infections (36, 50). Consistent with published work (19, 51), the IL-4gfp$^+$ Th2 cells increased during the later stages of infection. Agonistic anti-GITR treatment given over the first 12 d of infection increased the quantity of IL-4gfp$^+$ Th2 cells at

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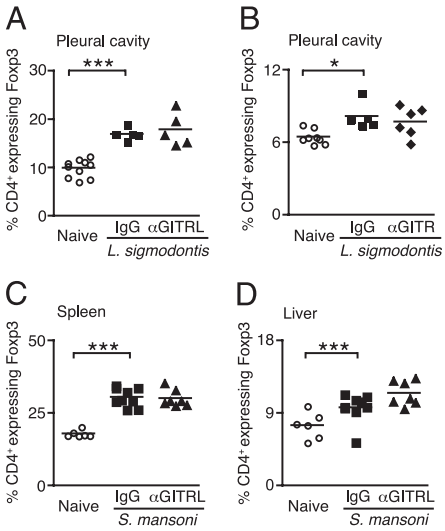

FIGURE 7. GITR–GITRL interactions are not required for expansion of Foxp3$^+$ Tregs during helminth infection. Percentage of CD4$^+$ T cells expressing Foxp3 from the pleural cavity of resistant C57BL/6 (*A*) and susceptible BALB/c (*B*) mice infected with *L. sigmodontis* and treated with a blocking anti-GITRL mAb (▲) or agonistic anti-GITR mAb (♦), respectively, over the first 12 d of infection. Naive mice are represented by circles, and IgG-treated mice are represented by squares. Percentage of CD4$^+$ T cells expressing Foxp3 in the spleen (*C*) and liver (*D*) of *S. mansoni*-infected BALB/c IL-4gfp reporter mice treated with a blocking anti-GITRL mAb (▲) or rat IgG (■) from week 5 p.i. to autopsy at week 8. Open symbols represent naive controls. One representative experiment shown of at least two for all panels. Symbols represent individual mice, and lines represent mean values. $*p < 0.05$, $***p < 0.001$, two-way ANOVA, followed by Tukey's post hoc test based on combined data from at least two experiments.

the infection site, as well as their functional quality, as represented by an increased ability of the IL-4gfp$^+$ Th2 cells to secrete Th2 cytokines. Thus, GITR costimulation can be used therapeutically to boost both the quantity and quality of Th2 responses toward infection; however, this was not sufficient to enhance killing of *L. sigmodontis*.

The potential for using superphysiological GITR costimulation as a therapeutic adjuvant has been successfully applied in a variety of tumor (52–54) and infection settings (18, 20). In these contexts, the protective effects were seen within or shortly after the duration of anti-GITR treatment, and only one study has addressed the long-term effects of superphysiological GITR costimulation (25). Immune therapies that successfully improve protection toward *L. sigmodontis* often result in relatively slow killing, taking 1 or 2 mo for the effects on parasite survival to become detectable, and are associated with a long-term enhancement of Th2 immunity (19, 36, 37). Our data with *L. sigmodontis* indicated that the Th2-boosting effects of agonistic anti-GITR treatment are temporary. Thus, the lack of efficacy of superphysiological GITR costimulation on *L. sigmodontis* infection is likely related to the temporary nature of its Th2-promoting effect, suggesting that filarial parasites are relatively resistant to short-term enhancement of Th2 responses. This indicates that a successful therapy needs to be able to induce a long-term change in the protective Th2 response.

Agonistic anti-GITR treatment has been used as an adjuvant to induce long-term Ab responses toward viral Ags (25), raising a question about why the enhancing effect on Th2 immunity toward *L. sigmodontis* was temporary. Filarial parasites induce a variety of regulatory mechanisms to inhibit host immunity (1–3), in particular inducing strong Foxp3$^+$ Treg responses (19, 36, 37, 50, 55, 56). One possibility is that after anti-GITR treatment is discontinued, parasite-induced immune suppression reasserts itself, countering the enhanced Th2 response. In support of this, we found that during established *L. sigmodontis* infection, the efficacy of superphysiological GITR costimulation can be enhanced by simultaneously depleting CD25$^+$Foxp3$^+$ Tregs (19). Alternatively, anti-GITR treatment may have differential effects on Th2 versus Th1 responses. Despite the mainly Th2 nature of *L. sigmodontis* infection, agonistic anti-GITR treatment resulted in a long-term elevation (up to d 60 p.i.) of Th1-associated *L. sigmodontis*-specific IgG2a, contrasting with the temporary Th2 enhancement. Thus, although GITR costimulation is capable of promoting both Th1 and Th2 responses (57), it may have a more dominant effect on Th1 immunity.

Under physiological conditions, the ablation of GITR–GITRL signaling is known to impair the generation of Th1 responses in a variety of settings (13, 21, 31–33), but its requirement for Th2 responses is unknown. Increased expression of GITR is associated with Th2 responses and resistance to *L. sigmodontis* both during immune priming in resistant C57BL/6 mice and in late-phase killing in susceptible BALB/c mice (19, 37). Because GITR costimulation is associated with the enhancement of immunity and abrogation of Treg responses (14, 17, 28), this implied that GITR costimulation might be an important factor in the generation of Th2 responses and natural resistance against *L. sigmodontis*. Although superphysiological GITR costimulation is capable of promoting Th2 responses toward *L. sigmodontis*, blockade of GITR–GITRL interactions using a neutralizing anti-GITRL mAb did not inhibit the initiation of Th2 responses toward *L. sigmodontis* in resistant C57BL/6 mice. Similarly, GITR–GITRL interactions were not required for the maintenance of Th2 responses or late-phase killing in susceptible BALB/c mice. Thus, despite its therapeutic potential, under physiological conditions GITR–GITRL interactions do not seem to be required for protective immunity toward *L. sigmodontis*.

In contrast with *L. sigmodontis* infection, GITR–GITRL interactions were found to play a partial role in the development of both Th2 and Th1 responses against challenge with *S. mansoni*, with reduced numbers of IL-4–secreting IL-4gfp$^+$ Th2 cells and IFN-γ–secreting CD4$^+$ T cells in the spleen of *S. mansoni*-infected mice following GITRL blockade. This seemed to be related more to maintenance rather than to priming of Th2 cells, because GITR–GITRL blockade did not impact upon Th2 responses following s.c. injection of *S. mansoni* eggs. The effect of the antagonistic anti-GITRL mAb was relatively minor and did not impact on Th2 cells within the liver or on the formation of Th2-induced egg granulomas, indicating that GITR–GITRL signaling is not a prominent determinant of *S. mansoni*-induced pathology. Thus, in contrast to their reported roles in the development of Th1 responses (13, 21, 31–33), GITR–GITRL interactions seem to be largely redundant for the initiation and maintenance of Th2 immunity toward parasitic helminths.

In addition to costimulation of CD4$^+$ Teffs, GITR stimulation was shown to expand Tregs in vitro (14, 43, 47, 58), although in vivo studies showed conflicting results regarding Foxp3$^+$ Treg requirements for GITR–GITRL interactions (14, 18, 23, 47, 59, 60). Although infection with *L. sigmodontis* results in the rapid expansion of CD4$^+$Foxp3$^+$GITR$^+$ Tregs at the infection site (36), agonistic anti-GITR treatment or blocking GITR–GITRL interactions had no impact upon CD4$^+$Foxp3$^+$ Treg expansion. Similarly GITR–GITRL interactions were not required for expansion of Foxp3$^+$ Tregs during the acute egg phase of *S. mansoni* infection. Thus, GITR–GITRL interactions are not required for expansion of Foxp3$^+$ Tregs during either *L. sigmodontis* or *S. mansoni* infections.

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In summary, this work indicates that superphysiological GITR costimulation can be used to therapeutically enhance the quantity and quality of Th2 responses during helminth infection. However, the temporary nature of its boosting effect is a potential limit to its efficacy, and more work is needed to identify optimal treatment regimens to promote long-term changes in immunity. Although agonistic anti-GITR treatment enhanced Th2 responses, physiological GITR–GITRL interactions were not fundamentally required for the priming or maintenance of Th2 responses toward either *L. sigmodontis* or *S. mansoni*. Thus, the potent immune stimulatory properties that have been reported for superphysiological GITR costimulation may not accurately reflect its actual physiological roles.

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Disclosures

The authors have no financial conflicts of interest.

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