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CTLA-4 and CD4$^+$CD25$^+$ Regulatory T Cells Inhibit Protective Immunity to Filarial Parasites In Vivo[1]

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The T cell coinhibitory receptor CTLA-4 has been implicated in the down-regulation of T cell function that is a quintessential feature of chronic human filarial infections. In a laboratory model of filariasis, *Litomosoides sigmodontis* infection of susceptible BALB/c mice, we have previously shown that susceptibility is linked both to a CD4$^+$CD25$^+$ regulatory T (Treg) cell response, and to the development of hyporesponsive CD4$^+$ T cells at the infection site, the pleural cavity. We now provide evidence that *L. sigmodontis* infection drives the proliferation and activation of CD4$^+$Foxp3$^+$ Treg cells in vivo, demonstrated by increased uptake of BrdU and increased expression of CTLA-4, Foxp3, GITR, and CD25 compared with naive controls. The greatest increases in CTLA-4 expression were, however, seen in the CD4$^+$Foxp3$^-$ effector T cell population which contained 78% of all CD4$^+$CTLA-4$^+$ cells in the pleural cavity. Depletion of CD25$^+$ cells from the pleural CD4$^+$ T cell population did not increase their Ag-specific proliferative response in vitro, suggesting that their hyporesponsive phenotype is not directly mediated by CD4$^+$CD25$^+$ Treg cells. Once infection had established, killing of adult parasites could be enhanced by neutralization of CTLA-4 in vivo, but only if performed in combination with the depletion of CD25$^+$ Treg cells. This work suggests that during filarial infection CTLA-4 coinhibition and CD4$^+$CD25$^+$ Treg cells form complementary components of immune regulation that inhibit protective immunity in vivo. ***The Journal of Immunology,*** **2007, 179: 4626–4634.**

Down-regulation of T cell function resulting in immune suppression is a quintessential feature of chronic helminth infections (1–7). In human filariasis, immunosuppression is characterized by the impairment of both Ag-specific Th1 and Th2 immune responses (8–10). Filarial parasites suppress host immunity through a variety of immunoregulatory layers involving T cells, macrophages, and dendritic cells (DC)[3] (6, 11–15). Of these layers, regulation within the CD4$^+$ T cell compartment plays a vital role in determining the outcome of infection (16), combining the induction of regulatory T (Treg) cell responses (10, 16, 17) and the development of intrinsically hyporesponsive or "conditioned" effector T (Teff) cells (10, 16). The observations that the T cell inhibitory receptor CTLA-4, linked with both CD4$^+$ Treg cell responses and Teff cell tolerance (18, 19), plays an in vitro role in filarial-induced suppression in humans has led to the hypothesis that it is an important element in controlling susceptibility to filariasis (10, 20).

CTLA-4 is a potent inhibitor of T cells and is involved in mediating T cell anergy and tolerance (18, 19). A consequence of its importance is illustrated by CTLA-4-deficient mice that die of lymphoproliferative disease within 3–4 wk of age (21, 22). CTLA-4 is constitutively expressed by CD25$^+$Foxp3$^+$ Treg cells (23, 24) and is up-regulated by CD4$^+$ Teff cells following antigenic stimulation. CTLA-4 can suppress immunity through a surprisingly diverse range of mechanisms, the most direct of which are competition with the positive costimulatory molecule CD28 for common ligands, along with transmission of a cell-intrinsic negative signal that inhibits T cell activation (25–27). One effect of signaling through CTLA-4 may be to abbreviate the dwell time between T cell and APC; therefore, further reducing the strength of TCR-mediated stimulation (28). Indirect mechanisms include stimulating the production of the regulatory cytokine TGF-β (29–31) and "back signaling" through CD80/CD86, causing up-regulation of indoleamine 2,3-dioxygenase resulting in the development of DC with inhibitory properties (32, 33). Although many of the regulatory roles of CTLA-4 are independent of and can be complementary to Treg cells (34, 35), CTLA-4 is also associated with the development and suppressive function of Treg cell responses (23, 24, 36, 37).

The inhibitory effects of CTLA-4 impede immunity to a variety of different infections (38–41). CTLA-4 is believed to preferentially modify Th2 responses (42) and has been shown to down-regulate Th2 immune responses to helminth infections, such that neutralizing CTLA-4 during *Nippostrongylus brasiliensis* infection enhances protective immunity (43). Inhibition through CTLA-4, therefore, makes a strong candidate for a mechanism of filarial immunosuppression. In both human filariasis and in murine infection models, expression of CTLA-4 by CD4$^+$ T cells is up-regulated upon infection (10, 16, 20) and is linked to CD4$^+$CD25$^+$ Treg and Tr1 cell responses (16, 17, 20, 44). In humans, CTLA-4 has been shown to play a role in the in vitro Ag unresponsiveness of PBMC, but rather than acting through CD4$^+$ Treg cells, CTLA-4 appears to mediate inhibition by inducing the expression of T cell anergy factors (10, 20). Similar observations have been noted during *Helicobacter pylori* infection, in which T cell unresponsiveness was due to CTLA-4 mediated T cell anergy rather than active suppression through CD4$^+$CD25$^+$ Treg cells (41).

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[3] Abbreviations used in this paper: DC, dendritic cell; Treg, regulatory T; Teff, effector T; Mf, microfilariae; LsAg, *Litomosoides sigmodontis* whole worm Ag; tLN, thoracic lymph node; PleC, pleural cavity cell; FC, flow cytometry; GLM, generalized linear model; pleCD4$^+$, pleural cavity CD4$^+$.

Using the model filarial nematode *Litomosoides sigmodontis* (45, 46), we have previously shown that $CD4^+$ T cell regulation is a major determinant of susceptibility to *L. sigmodontis* infection (16), allowing the development of patent infections in BALB/c mice. Patency is defined by the appearance of transmission stage microfilariae (Mf) in the bloodstream 55 days after infection. $CD4^+$ T cell regulation was denoted by a $CD4^+CD25^+Foxp3^+$ Treg cell response and a loss of $CD4^+$ T cell Ag-responsiveness at the site of infection (the pleural cavity) between 40 and 60 days after infection (16). Despite a role for $CD4^+CD25^+$ Tregs in inhibiting protective immunity to *L. sigmodontis*, the loss of $CD4^+$ T cell Ag responsiveness was not associated with increased expression of *Foxp3* mRNA, but was associated with dramatically up-regulated expression of the coinhibitory receptor CTLA-4 and the costimulatory molecule GITR. Simultaneously depleting $CD25^+$ Treg cells along with providing costimulatory signals through GITR restored T cell Ag responsiveness and resulted in enhanced killing of an established infection. These results led to our hypothesis that *L. sigmodontis* infection induces two distinct levels of T cell regulation, conventional $CD4^+Foxp3^+CD25^+$ Tregs and the development of a newly recognized intrinsically hyporesponsive phenotype within the $CD4^+CTLA\text{-}4^+GITR^{high}$ Teff cells (16). The Ag responsiveness of the $CD4^+$ Teff cells is controlled by the balance of signals received through their costimulatory and coinhibitory receptors.

We now demonstrate that CTLA-4 complements $CD25^+$ Treg cells in inhibiting protective immunity to filarial infection in vivo. We show that *L. sigmodontis* infection drove the proliferation and activation of $CD4^+Foxp3^+$ Treg cells, but provide evidence that $CD4^+$ Teff cell hyporesponsiveness was not due to active suppression by $CD4^+CD25^+$ Treg cells. Although infection up-regulated the expression of CTLA-4 on $CD4^+Foxp3^+$ Treg cells, the greatest increase in CTLA-4 expression was seen on the $CD4^+Foxp3^-$ Teff cells, suggesting that CTLA-4 is directly inhibiting $CD4^+$ Teff cells. Once infection had established, parasite killing could only be enhanced by simultaneously targeting both levels of T cell regulation: blockade of inhibitory signals through CTLA-4 in combination with $CD25^+$ Treg cell depletion.

Materials and Methods

Mice and parasites

Female BALB/c mice were used at 6–8 wk of age and maintained in individually ventilated cages. The *L. sigmodontis* life cycle was maintained in gerbils using the mite vector *Ornithonyssus bacoti* (46). Infective larvae (L3) were recovered from mites 13 days postfeeding by dissection in RPMI 1640 supplemented with 10% FBS (Invitrogen Life Technologies), and mice were infected s.c. on the upper back. Pleural lavage with 10 ml of cold AIM V medium was used to recover parasites. Recovered parasites were fixed in hot 70% ethanol for detailed morphological analysis including length, uterine contents, developmental state, and physiological condition (46). *L. sigmodontis* whole worm Ag (LsAg) was prepared by collecting the PBS-soluble fraction of homogenized adult male and female worms.

In vivo Ab and BrdU treatments

In vivo Ab treatments utilized anti-CTLA-4 (UC10-4F10-11) and anti-CD25 (PC61). Mice received either 1 mg of anti-CTLA-4 in PBS on days 27 and 32 alone or in combination with 1 mg of anti-CD25 on day 27. An equivalent dose of rat IgG was used as a control. For BrdU treatment, mice received i.p. injections of 0.8 mg of BrdU (Sigma-Aldrich) in PBS 24 and 16 h before autopsy.

Cell purifications and in vitro restimulations

The parathymic, posterior mediastinal, and paravertebral lymph nodes draining the thoracic cavity (tLN) were dissociated and washed in AIM V medium before being resuspended in RPMI 1640 with 0.5% mouse sera (Caltag Laboratories-Medsystems), 100 U/ml penicillin/100 μg/ml streptomycin, and 2 mM L-glutamine. Pleural cavity cells (PleC) were isolated from lavage fluid. For $CD4^+$ and $CD4^+CD25^-$ T cell purifications, PleC were adhered to plastic for 2 h at 37° and the nonadherent population was taken. CD4 purifications were performed using CD4 MicroBead magnetic cell sorting (Miltenyi Biotec) per the manufacturer's instructions, except that HBSS/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. To purify $CD4^+CD25^-$ T cells, CD25 depletion was performed before the CD4 purification using biotinylated anti-CD25 (PC61; BD Pharmingen) in combination with streptavidin MicroBead magnetic sorting (Miltenyi Biotec). $CD4^+$ T cell purities were 75.1–81.2% with 41.5–47% of the $CD4^+$ T cells from infected mice expressing CD25. $CD4^+CD25^-$ T cell purifications contained 85.2–89.2% $CD4^+$ T cells, of which 12–22% expressed low levels of CD25. Irradiated (30 Gy) splenic APC were added to 96-well round-bottom plates at 1 $\times$ 10^6 cells/well, along with 1 $\times$ 10^5 $CD4^+$ T cells/well. Whole tLN cells were used at 5 $\times$ 10^5 cells/well. Cultures were stimulated with medium alone or 10 μg/ml LsAg. Supernatants were sampled at 72 h for cytokine analysis, and 1 μCi/well [*methyl*-^{3}H]thymidine was added for 16 h to measure proliferation.

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. Biotin detection Abs were used with ExtrAvidin/alkaline phosphatase conjugate (Sigma-Aldrich) and Sigma-Aldrich FastTM *p*-nitrophenyl phosphate substrate. For flow cytometry (FC), nonspecific binding was blocked with 4 μg of rat IgG/1 $\times$ 10^6 cells and the following Abs applied: PE-conjugated anti-CTLA-4 (UC10-4F10-11), peridinin chlorophyll protein-conjugated streptavidin, allophycocyanin and FITC-conjugated anti-CD4 (RM4-5), biotinylated anti-CD25 (7D4), and FITC-conjugated anti-GITR (DTA-1, in house). Staining for Foxp3 was performed using FITC-, PE-, and allophycocyanin-conjugated anti-Foxp3 (FJK-16s; eBioscience) per the manufacturer's instructions. BrdU staining was performed after the final Foxp3 staining step using FITC-conjugated anti-BrdU with DNase according to the manufacturer's instructions (BD Pharmingen). Staining was compared against the relevant isotype controls to verify specificity. Because the majority of $CD4^+$ T cells expressed low levels of GITR when compared with the isotype control, FC plots were gated on $CD4^+GITR^{high}$ cells. To measure intracellular CTLA-4, cells were permeabilized with a BD Pharmingen Cytofix/Cytoperm kit or with a eBioscience Foxp3 permeabilization kit. Flow cytometric acquisition was performed using a FACSCalibur running CellQuest Pro software and a LSR 2 running FACSDiva software (BD Biosciences). Analysis was performed using CellQuest Pro and FlowJo (Tree star). Reagents were obtained from BD Biosciences unless otherwise stated.

L. sigmodontis-*specific Ab ELISA*

Serum was isolated from whole blood using SeraSieve (Hughes and Hughes). ELISA plates (Nunc) were coated with LsAg at a concentration of 5 μg/ml in 0.45 M $NaHCO_3$ (Sigma-Aldrich)/0.18 M Na_2CO_3 (Sigma-Aldrich). Plates were incubated with eight serial dilutions (1/200–1/25600) of mouse sera, and a representative dilution from the linear section of the dilution curve was selected for each isotype (1/800 for IgG1 and 1/200 for IgG2a and IgG2b). Detection of Ab isotypes was performed using HRP-conjugated anti-mouse IgG1, IgG2a, and IgG2b (Southern Biotechnology Associates) in combination with an ABTS peroxidase substrate system (KPL).

Statistics

Statistical analysis was performed using JMP version 6 (SAS). Analysis of combined data from multiple repeat experiments, or of experiments containing multiple groups, was performed using one-way or general linear model (GLM) ANOVA. When using GLM to combine data from multiple experiments, it was first verified that there were no significant differences between experiments before treatment effects were analyzed. Pairwise comparison between means was performed using the Wilcoxon rank test.

Results

$CD4^+Foxp3^+$ Treg cells actively respond to L. sigmodontis *infection, but are not directly associated with the in vitro $CD4^+$ T cell hyporesponsiveness*

Our previous work led to the hypothesis that *L. sigmodontis* infection induces two distinct components of T cell regulation within the pleural cavity: suppressive $CD4^+CD25^+$ Tregs and intrinsically hyporesponsive $CD4^+CTLA\text{-}4^+GITR^{high}$ Teff cells. To test

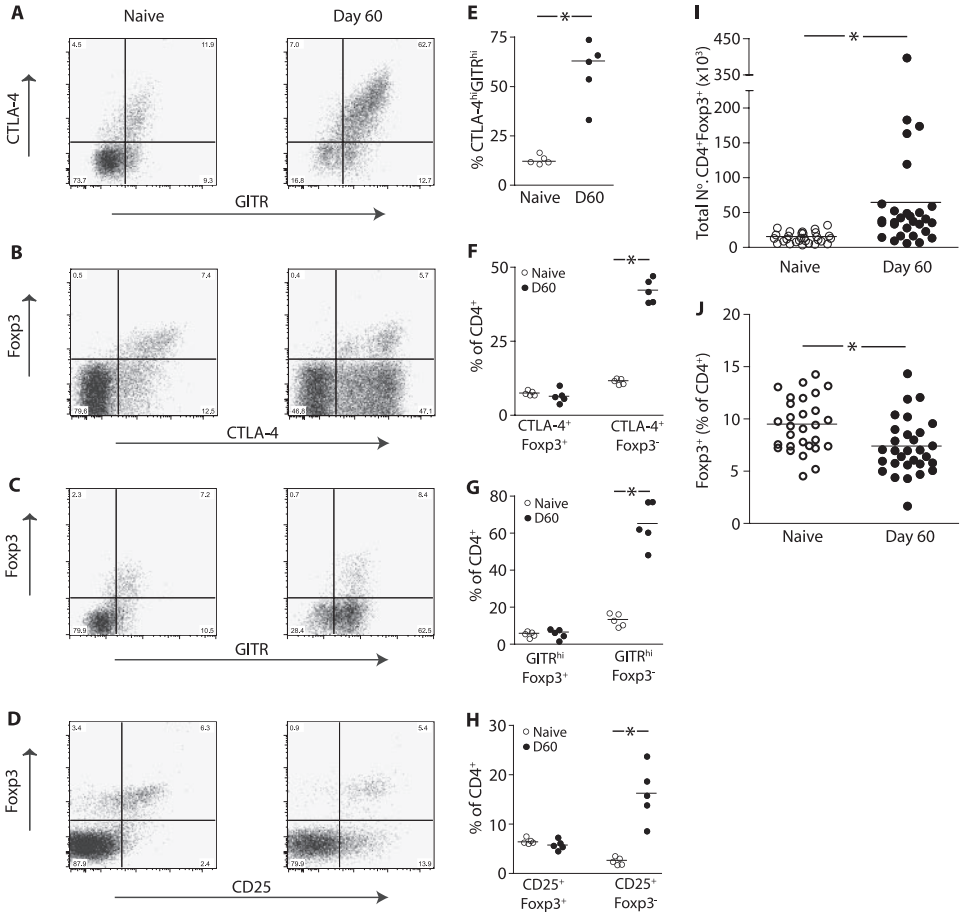

FIGURE 1. The majority of pleCD4$^+$CTLA-4$^+$GITR^{high} T cells do not express Foxp3. PleC from naive and *L. sigmodontis*- infected BALB/c mice were isolated 60 days postinfection and FC was used to analyze the expression of Foxp3, CD25, GITR, and CTLA-4 within the CD4$^+$ T cell population. *A–D,* Dot plots, gated on CD4$^+$ T cells, demonstrating expression of GITR vs CTLA-4 (*A*), Foxp3 vs CTLA-4 (*B*), Foxp3 vs GITR (*C*), and Foxp3 vs CD25 (*D*). Experiments were performed at least twice with four to six mice per group; data are from one representative mouse per group. *E,* Percentage of pleCD4$^+$ T cells from naive (○) and 60-day infected mice (●) coexpressing high levels of CTLA-4 and GITR. Symbols represent individual animals, and the line represents the median (∗, $p < 0.05$; Wilcoxon rank test). Results are representative of eight independent experiments. *F,* Percentage of CTLA-4$^+$Foxp3$^+$ and CTLA-4$^+$Foxp3$^-$ cells within the pleCD4$^+$ T cell population from naive (○) and 60-day infected mice (●). Symbols represent individual animals, and the line represents the median (∗, $p < 0.05$; Wilcoxon rank test). Results are representative of two independent experiments. *G,* Percentage of GITR^{high}Foxp3$^+$ and GITR^{high}Foxp3$^-$ cells within the pleCD4$^+$ T cell population from naive (○) and 60-day infected mice (●). Symbols represent individual animals, and the line represents the median (∗, $p < 0.05$; Wilcoxon rank test). Results are representative of two independent experiments. *H,* Percentage of CD25$^+$Foxp3$^+$ and CD25$^+$Foxp3$^-$ cells within the pleCD4$^+$ T cell population from naive (○) and 60-day infected mice (●). Symbols represent individual animals, and the line represents the median (∗, $p < 0.05$; Wilcoxon rank test). Results are representative of three independent experiments. *I,* Total number of CD4$^+$Foxp3$^+$ T cells within the pleural cavity of naive (○) and infected (●) BALB/c mice 60 days postinfection. Shown are combined data from six separate experiments, with symbols representing individual mice and the line representing the mean value (∗, $p < 0.01$; GLM). *J,* Percentage of CD4$^+$ T cells expressing Foxp3$^+$ within the pleural cavity of naive (○) and infected (●) BALB/c mice 60 days postinfection. Shown are combined data from six separate experiments, with symbols representing individual mice and the line representing the mean value (∗, $p < 0.01$; GLM).

whether the latter subset represents a Foxp$^-$ nonregulatory cell population, we stained the pleural cavity CD4$^+$ (pleCD4$^+$) T cells from naive and *L. sigmodontis*-infected BALB/c mice 60 days postinfection for intracellular Foxp3 and CTLA-4 and surface CD25 and GITR. In confirmation of our previous results, 50–70% of the pleCD4$^+$ T cell population from infected mice coexpressed high levels of CTLA-4 and GITR (Fig. 1, *A* and *E*). The pleCD4$^+$ T cells from infected mice also showed higher forward scatter and side scatter profiles than those from naive mice, indicative of a blasting phenotype (data not shown). Costaining for Foxp3 demonstrated that the majority of CD4$^+$CTLA-4$^+$GITR^{high} cells were Foxp3$^-$ and thus appear to represent an activated CD4$^+$ Teff cell population rather than a CD4$^+$Foxp3$^+$ Treg cell population (CTLA-4; Fig. 1, *B* and *F*; GITR, Fig. 1, *C* and *G*). Overall, in infection the majority of CD4$^+$CD25$^+$ T cells were also Foxp3$^-$ (Fig. 1, *D* and *H*). In fact, although there was a significant expansion in the absolute numbers of CD4$^+$Foxp3$^+$ Tregs in the pleural cavity 60 days after infection (Fig. 1*I*), their proportion within the CD4$^+$ T cell population was significantly decreased compared with naive mice (Fig. 1*J*).

Since total numbers of pleCD4$^+$Foxp3$^+$ Treg cells were increased, we tested whether infection was driving their expansion by using BrdU incorporation to assess the proliferation of different pleCD4$^+$ T cell subsets in vivo. Naive and infected mice were injected with BrdU on day 59 of infection and 24 h later ex vivo FC was used to assess BrdU uptake by Foxp3$^+$ and Foxp3$^-$CD4$^+$ T cells. In both the naive and infected mice, the CD4$^+$Foxp3$^+$ Treg population contained a significantly higher percentage of divided cells compared with the CD4$^+$Foxp3$^-$ Teff population, indicating that the CD4$^+$Foxp3$^+$ Treg cells generally show a higher

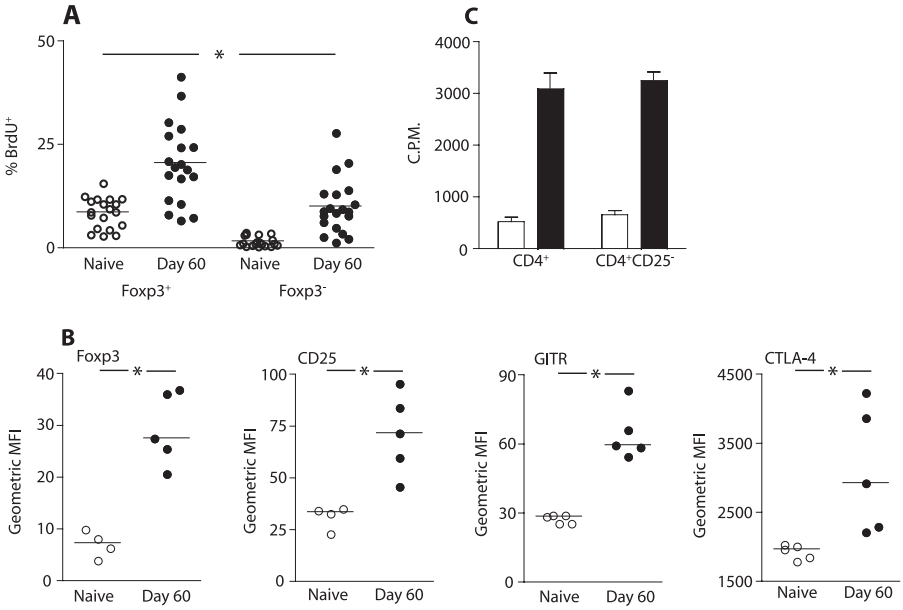

FIGURE 2. *L. sigmodontis* infection induces the proliferation and activation of $CD4^+Foxp3^+$ Treg cells. *A,* Naive (○) and infected (●) BALB/c mice were injected with BrdU on day 59 of infection, and on day 60 their PleC were isolated and stained ex vivo for CD4, Foxp3, and BrdU. Results are expressed as the percentage of $BrdU^+$ cells within the $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ populations. Shown are pooled data from four independent experiments. Symbols denote individual mice and lines represent the mean value (∗, the percentage of cells incorporating BrdU was significantly different between $Foxp3^+$ and $Foxp3^-$ cells independent of infection status ($p < 0.01$) and between naive and infected mice independent of Foxp3 expression ($p < 0.01$; GLM)). *B,* Pleural exudate cells from naive (○) and infected (●) were isolated 60 days postinfection and FC was used to determine the geometric mean fluorescence intensity of Foxp3, CD25, GITR, and CTLA-4 expression on $CD4^+Foxp3^+$ T cells. Symbols denote individual mice and lines represent the median values (∗, $p < 0.05$; Wilcoxon rank test). Results are representative of at least two independent experiments. *C,* $CD4^+$ and $CD4^+CD25^-$ T cells were purified from the pleural cavity of *L. sigmodontis*-infected mice 60 days postinfection and their proliferative responses were measured in vitro following restimulation with medium alone (□) and LsAg (■). Cultures were performed using cells pooled from at least five mice. Shown are mean and SD of triplicate cultures. Results are representative of two independent experiments.

rate of cell division in vivo (Fig. 2*A*). Infection did drive the proliferation of $CD4^+Foxp3^+$ Treg cells because there was a significant 2-fold increase in the percentage of dividing $CD4^+Foxp3^+$ Treg cells compared with naive mice. The $CD4^+Foxp3^-$ Teff cells from infected mice also showed significantly higher levels of proliferation in vivo with a 6-fold increase over the naive controls.

Alongside the increased proliferative response, the $CD4^+Foxp3^+$ Tregs from infected mice showed a more activated phenotype than those from naive mice, with significantly increased levels of expression of Foxp3, CD25, GITR, and CTLA-4 over the naive controls (Fig. 2*B*). Given the activated phenotype of the $CD4^+Foxp3^+CD25^+$ Treg cells, we tested whether they were inhibiting the Ag-specific responsiveness of the $pleCD4^+$ T cell population. $CD4^+$ T cells and $CD4^+CD25^-$ T cells were purified from the pleural cavity 60 days after infection and their ability to respond to LsAg in the presence of irradiated spleen cells from naive animals was compared in vitro. Depleting the $CD4^+CD25^+$ T cells from the whole $CD4^+$ T cell population did not increase their Ag-specific proliferative responses (Fig. 2*C*). Thus, infection with *L. sigmodontis* drives the activation and proliferation of $CD4^+Foxp3^+CD25^+$ Treg cells; however, the $CD4^+Foxp3^+CD25^+$ Treg cells do not appear to be responsible for the previously documented loss of in vitro Ag responsiveness of the $pleCD4^+$ T cells (16) since their depletion did not increase Ag-specific proliferation.

In vivo neutralization of CTLA-4 enhances parasite killing when combined with the depletion of $CD25^+$ Treg cells

The high expression of intracellular CTLA-4 by the $pleCD4^+Foxp3^-$ Teff cells and the $pleCD4^+Foxp3^+$ Treg cells suggests a role for CTLA-4 in suppressing T cell responses during *L. sigmodontis* infection. To directly test whether CTLA-4 is acting to inhibit protective immunity to *L. sigmodontis*, either through or alongside $CD4^+CD25^+$ Treg cells, we treated *L. sigmodontis*-infected BALB/c mice with a neutralizing anti-CTLA-4 Ab alone or in combination with a depleting anti-CD25 Ab. We have previously shown that anti-CD25 treatment alone does not affect parasite recovery (16), and due to restrictions in parasite material this group was not included. Ab treatments were started 28 days postinfection, at the time of the final moult from the L4 to adult stage, and autopsies were performed at day 60 when infection normally becomes patent. Although neutralization of CTLA-4 alone had no effect on parasite recoveries, combined treatment with anti-

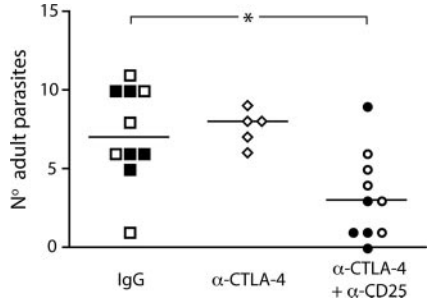

FIGURE 3. Neutralization of CTLA-4 in combination with CD25 depletion enhances parasite killing. *L. sigmodontis*-infected mice were treated with anti-CTLA-4 alone (◇), anti-CTLA-4 and anti-CD25 (●, ○), or controls treated with rat IgG (■, □). Anti-CTLA-4 was given on days 28 and 35 and anti-CD25 on day 28. Adult worm recoveries were assessed 60 days postinfection. Open and closed symbols, Two independent experiments. Symbols denote individual mice and lines represent median values (∗, $p <$ 0.01 between IgG and anti-CTLA-4/CD25-cotreated group; GLM).

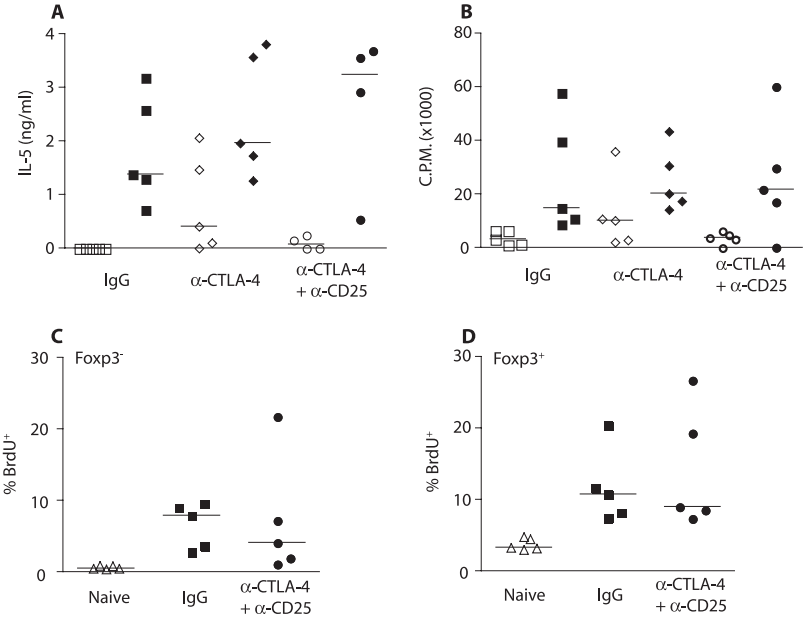

FIGURE 4. Parasite killing is not associated with increased cytokine or proliferative responses. tLN cells were isolated from IgG-treated (■, □), anti-CTLA-4-treated (◇, ♦), and anti-CTLA-4/anti-CD25-cotreated (●, ○) mice 60 days postinfection and restimulated in vitro with medium alone (□, ◇, ○) and LsAg (■, ♦, ●). Ag-specific production of IL-5 (*A*) and proliferation (*B*) were measured. Results are representative of two independent experiments. Symbols denote individual animals and lines represent the median value. *C* and *D*, Naive (△) and *L. sigmodontis*-infected mice (■, ●, ♦) were injected with BrdU on day 59 of infection and FC was used to assess BrdU incorporation by pleCD4$^+$Foxp3$^-$ (*C*) and pleCD4$^+$Foxp3$^+$ (*D*) T cells on day 60. Infected mice were either treated with control rat IgG (■, □) or cotreated with anti-CD25 and anti-CTLA-4 (●, ○). Symbols denote individual animals and lines represent the median values.

CTLA-4 and anti-CD25 significantly reduced adult parasite burden by 54% (Fig. 3). Despite the reduction in the number of adult worms, treatment had no detectable effect on the development of the surviving parasites, because there were no differences in growth, uterine embryogenesis, or the proportion of adult females with uterine Mf (data not shown). Treatment also had no affect on the prevalence of patent infections, because the percentage of mice with blood Mf was equivalent between groups (data not shown). Overall, this suggests that CTLA-4 and CD25$^+$ Treg cells synergize to inhibit protective immunity since parasite killing could only be promoted by neutralizing both suppressive components.

Enhanced parasite killing is not associated with increased cellular immune responsiveness

Previously, we have shown that the killing of *L. sigmodontis* adult parasites by combined anti-GITR and anti-CD25 treatments is associated with a long-term increase in Ag-specific immune responsiveness (16). To determine whether the increased parasite killing caused by anti-CTLA and anti-CD25 cotreatment was associated with a similar restoration of immune responsiveness, we measured the in vitro Ag-specific proliferative and cytokine responses of tLN cells following treatment. Neither anti-CTLA-4 treatment alone or in combination with anti-CD25 resulted in increased Ag-specific production of IL-5 (Fig. 4*A*) or of IL-4, IL-10, or IFN-γ (data not shown). Similarly, there was no increase in Ag-specific proliferation following a single treatment with CTLA-4 or cotreatment with anti-CD25 and anti-CTLA-4 (Fig. 4*B*). Because in vitro assays may not always reflect in vivo responses, BrdU incorporation was used to assess the proliferation of pleCD4$^+$Foxp3$^-$ or pleCD4$^+$Foxp3$^+$ populations in vivo. Treatment failed to affect the proliferation of either cell population within the pleural cavity (Fig. 4, *C* and *D*) or tLN (data not shown).

Alongside increases in immune responsiveness, combined anti-CD25 and anti-GITR treatments changed the activation phenotype of the pleCD4$^+$ T cells, resulting in reduced expression of CD25, CTLA-4, and GITR (16). Interestingly, despite performing a CD25

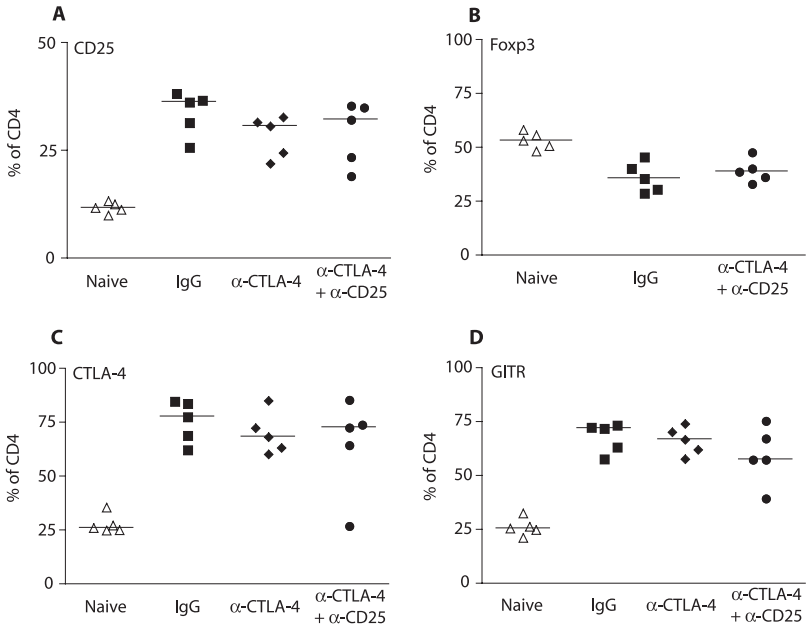

FIGURE 5. Treatment with anti-CTLA-4 and anti-CD25 does not induce a long-term change in the activation phenotype of the pleCD4$^+$ T cells. PleC were isolated from naive (△) and infected (■, ♦, ●) BALB/c mice 60 days after infection, and the expression of CD25 (*A*), Foxp3 (*B*), CTLA-4 (*C*), and GITR (*D*) by CD4$^+$ T cells was assessed using FC. Infected mice were either control treated with rat IgG (■), treated with anti-CTLA-4 alone (♦), or cotreated with anti-CTLA-4 and anti-CD25 (●). Results are representative of two independent experiments with the exception of Foxp3 that is based on a single experiment. Symbols denote individual animals and lines represent median values.

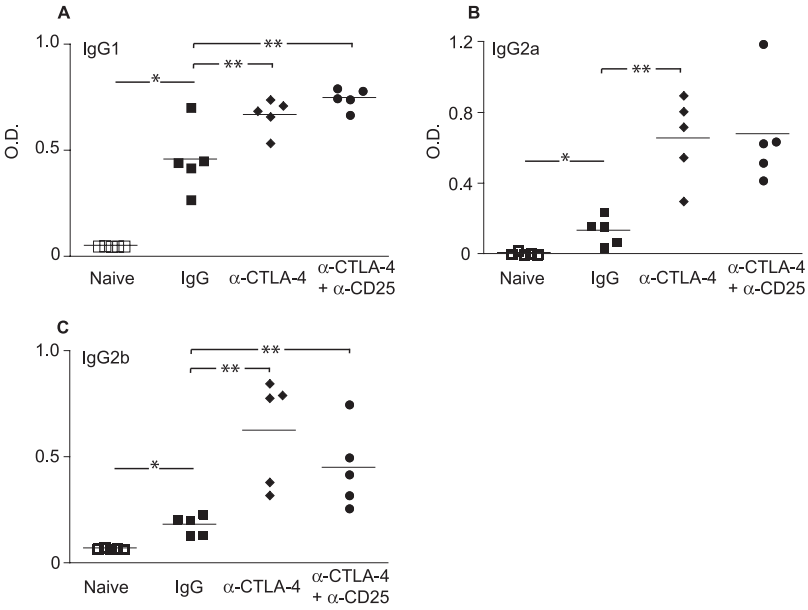

FIGURE 6. Treatment with anti-CTLA-4 increases *L. sigmodontis*-specific serum Abs independently of anti-CD25 treatment. *L. sigmodontis*-specific IgG1 (*A*), IgG2a (*B*), and IgG2b (*C*) were quantified in the sera of naive (□) and infected animals following treatment with IgG (■), anti-CTLA-4 (♦), and anti-CTLA-4 in combination with anti-CD25 (●). Symbols represent individual animals and lines represent mean values. Results are representative of two independent experiments. The naive and infected IgG groups were compared using Wilcoxon's rank test and significant differences between means ($p < 0.0125$) are indicated by ∗. The three infection and treatment groups were compared using one-way ANOVA with Tukey's means comparison. Significant differences between means are indicated by ∗∗.

depletion in combination with the CTLA-4 neutralization, expression of CD25 and Foxp3 by pleCD4$^+$ T cells was unchanged at day 60, suggesting that the Foxp3$^+$ Treg cell response had recovered by the time of autopsy (Fig. 5, *A* and *B*). The expression of CTLA-4 and GITR by pleCD4$^+$ T cells from anti-CTLA-4 and anti-CD25-cotreated mice was also unchanged at day 60 of infection (Fig. 5, *C* and *D*). Thus, although cotreatment with anti-CTLA-4 and anti-CD25 enhanced parasite killing, it did not induce a long-term change in cellular immune responsiveness or in the activation phenotype of the pleCD4$^+$ T cell population.

CTLA-4 blockade enhances parasite-specific Ab production independently of CD25 depletion

To test whether the anti-CTLA-4 and anti-CD25 treatments affect humoral immunity to *L. sigmodontis*, we measured LsAg-specific Ab responses at the time of autopsy. *L. sigmodontis*-specific IgG1, IgG2a, and IgG2b were detected in infected mice 60 days after infection, and all three isotype responses were significantly increased following treatment with anti-CTLA-4 alone (Fig. 6). Combining the anti-CTLA-4 treatment with CD25 depletion did not further increase parasite-specific isotype responses. *L. sigmodontis*-infected mice had only low levels of LsAg-specific IgG3, which were not increased by the Ab treatments (results not shown). Thus, although there were no detectable increases in cellular immune responses, anti-CTLA-4 treatment alone enhanced *L. sigmodontis*-specific humoral immunity.

Discussion

The T cell coinhibitory receptor CTLA-4 has been implicated as one of the mediators of T cell unresponsiveness that is commonly observed during chronic filarial infections in humans (10, 20). This hypothesis is strengthened by functional data showing that neutralization of CTLA-4 in vitro can increase the Ag-specific responsiveness of human PBMC from patients with lymphatic filariasis (10, 20). CTLA-4 has also been linked to filarial-induced regulatory T cell responses both in humans (20, 44) and in murine infection models (16, 17). We now provide in vivo evidence that CTLA-4 acts alongside or through CD4$^+$CD25$^+$ Treg cells to inhibit protective immunity to filarial parasites, and that neutralization of CTLA-4 in combination with the depletion of CD4$^+$CD25$^+$ Treg cells during an established filarial infection enhances killing of adult filariae.

Two important determinants of susceptibility to *L. sigmodontis* infection are the induction of a CD4$^+$CD25$^+$ Treg cell response and the development of a hyporesponsive CD4$^+$CTLA-4$^+$GITR^{high} T cell phenotype at the site of infection (16). Interestingly, despite a role for CD4$^+$CD25$^+$ Tregs in inhibiting protective immunity to *L. sigmodontis* and in controlling Ag-specific responses to *Brugia pahangi* (17), *L. sigmodontis*-induced CD4$^+$ T cell hyporesponsiveness does not appear to be linked to direct suppression by Foxp3$^+$ Treg cells, suggesting two independent levels of T cell regulation (6, 16). In confirmation of this, we have now demonstrated that the majority of pleCD4$^+$CTLA-4$^+$GITR^{high} T cells did not express Foxp3 and that removing CD4$^+$Foxp3$^+$CD25$^+$ Treg cells did not increase the Ag-specific responsiveness of the pleCD4$^+$ T cells. Thus, the Ag-specific hyporesponsiveness of the CD4$^+$CTLA-4$^+$GITR^{high} T cell population cannot be attributed to ongoing suppression by CD4$^+$Foxp3$^+$CD25$^+$ Treg cells, and instead suggests that the CD4$^+$CTLA-4$^+$GITR^{high} T cells predominantly represent a down-modulated Foxp3$^-$ Teff cell population. *L. sigmodontis* infection did, however, drive a CD4$^+$Foxp3$^+$ Treg cell response because the CD4$^+$Foxp3$^+$ Treg cells from infected animals showed increased proliferation in vivo and increased expression of activation markers compared with naive controls. Similar evidence for multiple levels of T cell regulation, including Treg cells and Teff cell hyporesponsiveness, is seen during chronic human filarial infection where immune down-regulation is associated both with increases in *Foxp3* mRNA expression and with increases in expression of T cell anergy factors (10).

The potent inhibitory properties of CTLA-4 make it a prime candidate for mediating filarial immunosuppression, either acting directly through CD4$^+$CD25$^+$CTLA-4$^+$ Treg cells (24) or forming an independent suppressive mechanism that may act in conjunction with CD4$^+$ Treg cells (34, 35, 47). Although neutralizing CTLA-4 alone during an established *L. sigmodontis* infection did not promote protective immunity, when CTLA-4 neutralization was performed in combination with depletion of CD25$^+$ Treg cells, parasite killing was enhanced. CTLA-4 was up-regulated on CD4$^+$Foxp3$^+$ T cells and thus could represent a mechanism by

which $CD4^+$ Treg cells inhibit immunity. The greatest increase in CTLA-4 expression, however, was seen in the $CD4^+Foxp3^-$ population, suggesting a more prominent role on the $CD4^+$ Teff population. If inhibition via CTLA-4 was entirely mediated through $CD4^+CD25^+$ Treg cells, then either CTLA-4 blockade or CD25 depletion alone would be expected to be sufficient to induce protection. Although neither regimen is absolute, the requirement for both treatments does indicate that they target two independent mechanisms of suppression. It is likely, therefore, that during *L. sigmodontis* infection CTLA-4 has a mode of action independent to, and synergistic with, $CD4^+CD25^+$ Treg cells. This is similar to recent work using *Schistosoma mansoni* infection in which a $CD25^+$ Treg independent role for CTLA-4 was shown (47). Despite suggesting a role for CTLA-4 on the $CD4^+$ Teff cells, our results with *L. sigmodontis* do not rule out a parallel role for CTLA-4 within the $CD4^+CD25^+$ Treg population.

One possibility is that coinhibition through CTLA-4 is responsible for maintaining the hyporesponsive $CD4^+$ T cell phenotype, since signaling through CTLA-4 can maintain Teff cell anergy (48) and has been shown to increase the expression of RAP1 and $p27^{kip1}$ that are implicated in T cell anergy and inhibition of cell cycle (49, 50). In human filariasis, T cell unresponsiveness is associated with increased expression of E3 ubiquitin ligases (c-cbl, cbl-b, Itch, and Nedd4) that are often up-regulated in anergic T cells (10). Neutralizing CTLA-4 during in vitro restimulation prevented increases in c-cbl, cbl-b, and Itch and restored Ag responsiveness. Thus, CTLA-4 appears to play a major role in controlling $CD4^+$ T cell hyporesponsiveness during filarial infections. In addition, CTLA-4 may suppress protective immunity by inducing regulatory DC that inhibit T cell responses through the production of indoleamine 2,3-dioxygenase (32, 51) or by inducing the expression of TGF-β which is often associated with filarial suppression (10, 29–31).

The elevated expression of both CTLA-4 and the costimulatory receptor GITR on the hyporesponsive $CD4^+$ Teff cells suggests that the net balance of opposing signals received through these two receptors will control how the $CD4^+$ Teff cell responds during *L. sigmodontis* infection. Using an agonistic anti-GITR Ab to provide costimulation during infection promotes protective immunity, but only when performed in combination with depletion of $CD25^+$ Treg cells (16). The similar requirements for two-step treatments when targeting either GITR or CTLA-4 suggest that both may be acting through the same mechanism. Thus, although anti-CD25 treatment depletes the Treg cell population, both the anti-CTLA-4 and the anti-GITR treatments may act to restore the function of the hyporesponsive $CD4^+$ Teff cells. In this scenario, neutralizing CTLA-4 will prevent the $CD4^+$ Teff cells from receiving the inhibitory signals that make them unresponsive, while providing additional costimulation through GITR may allow them to overcome the effects of coinhibition. Similar requirements for reawaking "exhausted" or unresponsive $CD8^+$ T cells have been demonstrated during chronic viral infections, where neutralizing the coinhibitory receptor PD-1 during lymphocytic choriomeningitis virus infection recovers $CD8^+$ T cell effector function, resulting in decreased viral loads (52).

Notably, although cotreatment with anti-CD25 and anti-CTLA-4 enhanced parasite killing, it did not result in any long-term change in cellular immunity. Increased parasite-specific Ab responses were seen following treatment with CTLA-4, but they were equally elevated in both the anti-CTLA-4 alone and anti-CTLA-4/anti-CD25-cotreated groups and thus their increase was not associated with parasite killing. The limited changes seen in immune responses at autopsy correlates with the fact that surviving adult parasites were still able to develop into a patent infection. This contrasts with cotreatment using anti-CD25 and anti-GITR which showed more effective parasite killing (73% reduction vs 54% with CTLA-4), although the infections for the GITR and CTLA-4 experiments were performed on different occasions, limiting their direct comparison. Treatment with anti-CD25 in combination with anti-GITR was also associated with increased Ag-specific cytokine and proliferative responses even though autopsy was performed 1 mo after treatment (16).

Since both anti-GITR and anti-CTLA-4 treatments resulted in parasite killing, it is unlikely that the increase in immune responsiveness seen following anti-GITR treatments is solely a by-product of parasite death, suggesting that targeting GITR is a more potent approach for inducing protective immunity than targeting CTLA-4. A possible explanation is the differing modes of action of the anti-CTLA-4 and anti-GITR Abs. While anti-GITR is an agonistic Ab and will actively provide a positive signal to the T cell, anti-CTLA-4 Ab is a neutralizing Ab and will only result in a temporary block in CTLA-4 signaling. Given the reported life span of the anti-CTLA-4 Ab in the blood (43), our treatment regimen would have blocked CTLA-4 activity for approximately a 2-wk period. The CD25 depletion is also only temporary and when depleted during infection $Foxp3^+$ Treg cells are found to recover more rapidly than in naive mice (53). Treatment may, therefore, have resulted in a temporary recovery in immune responsiveness sufficient to enhance parasite killing and Ab production. As the CTLA-4 blockade waned and the $CD4^+CD25^+Foxp3^+$ Treg population recovered, however, the immunosuppressive mechanisms would reassert themselves, turning cellular immunity off. The increased Ab response may be partly explained by their relatively long half-life within the blood, meaning that a temporary boost to the B cell response would still be detectable at day 60. Thus, for CTLA-4 blockade to be effective, it may need to be maintained until the infection has been completely cleared. In contrast, providing an agonistic signal through GITR may result in a long-term phenotype change in the Teff cell population permanently overcoming hyporesponsiveness. Certainly, the pleCD4$^+$ T cells showed a change in activation phenotype following anti-CD25/GITR treatment, including decreased expression of CTLA-4 that was not seen following anti-CD25/CTLA-4 treatment.

One important question is whether the regulatory mechanisms seen during *L. sigmodontis* infection are induced by the parasite to potentiate its own survival or whether they represent a natural response to chronic immune challenge. Both CTLA-4 and $CD4^+Foxp3^+$ Treg cells regulate immune responses to many different infectious agents (41, 54–58) and serve to protect the host from pathology caused by excessive immune responses (19, 59, 60). It is unlikely therefore that the CTLA-4 and $CD4^+CD25^+$ Treg cell responses are exclusively induced by *L. sigmodontis* to inhibit protective immunity, and as neutralizing their activity resulted in parasite killing they cannot be solely involved in controlling immune pathology. The trade-off to mounting a strong immune response to clear an infection is the induction of immune pathology. If the cost of clearing an infectious agent is high compared with the cost of the infection itself, then it may be more beneficial to the host to prevent immune pathology by down-regulating its own protective immune responses and accept the consequences of a persistent infection (55, 56). Thus, it is possible that the immune-regulation observed during *L. sigmodontis* infection is entirely provoked by the host, rather than representing immune manipulation by the parasite. Alternatively, the parasite may exploit the regulatory responses that the host uses to limit pathology while clearing infection, resulting in an inappropriate bias toward immune regulation. Distinguishing these two scenarios will be

important in the treatment of human infections where disrupting immune regulation may have serious consequences in terms of immune pathology.

Overall, the T cell coinhibitory receptor CTLA-4 and $CD25^+$ Treg cells appear to play complementary roles in suppressing protective immunity to filarial parasites, with CTLA-4 potentially responsible for maintaining hyporesponsiveness within the $CD4^+$ Teff cell compartment. Although it is possible to enhance protective immunity to an established infection, treatments are only effective if they simultaneously target both levels of T cell regulation. The CTLA-4 signaling pathway therefore provides a potential therapeutic target for inducing protective immunity to filarial parasites.

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Disclosures

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