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CTLA-4 and CD4+ CD25+ regulatory T cells inhibit protective immunity to filarial parasites in vivo

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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, *L. 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.

The inhibitory effects of CTLA-4 may impede immunity to a variety of different infections (38–41). CTLA-4 is believed to preferentially modify Th2 responses (42) and has shown to downregulate Th2 immune responses to filarial infections, such that neutralizing CTLA-4 during *Nippostrongylus brasilliensis* 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 upregulated 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).
The Journal of Immunology

4627

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 on CD4+ 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-4+GITRhigh 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 high 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 were infected s.c. on the upper back. Pleural lavage with 10 ml of cold L. sigmodontis (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°C 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 IgG1 × 106 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) spleen APC were added to 96-well round-bottom plates at 1 × 106 cells/well, along with 1 × 105 CD4+ T cells/well. Whole lN cells were used at 5 × 105 cells/well. Cultures were stimulated with parasite alone or 10 μg/ml LsAg. Supernatants were sampled at 72 h for cytokine analysis, and 1 μCi/well [methyl-3H]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 IgG1 × 106 cells and the following Abs applied: PE-conjugated anti-CTLA-4 (UC10-4F10-11), peridinin chlorophyll protein-conjugated streptavidin, alkaline phosphocynin 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 alkaline phosphocynin-conjugated anti-Foxp3 (FJK-I6c1; eBioscience) per the manufacturer’s instructions. BrdU staining was performed after the final Foxp3 staining step using FITC-conjugated anti-BrdU with DNsase 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 Ab staining were gated on CD4+CD25high cells. To measure intracellular expression of cytokines, CTLA-4, and Foxp3 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 FACS.Divana software (BD Biosciences). Analysis was performed using CellQuest Pro and FlowJo (Tree star). Agents 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 NaHCO3, 0.18 M Na2CO3 (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 Bio-technology 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, we 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-4+GITRhigh Teff cells. To test
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). Containing for Foxp3 demonstrated that the majority of CD4⁺ CTLA-4⁺ GITR⁺Foxp3⁺ cells were Foxp3⁻ and thus appear to represent an activated CD4⁺ T 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. 1I), their proportion within the CD4⁺ T cell population was significantly decreased compared with naive mice (Fig. 1J).

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⁻ Tcell population, indicating that the CD4⁺Foxp3⁻ Treg cells generally show a higher
rate of cell division in vivo (Fig. 2A). Infection did drive the proliferation of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells because there was a significant 2-fold increase in the percentage of dividing CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells compared with naive mice. The CD4<sup>+</sup>Foxp3<sup>+</sup> T 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<sup>+</sup>Foxp3<sup>+</sup> 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. 2B). Given the activated phenotype of the CD4<sup>+</sup>Foxp3<sup>+</sup>CD25<sup>+</sup> Treg cells, we tested whether they were inhibiting the Ag-specific responsiveness of the pleCD4<sup>+</sup> T cell population. CD4<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup> 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 triplcate cultures. Results are representative of two independent experiments.

In vivo neutralization of CTLA-4 enhances parasite killing when combined with the depletion of CD25<sup>+</sup> Treg cells

The high expression of intracellular CTLA-4 by the pleCD4<sup>+</sup>Foxp3<sup>+</sup> Teff cells and the pleCD4<sup>+</sup>Foxp3<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> 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 2](image)

**FIGURE 2.** L. sigmodontis infection induces the proliferation and activation of CD4<sup>+</sup>Foxp3<sup>+</sup> 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<sup>+</sup> cells within the CD4<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>+</sup> 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<sup>+</sup> and Foxp3<sup>+</sup> 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<sup>+</sup>Foxp3<sup>+</sup> 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<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> 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.

![Figure 3](image)

**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).
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 selectively inhibit protective immunity since parasite killing could 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. 4A) 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 (Fig. 4B). 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 T cells 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 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.

**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.
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+GITRhigh 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+GITRhigh 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+GITRhigh T cell population cannot be attributed to ongoing suppression by CD4+Foxp3+CD25+ Treg cells, and instead suggests that the CD4+CTLA-4+GITRhigh T cells predominantly represent a down-modulated Foxp3+ T 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+ 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 p27kip1 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, 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 filamentous suppression (10, 29–31).

The elevated expression of both CTLA-4 and the costimulatory receptor GITR on the hyporesponsive CD4+ T cell phenotype suggests that the net balance of opposing signals received through these two receptors will control how the CD4+ T 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 reawakening “exhausted” or unresponsive CD8+ T cells have been demonstrated during chronic viral infections, where neutralizing the inhibitory 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 co-inhibitory 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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