Removal of Regulatory T Cell Activity Reverses Hyporesponsiveness and Leads to Filarial Parasite Clearance In Vivo[1]

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Human filarial parasites cause chronic infection associated with long-term down-regulation of the host's immune response. We show here that $CD4^+$ T cell regulation is the main determinant of parasite survival. In a laboratory model of infection, using *Litomosoides sigmodontis* in BALB/c mice, parasites establish for >60 days in the thoracic cavity. During infection, $CD4^+$ T cells at this site express increasing levels of CD25, CTLA-4, and glucocorticoid-induced TNF receptor family-related gene (GITR), and by day 60, up to 70% are $CTLA\text{-}4^+GITR^{high}$, with a lesser fraction coexpressing CD25. Upon Ag stimulation, $CD4^+CTLA\text{-}4^+GITR^{high}$ cells are hyporesponsive for proliferation and cytokine production. To test the hypothesis that regulatory T cell activity maintains hyporesponsiveness and prolongs infection, we treated mice with Abs to CD25 and GITR. Combined Ab treatment was able to overcome an established infection, resulting in a 73% reduction in parasite numbers ($p < 0.01$). Parasite killing was accompanied by increased Ag-specific immune responses and markedly reduced levels of CTLA-4 expression. The action of the $CD25^+GITR^+$ cells was IL-10 independent as in vivo neutralization of IL-10R did not restore the ability of the immune system to kill parasites. These data suggest that regulatory T cells act, in an IL-10-independent manner, to suppress host immunity to filariasis. ***The Journal of Immunology,*** **2005, 174: 4924–4933.**

Filarial nematodes exert profound down-regulatory effects on the immune systems of their hosts. In seminal work over 20 years ago, the loss of Ag-specific T cell proliferative responses in infected humans was reported (1, 2). Substantial work has since confirmed that immune depression is not confined to a single arm of immunity: production of both Th1 (IFN-γ) and Th2 (IL-5) inflammatory cytokines are suppressed (3, 4), although IL-4 remains intact and IL-10 levels are generally increased (5–7). Indeed, not only do filariasis patients' peripheral blood leukocytes express higher levels of constitutive IL-10, but neutralizing Abs against IL-10 and/or TGF-β restore the ability of these cells to mount filarial-specific proliferative responses in vitro (5, 7). These observations, along with description of regulatory T cell (Treg)[4] populations that act through IL-10 and TGF-β (8–13), have led to the hypothesis that filarial infections induce Treg populations that suppress effector T cell responses, and thus promote parasite survival (14, 15).

Tregs were originally demonstrated to govern autoreactivity (8), but similar populations are now known to arise in response to exogenous Ags, including those presented by viral (16–18) and bacterial (19–22) pathogens. Moreover, $CD4^+CD25^+$ T cells curtail immunity to *Leishmania* (23, 24) and malaria (25) parasites in mouse models. Although impairing resistance against the pathogen, Tregs may also provide beneficial protection against immunopathology (26–29). There is also some suggestion that the pathogens themselves can manipulate the regulatory cells to immunosuppress the host and so potentiate their survival (16, 20).

Indications of Treg activity in human helminth infections are provided by the phenotype of T cell clones from onchocerciasis patients, which express regulatory cytokines (IL-10 and TGF-β) and CTLA-4 (30). In addition, CTLA-4 expressed on T cells from lymphatic filariasis patients can suppress IL-5 production, among other responses (31). More recently in *Schistosoma mansoni* infection, $CD4^+CD25^+$ T cells and IL-10 have been shown to inhibit both Th1 development (32) and egg-induced pathology (28). These studies suggest that Tregs are generated during helminth infections, but to date no experimental evidence has been provided to show that Tregs promote parasite survival by mediating the immune down-modulation seen during chronic infection (14, 15).

To address this hypothesis directly, we studied a unique model of filariasis, *Litomosoides sigmodontis* infection of permissive BALB/c mice (33). *L. sigmodontis* is a close relative of the *Brugia* and *Wuchereria* species, which are responsible for some 120 million cases of human lymphatic filariasis in the world today (34, 35). Intriguingly, resistant mouse strains (e.g., C57BL/6) are dependent on Th2 responses for resistance, yet susceptible mouse strains (e.g., BALB/c) also mount a highly skewed Th2 response (36). The lack of efficacy of the BALB/c Th2 response suggests that immunoregulatory factors, such as Treg populations, hamper its ability to protect against infection. Using *L. sigmodontis,* we provide the first experimental demonstration that Tregs are indeed

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[4] Abbreviations used in this paper: Treg, regulatory T cell; GITR, glucocorticoid-induced TNF receptor family-related gene; LsAg, *L. sigmodontis* whole worm Ag; tLN, thoracic lymph node; TC, thoracic cavity.

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responsible for susceptibility to a helminth parasite. Specifically, we demonstrate that infection results in increased expression of Foxp3 and the shutdown of $CD4^+$ T cell immunity, and that intervention against Tregs using Abs against CD25 and glucocorticoid-induced TNF receptor family-related gene (GITR), but not against IL-10R, provides an immunological cure for filarial infection.

Materials and Methods

Mice and parasites

Female BALB/c mice were used at 6–8 wk of age and were maintained in individually ventilated cages. The *L. sigmodontis* life cycle was maintained in gerbils using the mite vector *Ornithonyssus bacoti*. Infective larvae (L3) were recovered from mites 13 days postfeeding by dissection in RPMI 1640 supplemented with 10% FBS (Invitrogen), and mice were infected s.c. on the upper back. Each mouse received a dose of 25 L3, chosen to give sufficient parasite recoveries to allow accurate numeration, but small enough to allow optimal survival and maturation of adult worms (37). Thoracic lavage with 10 ml of cold AIM V medium was used to recover parasites. *L. sigmodontis* whole worm Ag (LsAg) was prepared by collecting the PBS-soluble fraction of homogenized adult male and female worms.

Cell purifications and in vitro restimulations

The parathymic, posterior mediastinal, and paravertebral lymph nodes (thoracic lymph nodes (tLNs)) draining the thoracic cavity (TC) were dissociated and washed in AIM V medium before being resuspended in RPMI 1640 with 0.5% mouse serum (Caltag-Medsystems), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. TC cells were isolated from lavage fluid. For $CD4^+$ T cell purifications, TC cells were adhered to plastic for 2 h at 37°C and the nonadherent population was taken. $CD4^+$ T cells were then isolated by CD4 MicroBead magnetic cell sorting (Miltenyi Biotech) per the manufacturer's instructions, except that HBSS with 0.25% mouse serum was used as the separation medium and 15 μg of rat IgG/1 $\times$ 10^7 cells was used as a block. TC $CD4^+$ T cell purities were 78–90% depending on experiment, but did not vary within experiments. TC $CD4^+$ T cell populations were free of contaminating $F4/80^+$ alternatively activated macrophages. Irradiated (30 Gy) splenic APCs were added to 96-well round-bottom plates at 1×10^6 cells/well, together with 1×10^5 $CD4^+$ T cells/well. Whole tLN cells were used at 5×10^5 cells/well. Cultures were stimulated with medium alone or 10 μg/ml LsAg. Supernatatants were sampled at 72 h for cytokine analysis, and 1 μCi/well of methyl-[^{3}H]thymidine was added for 16 h to measure proliferation. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations were isolated from the TC by negative selection for CD4 cells using anti-CD8 (53-6.72), anti-B220 (RAB632), anti-MHC class II (M5/114.15.2), anti-CD11b (M1/70), anti-Gr1 (RB6-8C5), and anti-F4/80 (A3-1), with anti-rat IgG Dynal Beads (Dynal). CD25 purification was then performed using anti-CD25phycoerythrin in combination with anti-phycoerythrin microbeads (Miltenyi Biotech). The $CD4^+CD25^+$ fraction was 75.9% $CD4^+$ with 72.5% $CD4^+CD25^+$. The $CD4^+CD25^-$ fraction was 78.8% $CD4^+$ with 72.4% $CD4^+CD25^-$. Anti-CD3 (145C2.11) was added to the cultures at 0.125 μg/ml, and 1×10^5 irradiated naive spleen cells were used as APCs.

Abs and reagents

Ab pairs used for cytokine ELISAs were: IL-4 (11B11/BVD6-24G2), IL-5 (TRFK5/TRFK4), IL-10 (JES5–2A5/SXC-1), and IFN-γ (R4-6A2/XMG1.2). Recombinant murine IL-4, IFN-γ, IL-10, and IL-5 (Sigma Aldrich) were used as cytokine standards. Detection Abs were biotinylated and used with ExtrAvidin-alkaline phosphatase conjugate and Sigma FastTM *p*-nitrophenyl phosphate substrate. For flow cytometry, nonspecific binding was blocked with 4 μg of rat IgG/1 $\times$ 10^6 cells, and the following Abs were applied: PE-conjugated anti-CTLA-4 (UC10-4F10-11), APC-conjugated streptavidin, Cy-Chrome-conjugated anti-CD4 (RM4-5), biotinylated anti-CD25 (7D4), anti-GITR (DTA-1), and FITC-conjugated goat $F(ab')_2$ anti-rat IgG (Caltag-Medsystems). All staining was compared against the relevant isotype controls to verify specificity. To measure intracellular CTLA-4, cells were permeabilized and stained with BD PharMingen's Cytofix/Cytoperm kit. Flow cytometric acquisition and analysis was performed using a FACSCalibur running CellQuest Pro software. ELISA detection was performed using ExtrAvidin-alkaline phosphatase conjugate (Sigma-Aldrich) in conjunction with Sigma FastTM *p*-nitrophenyl phosphate tablet substrate (Sigma-Aldrich). Reagents were obtained from BD Biosciences or American Type Culture Collection unless otherwise stated.

TGF-β bioassay

TC TGF-β levels were measured in the first milliliter of thoracic lavage fluid using a bioassay as previously described (38). In brief, 1.6×10^5 mink lung epithelial cells, modified to express a luciferase reporter gene under the control of a TGF-β-responsive promoter, were added to 96-well flat-bottom white FluoroNunc plates (Nunc) in 50 μl of RPMI 1640 supplemented with 0.5% mouse serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were allowed to adhere for 2 h, after which the medium was replaced with sample. The bioassay only measures the active form of TGF-β, thus samples were added directly to the plates to measure endogenously active TGF-β, and to measure total levels of TGF-β the samples were first heat activated for 5 min at 80°C. Recombinant human TGF-β1 (Roche Diagnostics) was used as a standard. The plates were incubated for 16 h at 37°C, 5% CO_2, and the Bright-Glo Luciferase Assay System (Promega) was used to quantify luciferase expression on a LUMIstar (BMG Labtech) per the manufacturer's instructions.

Real-time PCR

$CD4^+$ T cells were purified from the tLN, brachial LN, and TC of individual *L. sigmodontis*-infected mice and from pooled (five per group) naive mice. Purified $CD4^+$ T cells were resuspended in TRIzol (Invitrogen) and RNA extraction was performed per the manufacturer's instructions. After treatment with DNase1 (Ambion), cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Stratagene). Quantification of *Foxp3* mRNA was performed by real-time PCR using the LightCycler (Roche Diagnostics). The *Foxp3* forward and reverse primers were CCT GGCCTGCCACCTGGGATCAA (exon 3/4 junction) and TTCTCA CAACCAGGCCACTTG (exon 5), respectively. PCR amplifications were performed in 10 μl, containing 1 μl of cDNA, 0.3 mM primers and 5 μl of Qiagen hotstart Sybergreen using the following conditions: 15-min hotstart at 95°C, 15-s denaturation at 95°C, 20-s annealing of primers at 54°C, and 15-s elongation at 72°C, for 40–60 cycles. The fluorescent DNA binding dye was monitored after each cycle at 82°C. *Foxp3* expression was normalized against β-actin; forward and reverse primer sequences were TGGAATCCTGTGGCATCCATGAA and TAAAACGCAGCTCAGT AACAGTC, respectively. β-Actin PCR was performed as above, except that LightCycler-DNA SYBR green I mix (Roche Molecular Biochemicals) was used without hotstart, and the DNA binding dye was monitored at 86°C. Eight serial dilutions of cDNA pooled from each sample were used to create a standard curve (in arbitrary units) against which β-actin and *Foxp3* mRNA expression were normalized. *Foxp3* mRNA levels were then expressed as a ratio to β-actin mRNA expression. Two cDNA templates were produced from each sample, and PCR was performed twice from each cDNA template. Results are displayed as one representative run from each experiment.

In vivo Ab treatment

In vivo treatments used anti-GITR (DTA-1), anti-mouse CD25 (PC61), or anti-mouse IL-10R (1B1.3a). Mice received 1 mg of anti-CD25 in PBS on day 27 of infection or 1 mg of anti-GITR on days 27 and 34 for single treatments. For combined anti-CD25/GITR treatment, mice received 1 mg of each Ab on day 27. Mice received 1 mg of anti-IL-10R on day 27, followed by three doses of 0.5 mg (1 mg in second experiment) every 3 days. An equivalent dose of rat IgG was used as a control.

Statistics

Statistical analysis was performed using Minitab version 14. When combining experimental data using ANOVA, it was first demonstrated that there were no significant interactions due to experimental differences.

Results

L. sigmodontis *infection in mice recapitulates the loss of T cell proliferative responses in human filariasis*

To investigate whether the immune regulation, evident in human filariasis, would be reproduced within a mouse model, susceptible BALB/c mice were infected with *L. sigmondontis* L3 larvae and their parasite-specific immune responses were followed for a 60-day period. Fig. 1*A* represents the course of *L. sigmodontis* development within this time period, in which parasites migrate through the lymphatic system to the TC. After ~55 days, infection reaches patency, defined as the release of newborn microfilarial parasites into the bloodstream (39). To assay local immune responses to

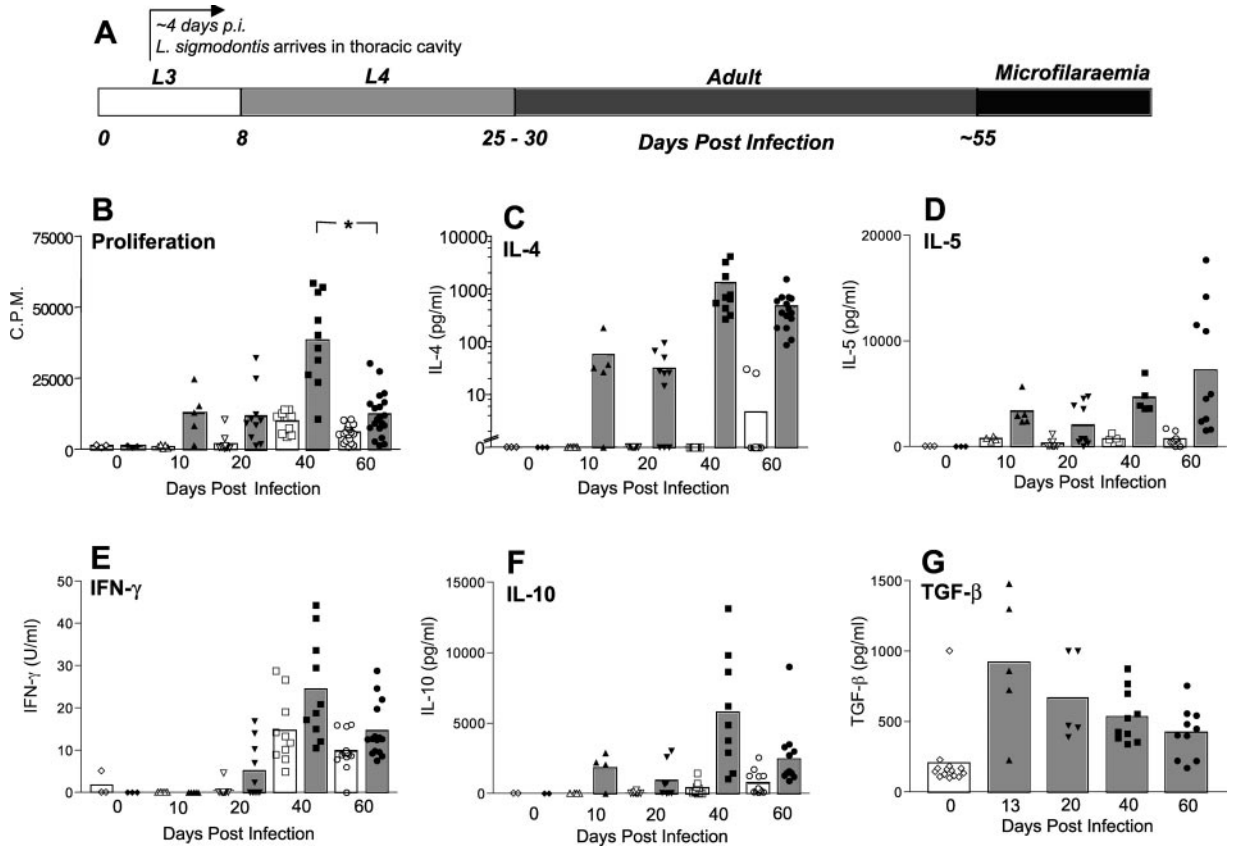

FIGURE 1. Time course of cellular immune responses to *L. sigmodontis* infection in susceptible BALB/c mice. *A*, Timeline showing the development of *L. sigmodontis* in BALB/c mice. Approximate times of molts between larval stages are indicated. *B–F*, Ag-specific immune responses. Thoracic lymph node cells were sampled on days 10 (triangles), 20 (inverted triangles), 40 (squares), and 60 (circles) postinfection and from naive mice (day 0, diamonds) and were restimulated with LsAg (filled symbols) or medium alone (open symbols). Bars represent mean values. Lymph nodes from naive animals were pooled (day 0) within experiments, with each symbol representing one experiment; at all other time points symbols represent individual mice. Data for day 10 are representative of three experiments; for each of days 20–60 data are combined from two experiments. $*$, $p < 0.05$ (Mann-Whitney). *B*, Ag-specific proliferation. *C*, IL-4 release in culture supernatants. *D*, IL-5 release in culture supernatants. *E*, IFN-γ release in culture supernatants. *F*, IL-10 release in culture supernatants. *G*, Total levels of TGF-β were measured, by bioassay reactive to all isoforms, in the thoracic lavage fluid of mice infected with *L. sigmodontis* or of naive controls. Each point represents one mouse, and results represent at least two experiments for each time point. Bars represent mean values.

infection, we isolated the parathymic, posterior mediastinal, and paravertebral lymph nodes (tLNs) that drain the TC and challenged them in vitro with soluble adult whole worm homogenate (LsAg).

Ag-specific proliferative responses were mounted by tLN cells from day 10 onward (Fig. 1*B*), reaching a peak 40 days postinfection, when the worms were adults but still immature. By day 60, once infection was fully patent, Ag-specific proliferative responses were significantly impaired (mean reduction 68%) compared with those 40 days postinfection, despite the continued survival of an equivalent number of adult parasites. The filarial-specific hyporesponsiveness observed during chronic human infection, therefore, appears to be paralleled by the loss of proliferative reactivity to Ag during murine infection with *L. sigmodontis*.

The type 2 cytokines IL-4 and IL-5 were produced by tLN cells in response to parasite Ag from an early point in infection (Fig. 1, *C* and *D*), whereas IFN-γ was released later and at relatively low levels (<50 U/ml) in a largely Ag-nonspecific manner (Fig. 1*E*). This profile of a Th2-dominated response to filarial nematode infection is consistent with previous reports of *L. sigmodontis* infection in BALB/c mice (40).

The two cytokines most strongly implicated in down-regulation of immune responses are IL-10 and TGF-β (9, 11). Thoracic lymph node cells showed Ag-specific IL-10 production from day 10, rising to a peak on day 40 (Fig. 1*F*). A 3- to 6-fold elevation of TGF-β was detectable in the thoracic lavage fluid of infected mice from day 13 postinfection, remaining elevated throughout (Fig. 1*G*). The majority of the TGF-β was in the latent form. Ag-specific production of TGF-β by tLN cells was not evident (data not shown). Thus, both Th2 and regulatory cytokines contribute to the early immune response and remain at significant levels throughout infection.

T cell responses are down-regulated at the site of infection

The localized nature of *L. sigmodontis* adult infection in the TC offered the opportunity to study T cell responses within the population in direct contact with the parasites. Therefore, we purified TC $CD4^+$ T cells, 40 and 60 days postinfection, with a mean recovery of 3.4×10^5 $CD4^+$ T cells per mouse. Infections were phased so that T cell assays coincided, and there were no significant differences between day 40 and day 60 worm recoveries (data not shown). $CD4^+$ T cells were restimulated in vitro with LsAg and Con A using irradiated spleen cells from naive mice as a source of APCs. As with tLN populations, patent infection was associated with a loss of Ag responsiveness by TC $CD4^+$ T cells, with those recovered 60 days postinfection showing lower proliferation to both LsAg (Fig. 2*A*) and Con A (data not shown) than those taken at day 40. Moreover, by day 60, there was a substantial

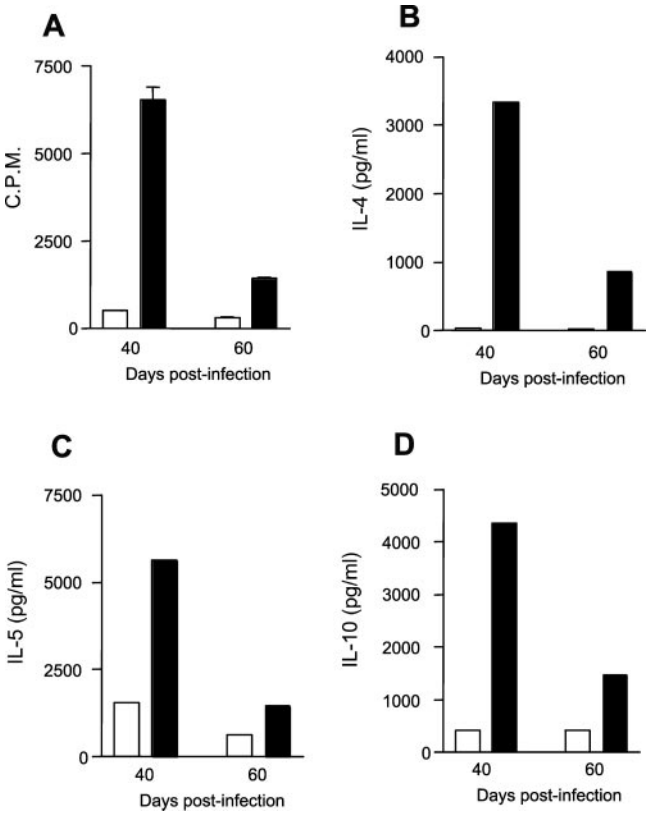

FIGURE 2. Decline of parasite-specific $CD4^+$ T cell responses at site of infection. $CD4^+$ T cells were purified from the TC 40 and 60 days postinfection and were cultured in vitro with medium alone (open bars) or LsAg (filled bars) with irradiated naive spleen cells. To directly compare Ag responsiveness, infections were phased to coincide at day 40 and 60 time points. Due to numbers of TC $CD4^+$ T cells, cells were pooled from at least 10 mice. Results are representative of two experiments. *A*, Proliferation (error bars represent the SD of triplicate cultures). *B*, IL-4 release. *C*, IL-5 release. *D*, IL-10 release.

decrease in Ag-specific IL-4, IL-5, and IL-10 production (Fig. 2, *B–D*).

Filarial infection up-regulates the expression of regulatory/activation markers

To study whether $CD4^+$ regulatory T cells are induced by filarial infection, we tested the expression of a set of markers (CD25, CTLA-4, and GITR), which in naive mice are constitutively expressed by Tregs. These markers are also found on recently activated $CD4^+$ T cells, but their up-regulation in chronic infection would be indicative of Treg activity warranting further functional testing. Therefore, we isolated cells from the TC, lymph nodes, and spleens of mice infected with *L. sigmodontis* and analyzed the expression of CD25, GITR, and intracellular CTLA-4 among the $CD4^+$ subset.

The phenotype of $CD4^+$ T cells from the TC was analyzed from day 13 onward, after the arrival of parasites in this compartment. Profiles changed little at early time points, other than significantly raised CTLA-4 at day 13 and CD25 at day 20 (data not shown). As infection progressed, however, the percentage of TC $CD4^+$ T cells expressing CTLA-4 and high levels of GITR ($GITR^{high}$) increased dramatically until the dominant phenotype became $CTLA\text{-}4^+GITR^{high}$, with 60–70% of the $CD4^+$ T cells double positive for these markers by day 60 (Fig. 3*A*). A minority of cells coexpressing CTLA-4 and $GITR^{high}$ were $CD25^+$, and these displayed significantly higher levels of CTLA-4 than did the $CD4^+CD25^-$ cells (medians of 50.5 and 11.3, respectively; $p < 0.05$, Mann-Whitney), and also of GITR (medians of 114.5 and 34.1, respectively; $p < 0.05$, Mann-Whitney) (Fig. 3*B*). Data from individual mice (Fig. 3*C*) emphasize the consistent up-regulation of these markers by TC $CD4^+$ T cells from day 40 onward. Thus, the hyporesponsive phenotype of the TC $CD4^+$ T cell population was associated with their increased expression of markers associated with T cell regulation.

During the later stages of infection, CTLA-4 and $GITR^{high}$ expression by $CD4^+$ T cells also rose at distal sites. In the tLN, CTLA-4 expression was significantly higher than in naive controls (mean increase, 43% of $CD4^+$ T cells at day 40 and 30% at day 60; $n = 5$ and $p < 0.05$ in each case, Mann-Whitney). $GITR^{high}$ expression also significantly increased at day 60 (36% above naive controls; $n = 5$ and $p < 0.05$). Similar changes were detected in splenic $CD4^+$ T cell populations at day 60 of infection (increases of 17% for CTLA-4, 33% for $GITR^{high}$; $n = 5$ and $p < 0.05$ in each case, Mann-Whitney). Interestingly, CD25 expression did not rise in the lymph nodes or spleen at any time point. Thus, at the time of most profound down-modulation of responsiveness, the dominant phenotype in the TC ($CD25^-GITR^{high}CTLA\text{-}4^+$) became more frequent in sites peripheral to the locus of infection.

Filarial infection induces early expression of Foxp3

Expression of the *Foxp3* gene has been shown to be critical for the development of particular populations of Tregs. These include natural $CD4^+CD25^+$ Tregs (41–43), with increasing evidence for a role in adaptive Treg populations (44, 45), although not all Treg populations express *Foxp3* (46). To determine whether filarial infection expands or induces Foxp3-dependent Treg activity, the expression of *Foxp3* mRNA by $CD4^+$ T cells from the TC and draining lymph nodes of infected mice was measured. Expression was measured in whole purified $CD4^+$ T cell populations, as CD25, CTLA-4, and GITR mark both Tregs and activated effector T cells, and even in the naive mouse, regulatory *Foxp3*-expressing T cells can be found in the $CD25^-$ fraction (13).

The expression of *Foxp3* was found to be increased in $CD4^+$ T cells isolated from the lymph nodes draining the initial s.c. infection site 12 days postinfection (Fig. 4*A*). At days 28 and 60 postinfection, the ratio of *Foxp3* to β-actin in the tLN $CD4^+$ T cell population was equivalent to that of the naive controls (Fig. 4, *B* and *C*). However, at these times, the total number of $CD4^+$ T cells in the tLN had increased substantially (Fig. 4*D*), indicating that there is expansion of both $Foxp3^+$ and $Foxp3^-$ populations maintaining the *Foxp3*:β-actin ratio at a steady state. Thus, the initial prominent increase in Foxp3 activity is balanced by expansion of effector cells ($Foxp3^-$), and once equilibrium is regained, both cell populations maintain homeostasis.

Unlike the tLN $CD4^+$ T cell population, $CD4^+$ T cells from the site of infection did not show any increases in the *Foxp3*:β-actin ratio, when tested at days 12, 28, and 60 (data not shown). Expansion of the TC $CD4^+$ T cell population does also occur (47), thus there appears to be proportional expansion of both $Foxp3^+$ and $Foxp3^-$ cells. Notably, the dominance of hyporesponsive $CD4^+CTLA\text{-}4^+GITR^+$ T cells at 60 days postinfection was not matched by an increase in the Foxp3:β-actin ratio, as would be expected if these cells represented a Treg population. Therefore, the $CD4^+CD25^-CTLA\text{-}4^+GITR^+$ T cells at the site of infection are likely to represent a down-modulated effector cell population.

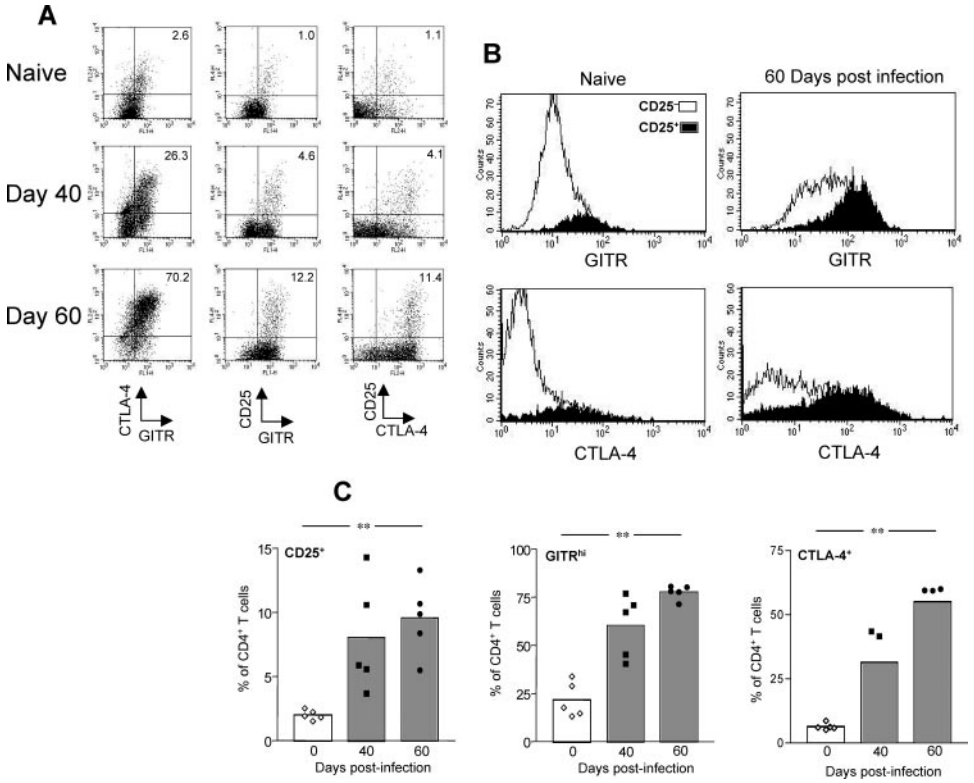

FIGURE 3. Treg-associated marker expression by $CD4^+$ T cells from infected mice. *A*, Expression of CD25, GITR, and intracellular CTLA-4 by TC $CD4^+$ T cells during *L. sigmodontis* infection. Data are from individual mice and are representative of two experiments. The naive, day 40 and day 60 samples shown, were analyzed on the same day. The percentage of double-positive cells is indicated for each plot. *B*, Expression levels of GITR and intracellular CTLA-4 by TC $CD4^+CD25^-$ (open histograms) and $CD4^+CD25^+$ cells (filled histograms) from naive and 60-day-infected BALB/c mice. Data are from individual mice, representative of five experiments. *C*, Percentages of TC $CD4^+$ T cells expressing CD25, $GITR^{high}$, and intracellular CTLA-4 in naive and 40- or 60-day-infected mice. Bars represent mean values. The figures shown are representative of five experiments. **, $p < 0.01$ between experimental groups (Kruskall-Wallis).

Neutralizing Tregs results in increased parasite clearance

The hyporesponsive phenotype of the $CD4^+$ T cell population, and the increases seen first in *Foxp3* expression and subsequently in Treg-associated surface markers, indicate that infection stimulates Treg activity. To directly test whether Tregs are acting to suppress the host's effector responses and so promote parasite survival, Abs against CD25 and GITR were used in vivo to intervene against regulatory control during an established infection. The anti-CD25 mAb PC61 has been shown to deplete Tregs expressing CD25, and in our hands the circulating $CD4^+CD25^+$ population was effectively removed for at least 7 days after treatment of naive mice (data not shown). The anti-GITR Ab is an agonistic Ab and has been shown to ablate regulatory activity of $CD4^+CD25^+$ T cells (13), to act as a costimulator toward activated $CD4^+$ effector T cells (48–51), and to render them resistant to suppression by Tregs (52).

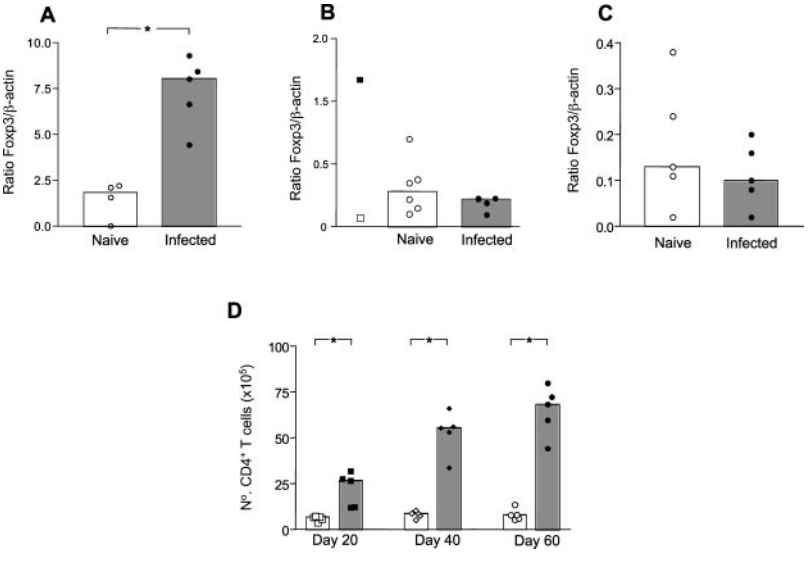

FIGURE 4. Filarial infection induces an early increase in *Foxp3* mRNA expression. $CD4^+$ T cells were purified from the brachial lymph node of naive (open symbols) and infected mice (closed symbols) 12 days postinfection (*A*) and from the tLN on days 28 (*B*) and 60 (*C*) postinfection. On day 28, $CD4^+CD25^+$ (■) and $CD4^+CD25^-$ (□) cells isolated from naive spleens were included as positive and negative controls, respectively. The relative expression of *Foxp3* mRNA was assessed as a ratio to *β-actin* mRNA using quantitative PCR. Bars represent median values. Results are representative of two independent experiments. *, $p < 0.02$ (Mann-Whitney). *D*, Total number of $CD4^+$ T cells within the tLN of naive (open symbols) and infected (closed symbols) mice. *, $p < 0.01$ between infected group and respective naive controls.

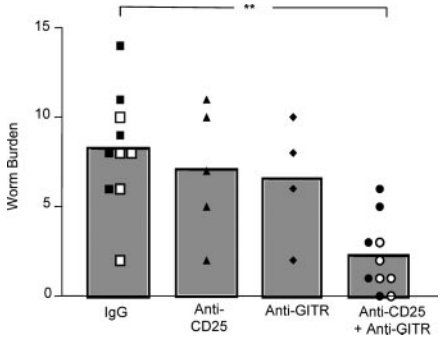

FIGURE 5. In vivo anti-CD25 and anti-GITR treatment of an established infection results in increased killing of parasites. Sixty-day worm burdens of infected mice treated with rat IgG, anti-CD25 alone, anti-GITR alone, or combined treatment with anti-CD25 and anti-GITR. Closed and open symbols represent results from two experiments. Bars represent mean values. **, Significant difference between the IgG group and the anti-CD25/GITR cotreated group. In experiment 1 (filled symbols), $p < 0.01$ (Mann-Whitney); in experiment 2 (open symbols), $p < 0.02$.

Ab treatments were given 27 days postinfection (the time of final molt to adult worms) to allow sufficient time for the parasite to establish its regulatory mechanisms within the host. Autopsies were performed before (day 40) and after (day 60) development of patent microfilaremia. Treatments with anti-CD25 or anti-GITR alone had no effect on worm burdens when assessed 40 or 60 days postinfection. Combining the two treatments, however, did result in a significant reduction in mean worm load of 73% compared with the IgG-treated control mice when quantified 60 days postinfection (Fig. 5).

The hypothesis that the anti-CD25/GITR treatment regime had broken a regulatory network was given credence by analysis of cell surface profiles and cytokine responses 60 days postinfection. At the site of infection, anti-CD25/GITR cotreatment resulted in a substantial depletion of $CD4^+CD25^+$ T cells (Fig. 6*A*). Notably, whereas expression of the costimulatory molecule GITR was only marginally affected (Fig. 6*B*), a marked decrease in the expression of the coinhibitory molecule CTLA-4 on $CD4^+$ T cells was observed (Fig. 6*C*). This demonstrated the long-term effect of Ab treatment on the $CD4^+$ T cell population. Although CD25 is also expressed by B cells, $CD8^+$ T cells, F4-80^+ macrophages, and dendritic cells during *L. sigmodontis* infection (our unpublished observations), B cells and $CD8^+$ T cells are unlikely to be responsible for regulation, as their depletion or absence does not reverse susceptibility (53, 54). We have also shown that dendritic cells isolated from the tLN of infected mice do not show suppressive activity in vitro (our unpublished observations). Infection does recruit suppressive F4/80^+ macrophages; however, this population was unaltered as a result of anti-CD25/GITR treatment (our unpublished observations). Thus, non-$CD4^+$ T cells do not appear to play a dominant regulatory role during *L. sigmodontis* infection.

Alongside the decrease in regulatory markers, we observed significantly increased in vitro Ag-specific responses by tLN cells isolated from anti-CD25/GITR cotreated mice, including higher levels of Ag-specific cytokines (Fig. 7) and proliferation (data not shown). Of particular interest was the increase in IL-5 production (Fig. 7*B*), as IL-5 is characteristically suppressed during chronic human filariasis (3, 4) and plays an important role in immunity toward *L. sigmodontis* (55, 56). Killing of *L. sigmodontis* by IL-5 has been linked to the encapsulation of worms by immune cells (54, 55), and encapsulated worms were present in the cotreated group (data not shown). The number of encapsulated worms was low, however, suggesting that the majority of worms had already been cleared by the immune system. Also of note was the increase in IL-10 production after neutralization of Tregs (Fig. 6*C*), implying that IL-10 is not the dominant suppressive mechanism. Costimulation of $CD4^+$ T cells by anti-GITR Ab has been noted to increase IL-10 expression elsewhere (50), and during *L. sigmodontis* infection it may be representative of an enhanced Th2 response rather than regulation. These experiments represent the first "immunological cure" of a filarial infection in which Abs to immune cell surface markers can reverse unresponsiveness and provoke parasite killing.

$CD4^+CD25^+$ T cells from the site of infection show poor responsiveness to Ag in vitro but do not suppress the proliferation of Th2 effector cells

Given the efficacy of anti-CD25/GITR cotreatment from 27 days postinfection, $CD4^+CD25^+$ T cells were purified from the site of infection during this time period to test their ability to inhibit the *L. sigmodontis*-specific response of $CD4^+CD25^-$ T cells. Fig. 8 demonstrates that TC $CD4^+CD25^-$ cells showed stronger proliferative responses toward both LsAg and anti-CD3 stimulation than did $CD4^+CD25^+$ T cells. Production of the effector cytokine IL-5 showed a similar pattern to that of proliferation (data not shown). When mixed at a 1:1 ratio, the $CD4^+CD25^+$ cells did not inhibit proliferation of the $CD4^+CD25^-$ cells (Fig. 8). In the same assay, and under the same conditions, naive $CD4^+CD25^+$ cells were able to inhibit the responses of naive $CD4^+CD25^-$ cells to anti-CD3 stimulation (data not shown). Th2 cells are known to be more resistant to in vitro suppression by $CD4^+CD25^+$ Tregs (57), as they can expand in the absence of IL-2 through responsiveness to Th2 growth factors such as IL-4, IL-7, and IL-9 (57, 58). Thus, although the majority of TC proliferating cells are of the $CD4^+CD25^-$ phenotype, their proliferation is not suppressed by the $CD4^+CD25^+$ fraction, either because the $CD4^+CD25^-$ cells are not IL-2 dependent or because the $CD4^+CD25^+$ cells do not exert classical in vitro Treg suppressive ability at this stage of the infection.

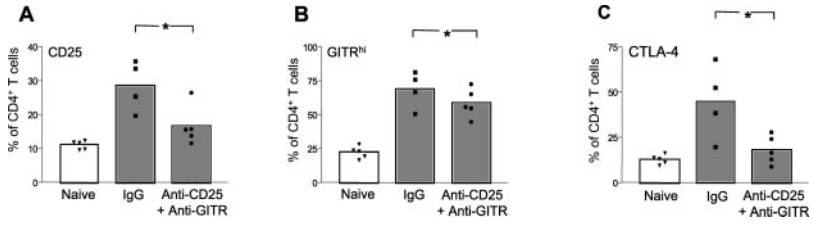

FIGURE 6. Anti-CD25 and anti-GITR treatment decreases expression of regulatory markers on $CD4^+$ T cells. CD25 (*A*), $GITR^{high}$ (*B*), and CTLA-4 (*C*) expression in TC $CD4^+$ T cells from naive mice and *L. sigmodontis*-infected IgG-treated or anti-CD25/GITR cotreated mice 60 days postinfection. Bars represent mean values. Figure shows one representative experiment. *, $p < 0.05$ between the IgG and anti-CD25/GITR cotreated groups. Analysis was performed using data from two experiments using a general linear model of ANOVA.

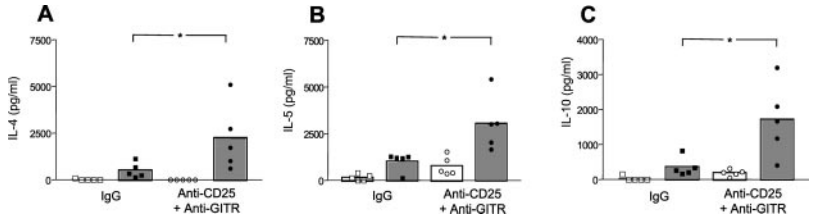

FIGURE 7. Anti-CD25 and anti-GITR treatment enhances immune responsiveness. IL-4 (*A*), IL-5 (*B*), and IL-10 (*C*) production by tLN cells from IgG control mice (squares) and anti-GITR/anti-CD25 cotreated mice (circles) restimulated with medium alone (open symbols) or LsAg (filled symbols). Bars represent mean values. Figures show one representative experiment. ∗, $p < 0.05$. Analysis was performed using data from two experiments using a general linear model of ANOVA.

IL-10 controls parasite-specific Th1 responses but does not play a primary role in filarial survival

Increased production of IL-10 occurs in chronic human filarial infections and has been associated with filarial immunosuppression (5, 7). IL-10 is therefore a potential mediator of $CD25^+GITR^+$ cell activity; however, IL-10 production declined as immunosuppression became established (Figs. 1*F* and 2*D*) and rose after negation of Treg activity (Fig. 7*C*). To determine whether IL-10 played a role in the observed T cell hyporesponsiveness, $CD4^+$ T cells were isolated from the TC 60 days postinfection and were restimulated with LsAg in the presence of a neutralizing anti-IL-10R Ab. The doses of anti-IL-10R chosen, 0.1 and 0.01 μg/ml, were based on those that gave maximal increases in IFN-γ production during in vitro culture of tLN cells from 60-day infected mice (data not shown). Fig. 9*A* demonstrates that neutralizing the IL-10R did not restore the proliferative capability of the TC $CD4^+$ T cells, although it did increase their production of IFN-γ (Fig. 9*B*). Therefore, IL-10 does not appear to mediate the hyporesponsive phenotype of the TC $CD4^+$ T cell population.

To test the role of IL-10 in the long-term survival of *L. sigmodontis* in vivo, mice were treated with four doses of anti-IL-10R Ab from the time of the final molt into adult worms (between days 27 and 36 postinfection). In two independent experiments, mice were autopsied on day 40 or 60. Neutralizing the IL-10R failed to alter worm burdens when assessed at either time point (Fig. 9*C*). Treatment did, however, significantly increase both background and Ag-specific IFN-γ production (Fig. 9*D*) without rescuing production of IL-4, IL-10, or IL-5 (data not shown). Thus, we conclude that IL-10 does not play a major role in controlling parasite recoveries.

Discussion

Human filarial parasites can survive for decades within normally immunocompetent hosts, with long-term survival being linked to suppression of the host protective immune response. Evidence for Treg activity in human helminth infections has been provided by T cell clones from onchocerciasis patients with a regulatory profile (30, 59) and the finding from lymphatic filariasis patients that CTLA-4^+ expressed on T cells suppresses IL-5 production (31). In mouse models, $CD4^+CD25^+$ T cells are active during *S. mansoni* infection, influencing Th subset development and the evolution of pathology (28, 32). These studies indicate that Tregs are generated during helminth infections and have led to the hypothesis that their activity down-modulates immune responsiveness during chronic infection, promoting parasite survival (14, 15, 60).

We now present the first experimental evidence that Tregs permit the establishment of a helminth infection. The initial infection of BALB/c mice by *L. sigmodontis* larvae resulted in an increase in expression of *Foxp3*, a gene critically associated with the development of Tregs (41–43), indicating that infection induces an early expansion of Treg activity. Once infection had become fully

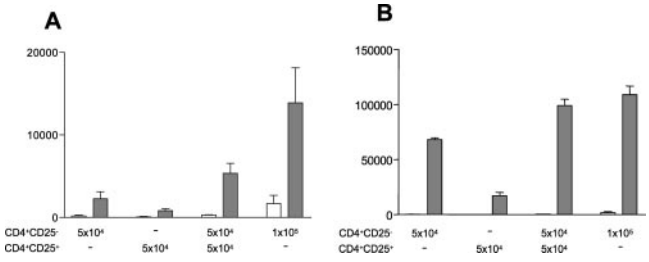

FIGURE 8. In vitro responses of TC $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified from the TC of infected mice 28 days postinfection. They were restimulated in vitro with LsAg (*A*) and anti-CD3 (*B*) as separate populations or mixed in a 1:1 ratio using the indicated cell numbers, and their proliferative responses were measured. Error bars represent SD of triplicate cultures. Results are representative of two independent experiments.

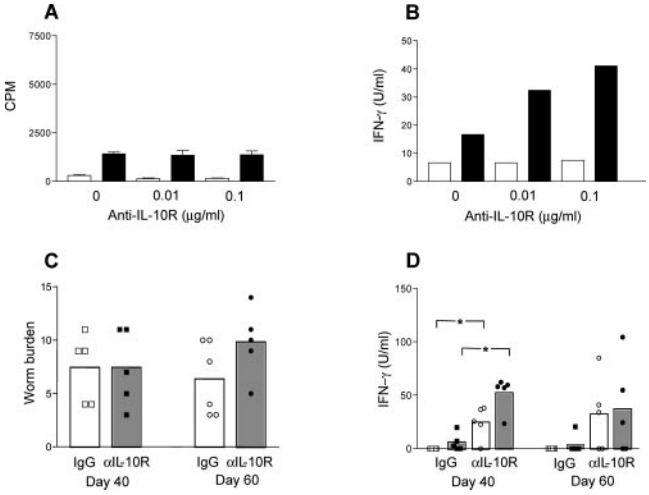

FIGURE 9. IL-10 does not play a major role in filarial survival. *A*, $CD4^+$ T cells were isolated from the TC of *L. sigmodontis*-infected mice 60 days postinfection, and their proliferative responses were measured after in vitro restimulation with medium alone (open bars) and LsAg (filled bars) in the presence of varying doses of anti-IL-10R. *B*, $CD4^+$ T cells were isolated from the TC of *L. sigmodontis*-infected mice 60 days postinfection, and their production of IFN-γ was measured after in vitro restimulation with medium alone (open bars) and LsAg (filled bars) in the presence of varying doses of anti-IL-10R. *C*, Worm recovery from *L. sigmodontis*-infected mice given 1 mg of anti-IL-10R (filled symbols) or rat IgG (open symbols) on days 27, 30, 33, and 36. In two independent experiments, worm burdens were assessed on day 40 (squares) or day 60 (circles). Bars represent mean values. Groups are not significantly different. *D*, Production of IFN-γ by tLN cells isolated from anti-IL-10R or IgG treated mice 40 days (squares) or 60 days (circles) postinfection and restimulated in vitro with medium (open symbols) or LsAg (filled symbols). Bars represent mean values. ∗, $p < 0.05$ (Mann-Whitney).

patent (defined as fully developed adult worms and blood microfilaremia), BALB/c mice showed a loss of parasite-specific immune responses, concordant with the filarial-induced suppression seen in humans. Functional hyporesponsiveness was associated with significant increases in expression of the Treg-associated markers CD25, CTLA-4, and GITR, which was most marked in the TC population in contact with parasites. Two subsets could be differentiated by CD25 expression once infection had reached patency. The majority of TC $CD4^+$ T cells coexpressed CTLA-4 and GITR but were CD25 negative. In contrast, the $CD25^+$ population had the highest levels of CTLA-4 and GITR.

To experimentally test the hypothesis that Tregs are responsible for filarial-induced suppression and worm survival, we treated infected BALB/c mice with anti-CD25 and anti-GITR. We demonstrated that if depletion of $CD25^+$ cells is combined with the use of an agonistic anti-GITR Ab, BALB/c mice with established infections are induced to kill their adult worms. Thus, we can conclude that a population of $CD25^+$ Tregs dampens protective immunity to filarial parasites and that ligation of GITR is also necessary for protection to be effective.

The requirement for cotreatment with anti-GITR and CD25 suggests that neither treatment alone is sufficient to fully neutralize regulation. A similar requirement for combined treatments has been observed during infection with murine leukemia virus (61), and combined anti-CD25 and anti-CTLA-4 treatments are more effective at breaking self-tolerance than either Ab alone (62). One explanation is that both Abs act synergistically on a single regulatory population. Alternatively, as anti-GITR treatment can act as a costimulatory molecule for effector T cells (49–51) and can make effector cells more resistant to suppression by Tregs (52), a second possibility is that the anti-GITR Ab is acting on a nonregulatory effector T cell. In agreement with this, during the adult stage of infection, the expression of *Foxp3* mRNA in the TC did not correlate with that of GITR or CTLA-4. This suggests that the majority of $CD4^+GITR^{high}CTLA\text{-}4^+$ T cells are effector cells and that their hyporesponsive phenotype seen at patency is not due to increased Foxp3-dependent Treg activity. The $CD4^+CD25^-GITR^{high}CTLA\text{-}4^+$ cells therefore might represent an effector cell population that has been "conditioned" toward a down-modulated or hyporesponsive phenotype over the course of infection, and GITR ligation may be delivering an activation, or anti-regulatory, signal to effector T cells (48–51, 63). Thus, the success of combined Ab treatment may be due to two separate actions on distinct target populations: the anti-CD25 Ab, which depletes the Treg population, and anti-GITR, which reactivates down-modulated anti-parasite effector cells, allowing them to reach their potential in eradicating infection.

In opposition to the costimulatory effects of GITR, signals through CTLA-4 are well documented to inhibit T cell responses (64). Expression of CTLA-4 is required for the hyporesponsive phenotype of $CD4^+$ T cells induced by oral tolerance (65) and plays a critical role in limiting Th2 responses (66). In relation to Th2-inducing parasites, CTLA-4 blockade during *Nippostrongylus brasiliensis* infection resulted in enhanced parasite-specific immunity, stronger Th2 cytokine production, and diminished parasite numbers (67). CTLA-4 expressed on $CD4^+CD25^-$ T cells during infection therefore might mediate their hyporesponsive phenotype, and its up-regulation may be a mechanism by which effector T cell response is turned off. Thus, during patent *L. sigmodontis* infection, the responsiveness of the $CD4^+CD25^-$ T cell population may reflect a balance between inhibitory signals through CTLA-4 and costimulatory signals through GITR. Interestingly, anti-CD25/GITR treatment caused a greater reduction in CTLA-4 expression than GITR expression, potentially rendering the $CD4^+CD25^-$ population more responsive to costimulatory than inhibitory signals.

The killing of adult *L. sigmodontis* worms induced by combined anti-CD25/GITR treatment was associated with a general enhancement of parasite-specific immunity. Of particular note was the increase in IL-5, a key cytokine in the development of immunity to *L. sigmodontis* larval stages, both in vaccination models and in primary infections (54–56, 68). IL-5 is also strongly involved in the lethal response to adult *L. sigmodontis* worms after the establishment of patent infections (55, 68). The increased production of IL-5 is therefore a likely candidate for orchestrating the clearance of adult worms after Ab treatments. In relation to the human infection, IL-5 is one of the cytokines characteristically suppressed in individuals with chronic filarial infection (3, 4), and thus it is likely that the down-regulation of IL-5 during infection is an important dictate of parasite survival.

Although IL-10 is implicated in mediating proliferative suppression in filariasis patient-derived T cells in vitro (69), our data do not demonstrate a regulatory role on the Th2 response. After an early peak, IL-10 production declined during *L. sigmondontis* infection and was actually increased upon successful treatment of infection; moreover, Ab-mediated neutralization of IL-10R in vitro did not restore T cell responsiveness and in vivo failed to restore protective immunity or to change the intensity of the Th2 response. In resistant mouse strains, IL-10 deficiency alone failed to influence resistance or susceptibility, although a regulatory role for IL-10 did come to light in the absence of IL-4, indicating a role in regulating non-Th2 components (70). IL-10, therefore, might still play a role in regulating *L. sigmodontis* infection, possibly acting in conjunction with other cytokines such as TGF-β (71), but our study demonstrates that it is not the key or sole mechanism for $CD4^+CD25^+$ Treg action.

This outcome contrasts with the prominent role of Treg-derived IL-10 in infection models where Th1 immune responses are protective (72). A prime example is *Leishmania major* infection, in which IL-10 is the pivotal down-regulatory mediator produced by $CD4^+CD25^+$ T cells and acts to inhibit the protective Th1 response (23, 73). It is important to note, however, that IL-10 generally exerts a more profound suppression of Th1 than Th2 responses (32). In infections with Th2-inducing helminth parasites, IL-10 is in fact required to promote Th2 responses, such that $IL\text{-}10^{-/-}$ mice fail to mount protective Th2 responses toward *Trichuris muris* and develop chronic infections (74). In the immune response to *S. mansoni* infection, which contains both Th1 and Th2 components, IL-10 production by $CD4^+CD25^+$ Tregs is linked to the inhibition of Th1 immunity and subsequent augmentation of Th2 (28, 32). Similarly, differential effects of IL-10 on Th1 and Th2 responses have been noted during infection with *Trichinella spiralis*, where IL-10 deficiency has been found to inhibit Th2-mediated killing of the adult worms, while simultaneously promoting the Th1-mediated killing of muscle larvae (75). We conclude that although IL-10 does dampen Th1 immunity during *L. sigmodontis* infection, it does not play a key role in promoting parasite survival in the largely Th2-dependent context of immunity to *L. sigmodontis*.

A fascinating issue for future study is how nematodes induce Treg activity. One possibility is that long-lived parasites actively induce Tregs, for example by signaling through particular TLRs to induce the production of regulatory cytokines (76) or by secretion of TGF-β homologues (38). If the parasites themselves are driving Treg activity, then a central question will be the lineage of the activated Treg population: do parasites recruit pre-existing "natural" Tregs or do they induce development of Treg from Th0 precursors? Tregs responding to *L. major* and certain viral infections

do appear to be recruited from the pre-existing populations (17, 23), whereas *Bordetella pertussis* infection induces naive Th0 precursor cells to become Tr1 cells (20). The expression of *Foxp3* mRNA, a marker for natural Tregs, was increased during the early stages of *L. sigmodontis* infection, suggesting that a natural pre-existing Treg population is expanded in the presence of the parasite. However, Foxp3$^+$ regulatory T cells can develop from naive precursors under the influence of TGF-β (44, 45), and as *L. sigmodontis* provoked TGF-β production throughout infection, it is also possible that a new population of Tregs was actively induced by this route.

If the Tregs involved are of "natural" origins, a second question of equal importance is whether the Treg populations are specific for parasite Ags or if they act in a nonspecific manner to prevent overly exuberant immune responses. Helminth-induced Tregs are capable of Ag-nonspecific suppression, as CD4$^+$CD25$^+$ T cells isolated from mice infected with *Heligmosoides polygyrus* are able to inhibit inflammation in a murine model of allergy.[5] Equally, the presence of a patent *L. sigmodontis* infection down-modulates inflammation invoked during allergic airway infiltration (M. S. Wilson, M. D. Taylor, and R. M. Maizels, unpublished observations), demonstrating that whether or not Tregs are parasite-specific, they can act in an Ag-nonspecific manner. Definition of their Ag specificity will be important to allow therapeutic interventions to be targeted solely against the Tregs involved in infection, rather than indiscriminately against whole Treg populations, where treatment could also potentially disrupt self-tolerance.

In conclusion, we offer a wider perspective for the role of Tregs in chronic helminth infections, which afflict >1 billion people worldwide. Although it is not surprising that the response to infection should engender a regulatory component, our data argue that Tregs are essential to continued parasite survival. These findings provide a conceptual framework to understand helminth parasitism, in which host regulatory pathways are recruited or exploited by the pathogens to aid their own survival. This understanding, in turn, will suggest therapeutic pathways to boost host immunity (either naturally or in the context of vaccination) to ensure that effector mechanisms gain precedence over the inappropriately invoked down-regulation.

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Disclosures

The authors have no financial conflict of interest.

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