Th2 responses to helminth parasites can be therapeutically enhanced by, but are not dependent upon, GITR-GITRL co-stimulation *in vivo*[1]

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Abstract

The immune suppression that characterizes human helminth infections can hinder the development of protective immunity or help reduce pathogenic inflammation. Signaling through the T cell co-stimulator GITR counteracts immune down-regulation by augmenting effector T cell responses and abrogating suppression by Foxp3^{+} regulatory T cells. Thus, super-physiological antibody-mediated GITR co-stimulation represents a novel therapy for promoting protective immunity towards parasitic helminths, whilst blocking physiological GITR-GITRL interactions may provide a mechanism for dampening pathogenic Th2 inflammation. We investigated the super-physiological and physiological role 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 super-physiological GITR co-stimulation using an agonistic anti-GITR mAb over the first 12 days 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 super-physiological GITR co-stimulation, antibody-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 super-physiological GITR co-stimulation 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.

Introduction

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 IL-5 and IFN-γ, and occasionally IL-4, production in response to parasite antigens (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. Strategies to induce long-term protective immunity are therefore needed that can counteract infection-induced immune regulation.

The Glucocorticoid-Induced TNF Receptor-related protein (GITR) is a co-stimulatory receptor and a member of the tumor necrosis factor receptor superfamily (9-11). It is constitutively expressed at high levels on Foxp3$^+$ regulatory T cells (Tregs) and at low levels on naïve CD4$^+$ T cells, and expression increases in both cell populations upon activation (12). The ligand for GITR (GITRL) is highly expressed on activated plasmatocytoid DC (13, 14) and macrophages (15). Co-stimulation through GITR augments CD4$^+$ effector T (Teff) cell responses (12, 16) and by enhancing CD4$^+$ Teff cell activation can abrogate Foxp3$^+$ Treg mediated suppression (14, 17). Therefore, GITR co-stimulation provides a potential strategy for counteracting immune regulation and boosting immune responses. We and others have demonstrated that agonistic anti-GITR mAb treatment can enhance immunity to helminth parasites (18, 19), and similar results have been found with other infections (20-22) and tumors (23, 24). As such there is growing interest in employing GITR co-stimulation as a novel vaccine adjuvant (25-28). On the counter side, 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 focussed on the effects of agonistic anti-GITR co-stimulation, yet these super-physiological effects of GITR ligation might be quite different from natural ligation through GITRL. Few studies have shown a physiological 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.

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 towards the highly immunogenic Th2-driving eggs that become trapped in tissue microvasculature, such as the liver, resulting in granuloma formation (35). We have 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, but in contrast they simultaneously present a more active CD4$^+$Foxp3$^-$ Teff cell response with upregulation of GITR. This raises an interesting question of 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 down-regulated immune phenotype leading to a fully developed patent infection 55 – 60 days p.i. (19, 36, 37), defined by the presence of transmission stage microfilariae (Mf) circulating within the blood (34), they do eventually overcome immune-regulation resulting in late-phase killing and clearance of *L. sigmodontis* after 70 days (34, 38, 39). GITR expression on BALB/c CD4$^+$Foxp3$^-$ T cells increases during the later stages of infection (19, 36), and super-physiological GITR co-stimulation can be used therapeutically alongside depleting CD4$^+$CD25$^+$Foxp3$^+$ Tregs to enhance parasite killing (19). Thus, GITR co-stimulation may also be involved in the reversal of immune-regulation and late-phase parasite killing following patency.

We tested the super-physiological 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 super-physiological GITR

co-stimulation during immune priming using an agonistic anti-GITR mAb increased Th2 responses towards *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, nor the late-phase parasite-clearance in susceptible BALB/c mice. Whilst 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 co-stimulation 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, antigen, and immunizations

Female BALB/c, C57BL/6 and BALB/c 4get IL-4gfp reporter mice (courtesy of Markus Mohrs, The Trudeau Institute) (40) were bred in-house and maintained under specific pathogen-free conditions at the University of Edinburgh. Mice were used at 6-12 weeks 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* antigen (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 approximately 30 *S. mansoni* cercariae. *S. mansoni* eggs were quantified following liver digestion in 4% potassium hydroxide buffer per gram 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 calculated using a compound microscope (LEICA) and LEICA analysis software (LAS). 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 antibody and BrdU treatments

Mice received i.p. injections of 1 mg agonistic anti-GITR mAb (DTA-1, in house) (17) on d0 and d7 of *L. sigmodontis* infection. To block GITRL, mice were given i.p. injections with 200 μg anti-GITRL mAb (MIH44, in house) (43) every three days. Treatment started on d0 or d59 of *L. sigmodontis* infection, d0 of *S. mansoni* egg injection, and d36 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 1mg BrdU (Sigma-Aldrich) 24 hours prior to autopsy.

Cell purifications and *in vitro* restimulations

The parathymic, posterior, mediastinal and paravertebral LN, were taken as a source of thoracic LN draining the pleural cavity (tLN). Pleural cavity cells were recovered by lavage. MLN, tLN, and spleen cells were dissociated and washed in RPMI-1640 (invitrogen) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2mM 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 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. To obtain a single-cell suspension the digest was passed through a 70 µm cell strainer (BD Biosciences). Leukocytes were isolated by resuspension in 33% isotonic Percoll (GE Healthcare) and centrifuged at 700 g. For restimulation with LsAg (10µg/ml) or *S. mansoni* SEA (15µg/ml) whole tLN cells were cultured at 5×10^5 cells/well and spleen cells 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) naïve splenocytes. Supernatants were harvested at 72 hours. To measure intra-cellular cytokines cells were stimulated for 4 hours with 0.5 µg/ml PMA (Sigma-Aldrich) and 1 µg/ml Ionomycin (Sigma-Aldrich), with 10 µg/ml Brefeldin A added for the final 2 hours (Sigma-Aldrich).

Flow cytometry

The following antibodies were used: Alexafluor700-conjugated anti-CD4 (RM4-5), polyclonal anti-GFP (Ebioscience), Alexafluor488-conjugated goat anti-rabbit IgG (Invitrogen), phycoerythrin-conjugated anti-IL-4 (11B11), Alexafluor647-conjugated anti-IL-13 (ebio13A, Ebioscience), allophycocyanin-conjugated anti-IL-5 (TRFK5), E450-conjugated anti-IFN-γ (XMG1.2, Ebioscience), Fluorescein isothiocyanate-conjugated anti-GITR (DTA-1, in house), Fluorescein isothiocyanate-conjugated anti-BrdU with DNase (B44) and allophycocyanin -conjugated anti-Foxp3 (FJK-16s, Ebioscience). Non-specific binding was blocked with 4 µg of 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 Stainkit (Molecular Probes), and the cells 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.

Cytokine and antibody ELISA

Antibody 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 antibodies were used with ExtrAvidin-alkaline phosphatase conjugate (Sigma-Aldrich) and Sigma FastTM *p*-nitrophenyl phosphate substrate (Sigma-Aldrich). To measure *L. sigmodontis* specific antibodies ELISA plates (NUNC) were coated with 5 µg/ml LsAg diluted in 0.45M NaHCO₃/0.18M Na₂CO₃ (Sigma-Aldrich). Plates were incubated with serial dilutions of serum, and a representative dilution from the linear section of the dilution curve selected for each isotype. Detection of Ab isotypes was performed using HRP-conjugated anti-mouse IgG1, IgG2a or IgM (Southern Biotechnology Associates) and ABTS 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 Tukey's post-hoc tests. 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 non-parametric data the unpaired Mann–Whitney test was used. Figures depict means when parametric tests were used and medians when non-parametric tests were used.

Results

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

Th2 responses are critical for resistance towards *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 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 d6 p.i., and only a weak, albeit significant, 2-fold increase in the proportion of IL-4gfp+ Th2 cells at d12 p.i. (Fig. 1A). The weak nature of the d12 increase was notable by the fact that it did not reach significance within every individual experiment, although 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 d40 p.i., when adult parasites have developed, and a 17-fold increase after the onset of patency at d60 p.i. (Fig 1A). A similar pattern was found within the tLN, with a modest initial expansion followed by a gradual increase to reach a 4-fold up-regulation at d60 p.i. (Fig. 1B). 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 have previously shown that resistance to *L. sigmodontis* infection in C57BL/6 mice is associated with increased expression of GITR on CD4+Foxp3− Teff cells during the early infection stages, whilst susceptible BALB/c mice fail to up-regulate GITR and show reduced CD4+Foxp3− Teff cell proliferation (36). To test the hypothesis that providing super-physiological GITR co-stimulation could be used to boost the initial Th2 responses and reverse susceptibility, we infected BALB/c IL-4gfp reporter mice with *L. sigmodontis* and treated them with agonistic anti-GITR mAb at d0 and d7 p.i. Treatment resulted in a 60% increase in the frequency of IL-4gfp+ Th2 cells in the pleural cavity 12 days p.i. (Fig. 2A). This increment was restricted to the infection site as although anti-GITR treatment increased the percentage of IL-4gfp+ Th2 cells in the tLN this was independent of infection (Fig. 2B). 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 intra-cellular staining for protein. Whilst infection alone at this early timepoint only caused a minor non-significant increase in the proportion of IL-4gfp+ Th2 cells producing IL-5 and IL-13 (Fig 2D-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. 2C-E). Although anti-GITR treatment did not increase the percentage of IL-4gfp+ Th2 cells in the

tLN in response to infection, it did result in increased *L. sigmodontis* Ag-specific IL-5 production following *in vitro* restimulation of tLN cells (Fig. 2F). 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 12 days p.i. (Fig. 2G).

To investigate whether anti-GITR treatment also boosts humoral immunity, we determined the levels of *L. sigmodontis* Ag-specific antibodies in the serum 12 days p.i.. Despite enhancing the cellular Th2 response, anti-GITR treatment did not result in increased production of the Th2 antibody isotype IgG1 (Fig. 2H). However, the increased production of IFN-γ was associated with elevated levels of serum IgG2a (Fig. 2I). Serum IgM, IgG2b, and IgG3 were unaffected by anti-GITR treatment (data not shown).

Thus, super-physiological GITR co-stimulation 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 d0 and d7 p.i. and autopsied at d60 when Mf are circulating in the bloodstream.

By d60 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 3A) compared to 5±5% at d12 (Fig. 1A). Despite the early boosting effect of anti-GITR treatment on Th2 responses there were no differences in the proportion (Fig. 3A-B) or number (results not shown) of IL-4gfp$^+$ Th2 cells between anti-GITR treated and IgG infected mice by 60 days p.i.. There were also no differences in the *L. sigmodontis* Ag-specific production of IL-5 (Fig. 3C), IL-4 or IL-13 (data not shown) by tLN 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 (Figs. 3D-E).

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. 3F). However, *L. sigmodontis*-specific IgG2a was still elevated in the sera at d60 p.i. (Fig. 3G), and IFN-γ production by purified tLN IL-4gfp$^-$ non-Th2 cells was enhanced, albeit in an antigen non-specific manner (Fig. 3H). Thus, the augmenting effects of anti-GITR treatment on Th2 priming appear to be temporary, and do not result in increased parasite killing. In contrast, even though *L. sigmodontis* infection mainly induces 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 up-regulate GITR on CD4$^+$Foxp3$^-$ Teff cells during the early stages of *L. sigmodontis* infection suggesting that their stronger initial Teff cell response and resistance may be related to GITR co-stimulation (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 days of infection with an anti-GITRL mAb that has been shown to block GITRL activity *in vivo* (13, 24, 43).

GITRL blockade failed to reduce the proportion of CD4+Foxp3− Teff cells proliferating *in vivo* in response to infection, thus GITR-GITRL interactions could not explain the enhanced effector T cell response of resistant C57BL/6 mice (Fig. 4A). Similarly, GITR-GITRL interactions were not required for the induction of Th2 responses as the production of *L. sigmodontis* Ag-specific IL-5 and IL-13 by tLN cells was unaffected (Fig. 4B, 4C). GITRL blockade also failed to impact on the resistant phenotype with both control and anti-GITRL treated mice showing similar parasite recoveries at d47 p.i. (data not shown). These data suggest that although super-physiological GITR co-stimulation 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 down-regulation (19, 36, 37), BALB/c mice show a late-phase killing response and clear infection after 70 days (34, 38, 39). We have found that expression of GITR on Foxp3− Teff cells at the infection site increases as infection progresses, and that treating infected mice with agonistic anti-GITR antibody 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 co-stimulation 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 d60-80 p.i.. Despite high levels of GITR expression (19, 37), blockade of GITRL was not found to impact on parasite burden or Mf levels 82 days p.i. (Fig. 5A-B). Accordingly, no differences were seen in the total number of IL-4gfp+ Th2 cells in the pleural cavity (Fig. 5C) or the tLN (Fig. 5D), or the proportion of pleural cavity IL-4gfp+ Th2 cells producing IL-4 and IL-5 protein (data not shown). *In vitro L. sigmodontis* Ag-specific production of IL-5 (Fig. 5E) and IL-4 (data not shown) by tLN cells also remained unaltered. Thus, GITR-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, resulting in immune inflammation and granuloma formation (35). We found that expression of GITR is strongly upregulated on CD4+Foxp3− Teff cells in the spleen and liver at the peak of egg-induced inflammation (week 8 p.i.) (Fig. 6A-B) suggesting that GITR signaling is potentiating the inflammatory Th2 response. We therefore tested whether blocking GITR-GITRL interactions can be used to moderate Th2 mediated inflammation and granuloma formation during *S. mansoni* infection.

As 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 10 days post-injection, however, GITRL blockade still failed to affect Th2 cell priming as the total number of IL-4gfp+ Th2 cells in

the draining popliteal LN (Fig. 6C), 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 (wk 5 p.i.) until the time of peak inflammation (wk 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. 6D). 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. 6E), and also no effect on the size of egg-induced liver granulomas (Fig. 6F). As well as 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. 6G) and liver (Fig. 6H). Thus, in contrast to *L. sigmodontis* infection, blockade of GITR-GITRL interactions partially reduces both Th2 and Th1 responses towards *S. mansoni*.

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 co-stimulation promotes Foxp3$^+$ Treg expansion (13, 18, 32, 43, 47) so to test whether GITR-GITRL interactions are responsible for the initial Foxp3$^+$ Treg expansion GITRL was blocked over the first 12 days 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. 7A). In the converse experiment, treating susceptible BALB/c mice with agonistic anti-GITR mAb on d0 and d7 p.i. also failed to affect the percentage of CD4$^+$ T cells expressing Foxp3 (Fig. 7B). Similarly, blockade of GITRL during the acute egg phase of *S. mansoni* infection (wk 5-8 p.i.) had no effect on Foxp3$^+$ Treg expansion in the spleen (Fig. 7C) or liver (Fig. 7D). Thus, GITR-GITRL interactions do not play a major role in the expansion of Foxp3$^+$ Tregs during *L. sigmodontis* or *S. mansoni* infections.

Discussion

Co-stimulation of GITR through agonistic mAb is known to augment effector T cell 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 super-physiological GITR co-stimulation, understanding of the physiological role of GITR is limited, particularly in Th2 settings. In the present study we investigated both the super-physiological 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 super-physiological GITR co-stimulation using agonistic anti-GITR mAb during immune priming initially augmented Th2 responses towards the filarial nematode *L. sigmodontis*. However, this effect was temporary with the Th2 response reverting to normal infection levels by the onset of patency (d60 p.i.) and 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 super-physiological GITR co-stimulation 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), and 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 LN of susceptible BALB/c mice over the first 12 days of infection. This contrasts with the rapid expansion of CD4^{+}Foxp3^{+} Tregs seen by d7 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 days of infection increased the quantity of IL-4gfp^{+} Th2 cells at the infection site, and also their functional quality as represented by an increased ability of the IL-4gfp^{+} Th2 cells to secrete Th2 cytokines. Thus, GITR co-stimulation can be used therapeutically to boost both the quantity and quality of Th2 responses towards infection; however, this was not sufficient to enhance killing of *L. sigmodontis*.

The potential for using super-physiological GITR co-stimulation 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 super-physiological GITR co-stimulation (25). Immune therapies that successfully improve protection towards *L. sigmodontis* often result in relatively slow killing, taking one or two months 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* indicates that the Th2 boosting effects of agonistic anti-GITR treatment are temporary. Thus, the lack of efficacy of super-physiological GITR co-stimulation 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 for a therapy to be successful it 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 antibody responses towards viral antigens (25), raising a question of why the enhancing effect on Th2 immunity towards *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 once anti-GITR treatment is discontinued parasite-induced immune suppression reasserts itself countering the enhanced Th2 response. In support of this we find that during established *L. sigmodontis* infection the efficacy of super-physiological GITR co-stimulation 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 d60 p.i.) of Th1-associated *L. sigmodontis* specific IgG2a, contrasting the temporary Th2 enhancement. Thus, although GITR co-stimulation 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). As GITR co-stimulation is associated with the enhancement of immunity and abrogation of regulatory T cell responses (14, 17, 28), this implies that GITR co-stimulation might be an important factor in the generation of Th2 responses and natural resistance against *L. sigmodontis*.

Although super-physiological GITR co-stimulation is capable of promoting Th2 responses towards *L. sigmodontis*, blockade of GITR-GITRL interactions using a neutralising anti-GITRL mAb did not inhibit the initiation of Th2 responses towards *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 appear to be required for protective immunity towards *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 appeared to be more related to maintenance rather than priming of Th2 cells as 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 its reported roles in the development of Th1 responses (13, 21, 31-33), GITR-GITRL interactions appear to be largely redundant for the initiation and maintenance of Th2 immunity towards parasitic helminths.

Alongside co-stimulation of CD4^{+} effector T cells, GITR stimulation has been shown to expand regulatory T cells *in vitro* (14, 43, 47, 58), although *in vivo* studies show conflicting results regarding Foxp3^{+} Treg requirements for GITR-GITRL interactions (14, 18, 23, 47, 59, 60). Even though 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.

In summary, this work indicates that super-physiological GITR co-stimulation 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 regimes 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 towards either *L. sigmodontis* or *S. mansoni*. Thus, the potent immune stimulatory properties that have been reported for super-physiological GITR co-stimulation may not accurately reflect its actual physiological roles.

Acknowledgments

Abbreviations used in this article

GITR   Glucocorticoid-Induced TNF receptor-related protein

Treg   Regulatory T cell

| **Teff** | effector T cell |
Mf	microfilarariae
p.i.	post-infection
LsAg	*L. sigmodontis* antigen
tLN	thoracic lymph nodes
MLN	mesenteric lymph nodes

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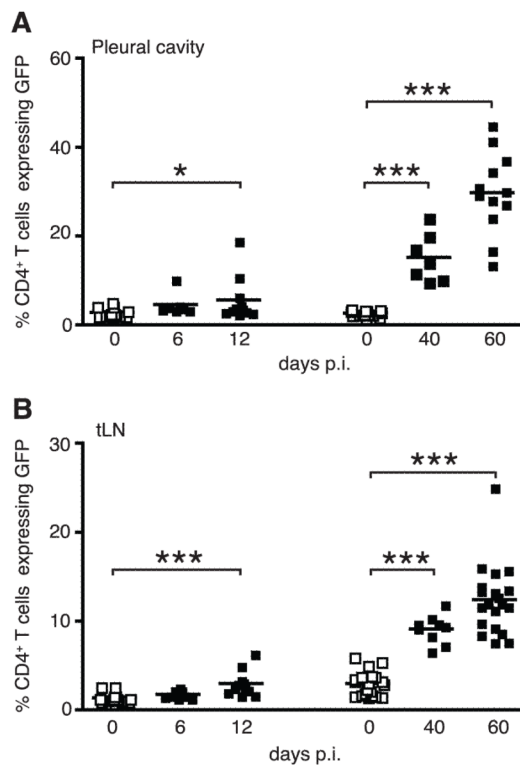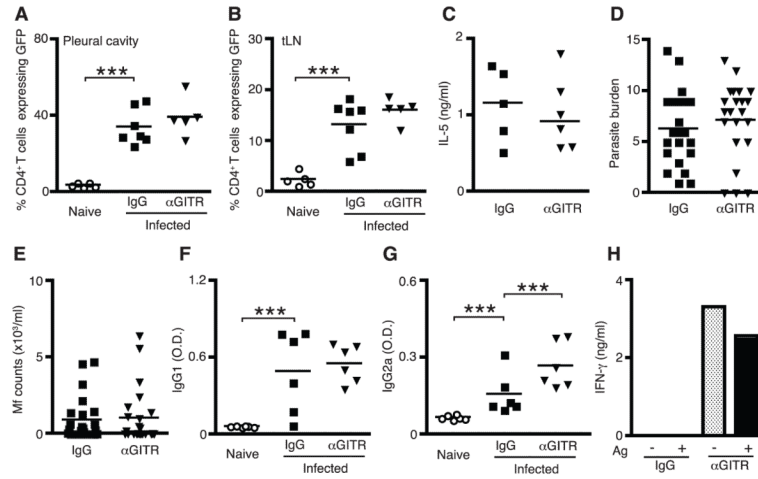
Figure 1.
Kinetics of Th2 cell induction following *L. sigmodontis* infection. IL-4gfp reporter mice were infected with *L. sigmodontis* (closed symbols), or left uninfected (open symbols), and the proportion of IL-4gfp^{+} Th2 cells in the pleural cavity (A) and tLN (B) quantified. Symbols represent individual mice. Lines represent means and figures show data of pooled experiments. *$p<0.05$, ***$p<0.001$ (1-way ANOVA followed by Tukey's HSD post-hoc tests based on combined data from at least 2 experiments per timepoint).
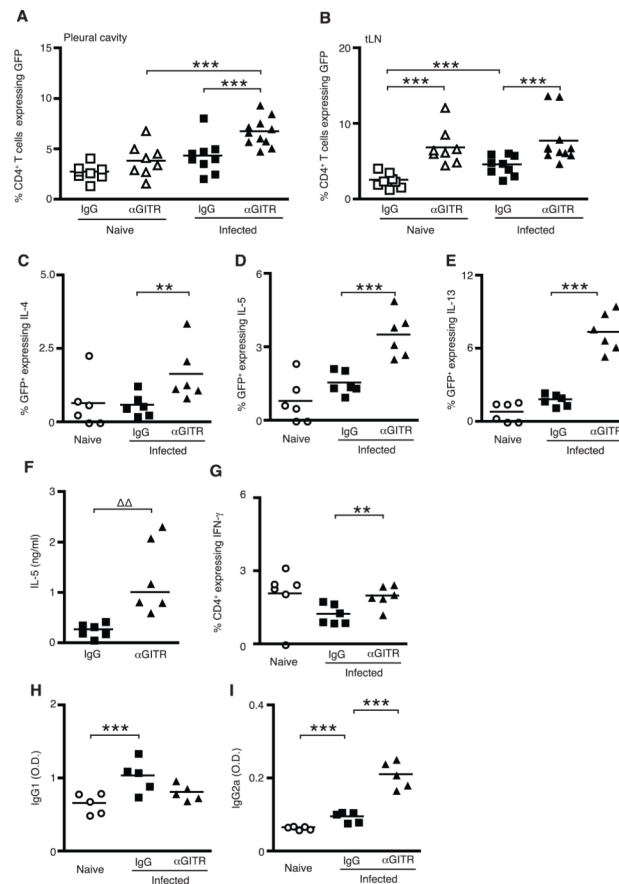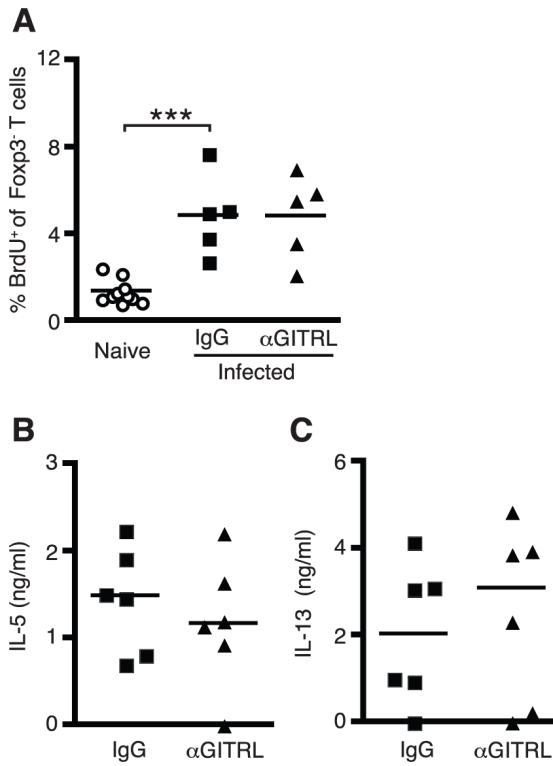
Figure 2.
Therapeutic administration of agonistic anti-GITR mAb enhances the magnitude of the Th2 response to *L. sigmodontis*. *L. sigmodontis* infected (closed symbols) BALB/c IL-4gfp reporter mice (A-E) or BALB/c mice (F-I) were treated with agonistic anti-GITR mAb (triangles) or rat IgG (squares) and autopsied 12 days p.i.. Open circles indicate naïve untreated mice. Lines show mean values unless otherwise stated and symbols represent individuals. (A-B) 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. (C-E) The proportion of pleural cavity CD4$^+$GFP$^+$ Th2 cells producing intracellular IL-4 (C), IL-5 (D), IL-13 (E). One representative experiment out of two shown. (F) *In vitro* production of IL-5 by tLN cells following restimulation with LsAg. One of four independent experiments shown. Lines show median values. (G) Proportion of CD4$^+$ T cells producing IFN-γ. (H-I) Serum levels of *L. sigmodontis* specific IgG1 (H) and IgG2a (I). One representative experiment of three shown. ***$p<0.001$, **$p<0.01$ (2-way ANOVA followed by Tukey's HSD post-hoc test based on pooled data from denoted experiments). ΔΔ $p<0.01$ (Mann Whitney).
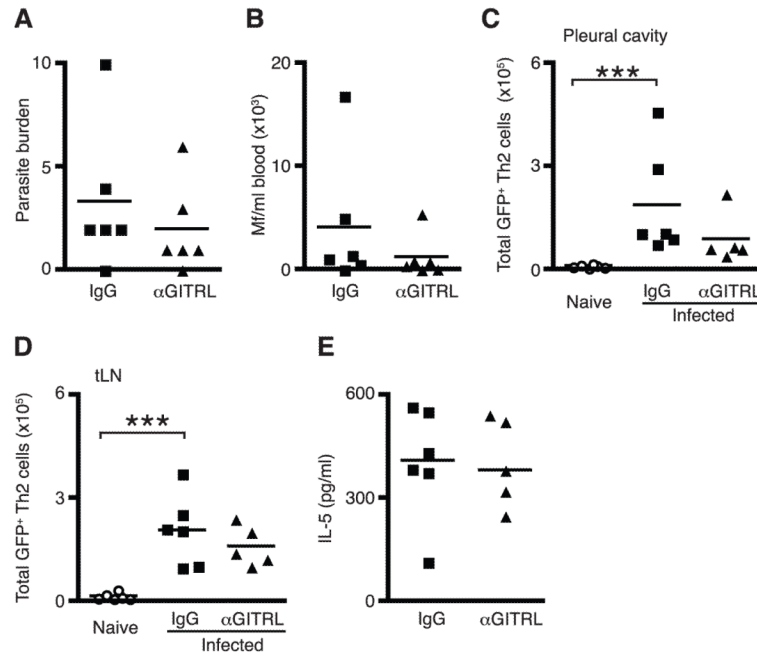
Figure 3.
Therapeutic administration of agonistic anti-GITR during immune priming does not enhance long-term protective Th2 immunity. *L. sigmodontis* infected (closed symbols) BALB/c IL-4gfp reporter mice were treated with an agonistic anti-GITR mAb (triangles) or rat IgG (squares) on d0 and d7 p.i. and autopsied at D60. Open circles represent naïve untreated mice. Symbols represent individual animals, and lines represent mean values unless otherwise stated. (A-B) The proportion of CD4$^+$ T cells expressing GFP in the pleural cavity (A) and tLN (B). One representative experiment of two shown. (C) *In vitro* production of IL-5 by tLN cells following restimulations with LsAg. One representative experiment of three shown, lines represent median values. (D-E) Adult parasite burden in the pleural cavity (D) and number of Mf/ml within the peripheral blood (E). Pooled data from four independent experiments shown. (F-G) Serum levels of *L. sigmodontis*-specific IgG1 (F) and IgG2a (G). One representative experiment of two 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 shown. ***p<0.001 (2-way ANOVA followed by Tukey's HSD post-hoc tests based on combined data from denoted experiments).
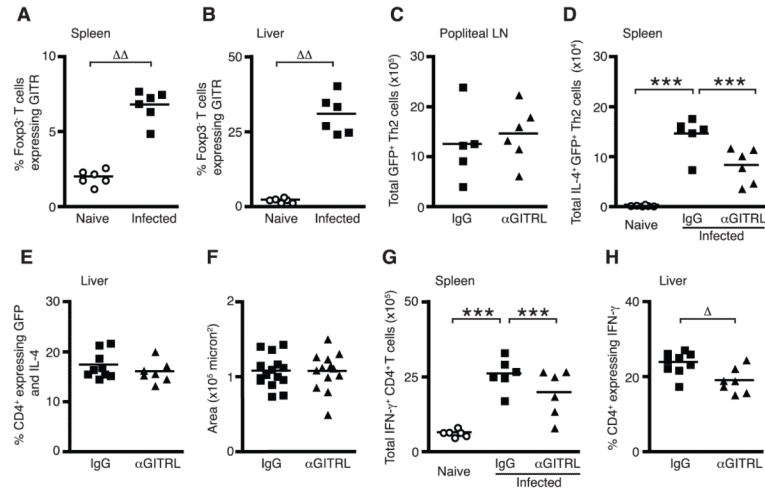
Figure 4.
GITRL blockade during immune priming does not affect Th2 development. *L. sigmodontis* infected (closed symbols) C57BL/6 mice were treated during immune priming with a blocking anti-GITRL mAb (triangles) or rat IgG (squares) over the first 12 days of infection. Open circles represent naïve untreated controls. (A) The proportion of Foxp3$^-$CD4$^+$ T cells that incorporated BrdU. One representative experiment of three shown. ***p<0.001 (2-way ANOVA followed by Tukey's HSD post-hoc tests based on combined data from three experiments). (B-C) *In vitro* production of IL-5 (B) and IL-13 (C) by tLN cells following restimulation with LsAg. One representative experiment of three shown. Symbols denote individual mice and lines show mean (A) or median (B-C) values.
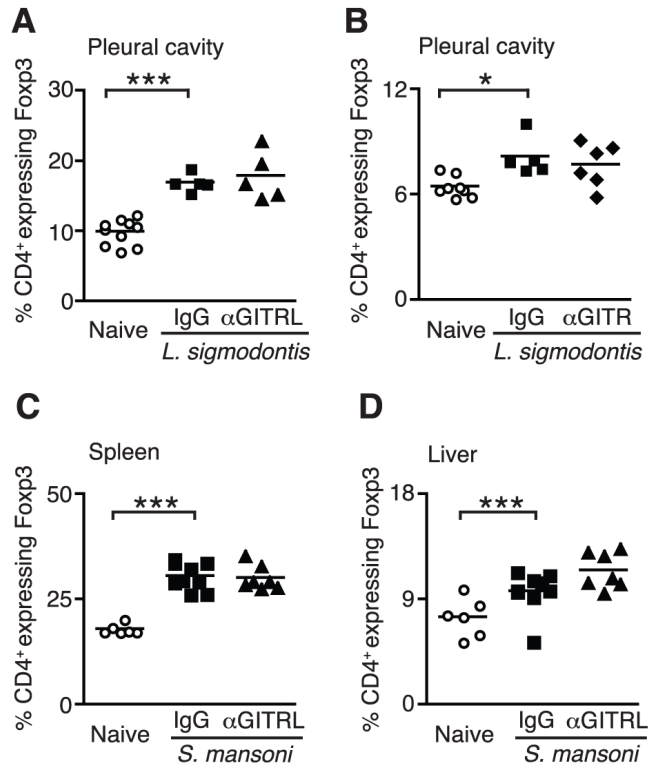
Figure 5.
GITR-GITRL interactions are not required for late-phase killing in susceptible BALB/c mice. *L. sigmodontis* infected (closed symbols) BALB/c WT or BALB/c IL-4gfp reporter mice were treated with a blocking anti-GITRL mAb (triangles) or rat IgG (squares) from d60 to d80 p.i., and mice were autopsied on d82. Circles represent naïve untreated mice, and symbols represent individual mice. (A-B) Adult parasite burden in the pleural cavity (A) and Mf/ml in peripheral blood (B). One representative experiment of three shown. Lines show mean values. (C-D) The number of CD4+GFP+ Th2 cells in the pleural cavity (C) and tLN (D). One representative experiment of two shown, lines denote mean. (E) *In vitro* production of IL-5 by tLN cells following restimulation with LsAg. One representative experiment out of three shown, lines show median values. ***p<0.0001. (2-way ANOVA followed by Tukey's HSD post-hoc tests based on combined data from three experiments).

Figure 6.

GITRL blockade impairs Th2 and Th1 responses towards *S. mansoni* eggs. (A-B) Percentage of CD4^{+}Foxp3^{−} T cells expressing GITR in the spleen (A) and liver (B) of *S. mansoni* infected (closed symbols) or naïve (open symbols) BALB/c mice 8 weeks p.i.. One representative experiment of two shown. (C) BALB/c IL-4gfp mice were injected s.c. in the footpad with *S. mansoni* eggs and treated with anti-GITRL mAb (triangles) or rat IgG (squares) for 12 days. The numbers of IL-4gfp^{+} Th2 cells in the popliteal LN were quantified at D12. One representative experiment of two shown. (D-H) *S. mansoni* infected IL-4gfp mice (closed symbols) were treated with anti-GITRL mAb (triangles) or rat IgG (squares) from wk 5 to autopsy at wk 8. Open symbols represent naïve untreated controls. One representative experiment of two 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. Δ $p<0.05$, ΔΔ $p<0.01$ (Mann-Whitney). ***$p<0.001$ (2-way ANOVA followed by Tukey's HSD post-hoc tests based on combined data from two experiments)

FIGURE 7.
GITR-GITRL interactions are not required for expansion of Foxp3^{+} Tregs during helminth infection. (A-B) 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 (triangles) or agonistic anti-GITR mAb (diamonds) respectively over the first 12 days of infection. Naïve mice are represented by circles and IgG treated mice by squares. (C-D) Percentage of CD4^{+} T cells expressing Foxp3 in the spleen (C) and liver (D) of *S. mansoni* infected (closed symbols) BALB/c IL-4gfp reporter mice treated with a blocking anti-GITRL mAb (triangles) or rat IgG (squares) from wk5 p.i. to autopsy at wk8. Open symbols represent naïve controls. One representative experiment shown out of at least two for all panels. Symbols represent individual mice and lines represent mean values. *p<0.05, ***p<0.001 (2-way ANOVA followed by Tukey's HSD post-hoc tests based on combined data from at least two experiments).