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The immunotherapeutic role of regulatory T cells in *Leishmania (Viannia) panamensis* infection

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Abstract

Leishmania (Viannia) parasites are etiological agents of cutaneous leishmaniasis in the New World. Infection is characterized by a mixed Th1/Th2 inflammatory response, which contributes to disease pathology. However, the role of T regulatory cells (Treg) in *Leishmania (Viannia)* disease pathogenesis is unclear. Using the mouse model of chronic *L. (V.) panamensis* infection, we have examined the hypothesis that Treg functionality contributes to control of pathogenesis. Upon infection, T regulatory cells (CD4⁺Foxp3⁺) presented with a dysregulated phenotype, in that they produced IFN- γ , expressed Tbet and had a reduced ability to suppress T cell proliferation *in vitro*. Targeted ablation of Tregs resulted in enlarged lesions, increased parasite load and enhanced production of IL-17 and IFN- γ with no change in IL-10 and IL-13 levels. Thus, indicating that an increased inflammatory response was commensurate with disease exacerbation and that the remaining impaired Treg cells were important in regulation of disease pathology. Conversely, adoptive transfer of Tregs from naïve mice halted disease progression, lowered parasite burden and reduced cytokine production (IL-10, IL-13, IL-17, IFN- γ). As Tregs appeared important for controlling infection, we hypothesized the expansion of Tregs could be used as an immunotherapeutic treatment approach. As a proof of principle, chronically infected mice were treated with rIL-2-anti-IL-2 antibody complex to expand Tregs. Treatment transiently increased numbers and percentage of Treg (draining lymph node, spleen), that resulted in reduced cytokine responses, ameliorated lesions and reduced parasite load (10⁵-fold). Thus, immunotherapy targeting Tregs could provide an alternate treatment strategy for leishmaniasis caused by *L. (Viannia)* parasites.

Keywords

Parasitic-Protozoan; inflammation; skin

INTRODUCTION

The members of the genus *Leishmania*, comprised of species of protozoan parasites (1), are the causative agents of leishmaniasis, which manifests as a wide spectrum of clinical diseases including cutaneous, mucocutaneous and visceral forms. The outcome of disease depends on the infecting parasite species, host, and environmental factors (2). *Leishmania* are taxonomically divided into two subgenera, *Leishmania* and *Viannia*, with the *Viannia* subgenus being predominant in Southern and Central Americas. In Colombia, *L. (V.) panamensis* is the agent responsible for the majority of leishmaniasis cases (3). Infection typically manifests as cutaneous disease, which can be chronic or recurrent; further, mucocutaneous forms have also been reported (4).

The immune response to *Leishmania* directly impacts the outcome of infection. The classic Th1/Th2 paradigm developed for *L. major*, however, does not consistently apply to the broad spectrum of species implicated in human disease (5–7). The control of disease can be subverted immunologically through multiple mechanisms, which include the Th2 cytokines but also Th1-IL-10⁺ CD4⁺ T cells, and the inhibition of macrophage activation through the production of TGF- β , IL-10 and other mediators (8). In the case of *Leishmania (Viannia) panamensis*, both the human disease and the mouse model present with a mixed cytokine response (9). Despite abundant IFN- γ production, parasite persistence and pathology still remain. Alongside IFN- γ , there is concomitant production of IL-10, IL-13, IL-17 and TNF- α . This imbalanced/mixed immune response may be partially responsible for disease pathology and indeed, in the mouse model, genetic depletion of IL-13 or IL-10 can result in disease resolution (9). However, excessive inflammation is known to play an important role in human pathology and infection with *Leishmania (Viannia)* organisms (10–12). An exaggerated immune response (high production of Th1 cytokines with reduced levels of IL-10) is associated with enhanced disease severity in infected patients (13–15). Additionally, there is a correlation with lesion size and the frequency of antigen specific cytokine producing cells (16); further, reductions in IFN- γ and TNF- α are found following disease resolution (17). From these findings, it follows that factors that control inflammation may improve the outcome of infection with *Leishmania* species.

Regulatory T cells (Tregs), characterized by the transcription factor Foxp3, are responsible for controlling aberrant immune responses through cell (CTLA-4, CD39, CD73) and cytokine mediated (IL-10, TGF- β) mechanisms (18, 19). Although Treg cells have been demonstrated to contribute to pathology and parasite persistence in leishmaniasis, these cells do not appear to play identical roles across *Leishmania* species. During *L. major* infection, Tregs prevent immune mediated parasite clearance leading to parasite persistence and potentially reactivation of disease (20). In the case of *L. donovani*, evidence indicates that the induction of T regulatory cells leads to disease exacerbation (21, 22). In contrast, in the *L. amazonensis* mouse model, it was found that Tregs have the opposite effect; these cells are beneficial to relieving a hyper-inflammatory state and aid in disease remediation (23).

Despite the increasing knowledge of immunopathological mechanisms that contribute to disease progression, the role of T regulatory cells during *Leishmania (Viannia)* infection has not been directly evaluated (24–27). Recently, it was found that *L. (V.) panamensis* infected

patients had improved Treg suppressive capacity following successful treatment (28). To determine whether Tregs play a beneficial role during infection with *L. (V.) panamensis*, we have utilized the mouse model which closely mimics the human mixed cytokine response (9), to investigate the impact of these cells on infection. We found that during infection, T regulatory cells in the draining lymph node display characteristics of dysregulation (express Tbet and IFN- γ) and have a reduced suppressive capacity in comparison to Tregs from naïve uninfected mice. Consequently, we sought to determine the impact of T regulatory cells on infection. These studies have revealed that these cells significantly modulate both the immune response (IL-10, IL-13, IL-17, IFN- γ) and parasite levels. The further experimental depletion of Tregs during infection leads to disease exacerbation, while temporal enhancement of T regulatory cells (through cell transfer or rIL-2/anti-IL-2 antibody therapy) leads to disease amelioration. Taken together, these results indicate that Tregs can be beneficial in resolving infection with *L. (V.) panamensis*.

MATERIALS AND METHODS

Animals

Female BALB/c mice were obtained from the National Cancer Institute (Frederic, MD, USA) and housed at the Yale University School of Medicine facilities, approved by the American Association for Accreditation of Laboratory Animal Care facilities. All animal procedures were performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and have been reviewed and approved by the Yale University Committee for the Use and Care of Animals. DERE (Foxp3-eGFP/DTR) mice employed were as previously described (29) and had been backcrossed for 10 generations onto the BALB/c background.

Parasite culture, infection, and parasite burden analyses

L. (V.) panamensis (strain MHOM/CO/1995/1989) were grown in Schneider's *Drosophila* medium supplemented with 20% heat-inactivated FCS and 17.5 $\mu\text{g/ml}$ gentamycin. The infection protocol has been described previously (9). Briefly, infective parasites were isolated from late stationary phase promastigotes from the 45/60% percoll gradient interface. Parasites (5×10^4) were injected intradermally into the top of a hind foot. Lesion development was monitored by measuring the foot thickness using a dial gauge caliper (Starrett Thickness Gauge) and calculating the ratio between the infected and the contralateral non-infected foot. At the termination of the experiment, parasites were quantified in infected tissue by limiting dilution assay, as previously described (6).

Indoleamine 2,3-dioxygenase (IDO) inhibition and in vivo depletion of T regulatory T cells

1-methyl-D-tryptophan (1-MT; Sigma-Aldrich) was formulated and administered to mice as previously described (30). Briefly, mice were treated with 2mg/ml 1-MT in their drinking water; starting 2 days post infection and continued for the duration of the experiment.

Depletion of Foxp3+ cells in DERE mice was performed as previously described (31). Briefly three weeks post infection, mice were administered 0.5 μg diphtheria toxin (DT; Enzo Life Sciences), intraperitoneally on 2 consecutive days per week for 2 weeks. PBMCs

were isolated from mice one day following the last DT injection; flow cytometry was employed to confirm T regulatory cell depletion.

Isolation of lymphocytes, cellular transfer and suppression assays

CD4⁺ and CD4⁺CD25⁺ cells were isolated from the spleen or draining lymph node of mice using the CD4⁺CD25⁺ regulatory T cell isolation kit (MACS Miltenyi Biotec) according to the manufacturer's protocol. CD4⁺CD25⁺ or CD4⁺CD25⁻ cells (3×10^5) were injected once intrasplenically in chronically infected mice (three to five weeks post infection) and infections monitored as indicated above.

For suppression assays, 5×10^4 isolated naïve CD4⁺CD25⁻ cells (Teff) were labeled with 5 μ M CFSE (eBioscience) and co-cultured with CD4⁺CD25⁺ cells (Treg) at varying ratios using 2×10^5 T cell depleted irradiated splenocytes as APCs. Cells were stimulated with 0.5 μ g/ml α CD3 clone 145-2C11 (16-0031, eBioscience). Treg suppressive capacity was assessed by examining CFSE dilution using flow cytometry. The percentage suppression was calculated as (% proliferation Teff alone - % proliferation Treg+Teff) / % proliferation Teff. The isolated CD4⁺ Tregs from both naïve and infected mice were found to have comparable levels of CD25 and Foxp3 expression (CD4⁺CD25⁺ purity was >90.0%).

Flow cytometry and cytokine analyses

Single cell suspensions were made from the draining lymph nodes and brought up to 5×10^6 cells/ml in RPMI supplemented with 10% FCS. Cells were cultured with PMA/Ionomycin (BD Pharmingen) for 4 hours, Fc receptors were blocked (α CD16/CD32, BD Pharmingen), and surface markers were stained with α CD3 (145-2C11, BD Pharmingen), α CD4 (RM4-5, BD Pharmingen), α CD8 α (53-6.7, BD Pharmingen), α CD11b (M1/70, BD Pharmingen); α CD11c (N418, eBioscience); α CD19 (1D3, eBioscience); α Ly6G (RB6-8C5, eBioscience); α CD25 (PC61.5, eBioscience), α CD39 (24DSM1, eBioscience), α CD73 (TY/23, BD Pharmingen), and/or α CTLA-4 (UC10-4F10-11, BD Pharmingen) with corresponding isotype controls (eBioscience or BD Pharmingen). Cells were fixed and permeabilized (Cytofix/Cytoperm, BD Pharmingen) for intracellular staining with the following antibodies: α Foxp3 (FJK-16s, eBioscience); α IFN- γ (XMG1.2, eBiosciences); α Tbet (4B10, eBioscience) and the corresponding isotype controls IgG2 κ , IgG1 κ (eBioscience). Alternatively, cells were cocultured for 72 hours with soluble leishmania antigen (SLA, *L. (V.) panamensis* freeze-thaw lysate). Supernatants were harvested and IFN- γ , IL-10, IL-13, IL-17, TNF- α and TGF β were analyzed by sandwich ELISA following the manufacturer's protocol (eBioscience).

Indoleamine 2,3-dioxygenase analyses

For the determination of indoleamine 2,3-dioxygenase (IDO), RNA was isolated from infected and naïve lymph nodes using Trizol (Invitrogen) and cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen) following the manufacturer's protocols. Real-time PCR was performed on the Mx3005P QPCR System (Stratagene) using iQ SYBR Green Supermix (Biorad) according to the manufacturer's instructions. IDO mRNA was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the comparative C_T method (32). The following primers were used: IDO fwd,

eggactgagaggacacaggttac; IDO rev, acacatacgccatggtgatgtac; GAPDH fwd, tgcaccaccaactgcttag; GAPDH rev, gatgcagggatgatgttc.

rIL-2/anti-IL-2 antibody complex treatment for Treg expansion

Anti-IL-2 antibody-rIL-2 complexes were formulated as previously described (33), using a 1:1 molar ratio. Briefly, mice with established lesions were given intraperitoneal injections of recombinant mouse IL-2 (PeproTech, 1 μ g) in complex with anti-IL-2 mAb (JES6-1, 5 μ g), every 3 days for a total of 4 doses. Due to the fact that rapamycin is a drug known to affect *Leishmania* (34) as *TOR1* and *TOR2* are essential genes, rapamycin together with the rIL-2/anti-IL-2 antibody was not employed. Two days and 8 days following the final treatment and at the end of the experiment, the percentages of CD4⁺, CD8⁺ and CD4⁺Foxp3⁺ cells were analyzed in the draining lymph node and spleen. Cytokine analysis was conducted at the same time point and at the termination of the experiment. Lesion size was monitored as described above.

Statistical analyses

Statistical analyses were conducted using the Students t-test or the Wilcoxon signed-rank test. P values ≤ 0.05 were considered significant.

RESULTS

Regulatory T cells are phenotypically altered in response to *L. (V.) panamensis* infection

Inflammation plays an important role in leishmaniasis caused by organisms of the *L. (Viannia)* subgenus (10–12). Understanding the contribution of T regulatory cells (Tregs) in modulating inflammation and thus disease outcome potentially could highlight areas for immunotherapeutic interventions. Tregs have been found to play variable roles in studies of the diverse species of *Leishmania*; these range across a wide spectrum, from preventing strong anti-parasitic Th1 responses, and limiting responses necessary for development of memory, thus aiding parasite persistence to the control of pathology and infection (23, 35–37). Consequently, we sought to directly examine the effects of T regulatory cells on a member of the *Viannia* subgenus, *L. (V.) panamensis*, using a mouse model of chronic disease. Previous experiments (9) in the *L. V. panamensis* mouse model indicated that in response to infection, as expected, there was an early expansion of T cells (CD4⁺, CD8⁺) and B cells in both susceptible BALB/c and resistance IL-13 deficient mice. Notably, the expansion of Treg cells was higher (2.4-fold) in the resistant IL-13 deficient mice than the overall T cell expansion (1.6-fold), while the expansion of T cells and Treg populations were comparable in the susceptible mice. These data suggested the immunological balance toward regulation might also contribute to disease control.

To further evaluate the Treg population present during infection, we initially examined whether the phenotypic markers associated with regulatory capacity might have been altered due to infection. Functional Tregs are known to act through the high affinity IL-2 receptor CD25, the co-stimulatory molecule CTLA-4 and/or the ectonucleases CD39 and CD73. However, the expression of the regulatory surface markers CTLA-4, CD25, CD39, and CD73 remained unchanged in the CD4⁺Foxp3⁺ population in infected mice in comparison

to naïve mice (Figure 1A). These results, suggested that these Treg functional mechanisms are unaltered and that these cells may remain capable of immunosuppression.

Recent findings have shown a functional plasticity of Treg populations in inflammatory environments (38–42). Although lineage specific transcription factors are more generally expressed independently, subpopulations expressing transcription factors which are markers of multiple CD4+ T cell lineages have been observed in distinct inflammatory states (43, 44). Following *ex vivo* stimulation with leishmanial antigen, a population of CD4+ cells expressing Foxp3 and Tbet was present and remained detectable throughout the course of infection (Figure 1B). Further, after PMA/ionomycin stimulation, $20.8 \pm 4.5\%$ of Foxp3+ cells from the draining lymph nodes of *L. (V.) panamensis* infected mice expressed IFN- γ (Figure 1C). IFN- γ producing Tregs have been found in both human and mouse models (45) of autoimmune (38, 45) and infectious diseases (46) and have been associated with Tregs taking on proinflammatory capabilities. Taken together, these data suggested functional dysregulation of Tregs during *L. (V.) panamensis* infection.

Regulatory T cells from *L. (V.) panamensis* infected mice are functionally impaired

Rodriguez-Pinto, et al. demonstrated that Tregs from *L. (V.) panamensis*-infected patients had diminished suppressive capacity that was restored after successful drug treatment (28). Considering that a significant portion of the Treg population have an altered phenotype (Foxp3+IFN- γ + Tbet+ cells) upon infection, this suggested that *L. (V.) panamensis* infection leads to diminished Treg functionality. To evaluate the regulatory capacity of Tregs from infected mice, the comparative ability of Tregs from naïve or infected mice to inhibit T effector proliferation *ex vivo* was assessed. Overall, the CD4+CD25+ (Tregs) cells isolated from the draining lymph node of *L. (V.) panamensis* infected mice were less effective than Tregs isolated from pooled peripheral lymph nodes of naïve mice (despite having equivalent Foxp3 expression) in inhibiting CD4+CD25– (Teff) proliferation in coculture with α CD3 and irradiated APCs (Figure 2A). Percentage suppression of Teff proliferation when cocultured with Tregs from infected mice was 26.6, 11.0, and 3.7 compared to 40.9, 27.5 and 13.5 when cocultured with Tregs from naïve mice at ratios of 2:1, 1:1 and 0.5:1 Treg:Teff, respectively (Figure 2B). Thus Tregs from *L. (V.) panamensis*-infected mice had a reduced suppressive capacity (as little as 27% that of naïve Treg cells) compared to Tregs from naïve mice. Therefore, despite maintenance of certain features consistent with Treg function (CD25, Foxp3, CTLA-4 and the surface ectonucleases CD39 and CD73), the Treg cells from *L. (V.) panamensis* infected mice were significantly less effective in inhibiting Teff proliferation. These results are consistent with those found for Treg cell function from human patients with active leishmaniasis cause by *L. (V.) panamensis*, where a reduced capacity to inhibit IFN- γ production by T effector cells was observed (28). Thus, in both human infection and the mouse model impaired Treg function is consistently observed, indicating that inflammatory response to infection may persist due to reduced Treg function.

Reduction of regulatory T cells during infection exacerbates disease caused by *L. (V.) panamensis*

To further examine the role of T regulatory cells during infection, the effect of Treg reduction on infection was examined. Initially, the chemical inhibitor of indoleamine-2,3-

deoxygenase (IDO), 1-methyltryptophan (1-MT), was utilized. IDO is known to be critical for Treg differentiation, enhancing Treg numbers and function, while suppressing T effector activation (47). It should be noted that the expression of indoleamine-2,3-dioxygenase (IDO) was significantly reduced in *L. panamensis* infected mice (25-fold relative to expression in peripheral lymph nodes of naïve mice; Supplementary Figure 1).

Therefore, IDO inhibition was utilized to further block the action of IDO and indirectly diminish T regulatory cell levels (48–50). Treatment of mice was initiated two days post-infection and was maintained for the duration of the experiment. As previously reported (48, 51), 1-MT treatment resulted in a significant but modest $17.3 \pm 1.7\%$ reduction in the level of Treg cells. Further, mice treated with 1-MT showed significantly larger lesions (Figure 3A) and over 100-fold increase in parasite numbers (Figure 3B). Notably, 1-MT treatment did not appear to skew the ongoing immune response toward either a Th2 or Th17 cytokine profile. Instead 1-MT treatment significantly increased the overall cytokine production in the draining lymph node, including IFN- γ , IL-10, IL-13 and IL-17 (Figure 3C). Thus, increased levels of both inflammatory and anti-inflammatory cytokines were observed in response to IDO inhibition. An elevated cytokine response is consistent, however, with previous observations that T regulatory cells modulate both Th1 and Th2 responses (52). These results support that reduction of T regulatory cells may be detrimental to disease control and that these cells modulate the mixed, hyper-cytokine response induced upon infection.

Although inhibition of IDO resulted in decreased levels of Treg cells and exacerbated disease, IDO can directly suppress the proliferation and differentiation of effector T cells, in part through tryptophan catabolism metabolites. Reducing IDO has been shown to promote the development of T effector cells in various systems (53–55). Therefore, a significant reduction in IDO is consistent both with T effector cell expansion and the findings of reduced functionality of Tregs in the *L. V. panamensis* infected mice. Further, the inhibitor 1-MT may potentially impact parasite survival through the buildup of tryptophan. Such a nutrient-rich environment could aid in the survival of the parasite, and has been reported that increased tryptophan levels improves trypanosome survival (a related kinetoplastid) (56). Tryptophan has been shown to be necessary for the growth of the promastigote stage of the parasite (57), although the effects on the intracellular amastigote stage are unclear and may vary dependent upon the origin (human versus mouse) of the macrophage (58). Consequently, an alternate approach was employed, using DEREK mice, where eGFP and diphtheria toxin receptor is under the control of the Foxp3 promoter, to allow the specific ablation of Foxp3+ cells (31).

As expected, Tregs from infected DEREK mice were completely depleted in response to diphtheria toxin (DT) treatment *in vivo* (data not shown). In response to DT-treatment, the Foxp3+ cell depleted mice developed exacerbated lesions (Figure 4A) and a 19-fold increase in parasite levels in comparison to control mice (Figure 4B). It should be mentioned that as previously reported (59, 60), infected BALB/c mice treated with diphtheria toxin developed less severe disease than either control infected mice or DT-treated DEREK mice (Supplemental Figure 2). An effect of DT toxin *in vivo* (although well below the known LD50 for mice (61)) has been noted recently (59, 60). This effect undoubtedly masks the

level of the exacerbatory effect of Treg depletion (Figure 4A). Further, the Treg-depleted DERE mice showed an enhanced production of the proinflammatory cytokines IL-17 and IFN- γ . However, interestingly there was no significant change in the level of IL-13 or IL-10 found at 9 weeks post-infection (Figure 4C). It has previously been shown (9) that both IL-10 and IL-13 together are necessary for parasite persistence and disease; however, the increases in Th1-Th17 responses alone in this case were not sufficient to achieve healing, but rather further exacerbated disease. Although the effects on Treg populations in the DERE mice is temporal (31), and the decrease in Treg cell levels due to 1-MT inhibition of IDO are modest, it is notable that these treatments have significant effects on disease progression. Taken together, these Treg depletion experiments show that reduction of T regulatory cells leads to enhanced pathogenicity and increased parasite levels that are accompanied by heightened cytokine responses.

Transfer of regulatory T cells during chronic infection ameliorates disease and reduces parasite levels

The detrimental effects of T regulatory cell reduction, led us to hypothesize that Tregs are beneficial for disease resolution by down modulating inflammation and the mixed cytokine response. To test this, Tregs were isolated from spleens of naïve mice and adoptively transferred into infected mice with established lesions. Two days post-transfer a modest and comparable increase in Tregs was found at both the lesion site and at the draining lymph node (15–17% increase). The transfer of T regulatory cells impaired lesion progression, with significant lesion reduction evident 5 days post-transfer in comparison to control mice receiving CD4+CD25⁻ T cells (Figure 5A). This impact upon lesion development was accompanied by a reduction in parasite burden (Figure 5B).

At the termination of the experiment, comparable reductions in the levels of production of IFN- γ , TNF- α , IL-10, IL-13 and IL-17 were observed (Figure 5C). Consistent with experiments demonstrating that depletion of T regulatory cells resulted in disease exacerbation, the reconstitution of T regulatory cells during established infection led to significant reductions in pathology and parasite levels. Overall, these results demonstrate the importance of T regulatory cells in controlling the immunopathologic state.

Treatment with rIL-2/anti-IL-2 antibody complexes induced regulatory T cells and ameliorated disease

As the transfer of CD4+CD25⁺ T regulatory cells mitigated disease progression, we aimed to determine whether induction/expansion of Tregs *in vivo* could provide an immunotherapeutic treatment strategy. As a proof of principle we utilized IL-2/anti-IL-2 antibody complex treatment (rIL-2/anti-IL-2 (JES6)) to target and promote the expansion of CD25⁺ Treg cells. Such treatment has been shown to preferentially amplify Tregs by blocking the medium affinity IL-2R $\beta\gamma$ receptor (expressed on CD8⁺ T cells and NK cells) binding site while the high affinity IL-2 receptor, CD25 (IL-2R $\alpha\beta\gamma$), binding site on Tregs remains exposed (33).

Following lesion development and establishment of chronic infection at 5 weeks post-infection, mice were systemically treated i.p. four times over a 9 day period (every three

days) with IL-2/anti-IL-2 complex. As expected (33, 62, 63), an expansion of total cells (spleen and DLN) was observed in response to treatment (Figure 6) at day 1 and 8 post-treatment. CD4⁺ and CD8⁺ T cells as well as CD4⁺FoxP3⁺ T cells responded to IL-2/anti-IL-2-complex treatment (Figures 6B–6D). However, the overall fold-increase of CD4⁺FoxP3⁺ cells in the DLN and spleen were higher than that found for total CD4⁺ or CD8⁺ T cells, with a 3-fold Treg increase versus a 1.5-fold CD4⁺ and CD8⁺ increase in the draining lymph node and a 5-fold increase in Tregs versus a 1.3 or 2.3-fold increase in CD4⁺ or CD8⁺, respectively, in the spleen at day 2 post-treatment were found. These results indicate a change in the overall balance of these cell populations. However, whether this translates into a modulation in the level of Treg at the site of infection remains to be determined. Concomitantly, an increase in markers of T regulatory capacity increased; the MFI of CD73, CTLA4, CD39, and CD25 significantly increased with treatment. These results are consistent with previous studies (63, 64) that established an enhanced regulatory capacity of IL-2/anti-IL-2 complex induced Treg cells. The reduced level of Tregs at 8 days post-treatment are consistent with the known half-life (24 h) of the IL-2 antibody complex (62) and earlier IL-2/anti-IL-2 complex studies (63) that demonstrated that increased Treg function and levels are temporary, waning quickly after cessation of treatment.

Further, IL-2/anti-IL-2 complex treatment regimen led to lesion resolution (Figure 7A) with a significant reduction (100,000-fold) in parasite burden (Figure 7B). This level of parasite reduction through rIL-2/anti-IL-2 antibody immunomodulation appears more effective than that for direct Treg cell transfer; however, this could be due to a larger, more sustained increase in Treg numbers. Interestingly, even given the short temporal enhancement of Treg cells, pronounced changes in parasite levels and pathology were found.

The successful expansion and enhancement of Treg cells led to a significant decrease in pro-inflammatory IL-17 cytokine production at day 2 post-treatment in both the draining lymph node and spleen (Figure 7C). Previous studies have shown that IL-2/anti-IL-2 complexes lead to increased levels of p-STAT5 and a reduction in pSTAT3, which are related to the development of Treg and Th17 cells, respectively (64). In the spleen, there was also a significant reduction in IFN γ , IL-10 and IL-13 that was not observed in the dLN; this may in part reflect the higher induction of CTLA4, CD39, CD25 and CD79 of Treg cells observed in the spleen and the intraperitoneal delivery route employed.

The successful expansion of Tregs subsequently led to a significant reduction in IFN- γ , IL-10, IL-13, IL-17 at the termination of the experiment (Figure7). Consequently, there was ultimately a down-regulation of inflammatory and anti-inflammatory responses. These results are consistent with those found for the transfer of Treg cells from naïve mice (Figures 5A, 5B) and may, in part, reflect the lower number of parasites found in the treated mice. Taken together, these experiments demonstrate that increasing T regulatory cells levels and/or function, even in a temporal fashion (Treg transfer or IL-2/anti-IL-2 complexes), can lead to the resolution of disease caused by *L. (V.) panamensis* and targeting these cells may provide a novel approach to disease treatment.

DISCUSSION

T regulatory cells are crucial for maintaining the balance of an appropriate immune response, preventing aberrant activation and minimizing collateral damage and immunopathology (65). Although initial studies indicated these cells had a negative impact on host defense, allowing pathogens to thrive by dampening immune system activation (66), further investigations have revealed a more complex dynamic that is dependent upon the disease state and pathogen (65, 66). Recent work utilizing Foxp3-DTR (DEREG) mice has allowed for specific ablation of Foxp3⁺ cells and increased insight into their role in disease following infection with various pathogens (67, 68). The results from these studies show temporal effects (68–70) and indicate that evaluation of Tregs on infection should be assessed in multiple approaches during infection to fully understand the ongoing dynamic and consequent potential as targets for therapy/treatment.

The role of T regulatory cells in the spectrum of diseases caused by leishmanial parasites appears to vary. This is not surprising, as species belonging to the *Leishmania* genus are taxonomically diverse (1) and the immunological mechanisms regulating infection and disease vary across the genus (5, 71, 72). In the case of *L. major*, Tregs play variable roles in the outcome of infection that is dependent upon the genetic background and thus the susceptibility of the mouse. Although a Th1 response can lead to healing, both a Th2 as well as multiple responses that down-regulate a Th1 response can prevent disease resolution (20). For the susceptible BALB/c mouse, CD4⁺CD25⁺ cells appear to constrain excessive Th2 responses (52, 73), facilitating healing. In C57BL/6 mice, an induction of IDO is observed (74) and the induced Tregs dampen the healing Th1 response and prevent sterile cure. The induction of Tregs has a two-fold impact. The presence of persistent parasites allows for memory and immunity to reinfection; however, the absence of sterile cure can allow for disease reactivation (66). In the case of *L. amazonensis*, T regulatory cells ameliorate disease; the reconstitution of RAG1-deficient C57BL/6 mice with naïve spleen cells depleted of CD4⁺CD25⁺ T cells before infection results in disease exacerbation in comparison to mice receiving whole spleen cells (23). This is consistent with earlier observations (5, 72, 75, 76) that pathology associated with *L. amazonensis* infection is dependent upon activation of T cells, B cells and an inflammatory response that fails to develop in immunodeficient mice (nude, RAG2⁻, CIITA⁻, MHCII-deficient). Hence a dampening of the immune response and cellular recruitment provides for parasite control.

Within the *L. (Viannia)* subgenus, however, the role of T regulatory cells in infection had not been fully evaluated. Disease caused by this subgenus is associated with inflammatory and mixed cytokine responses that sustain the recruitment of host macrophages and persistent infection (9, 11, 35, 77, 78). Although present at the site of infection and draining lymph node in the mouse model of *L. (V.) braziliensis*, Treg cells do not interfere with the development of protective immunity (27). In the non-human primate macaque model, lesion resolution was ascribed to the recruitment of regulatory T cells and suppression of an effector T-cell-mediated inflammatory response (26). In patient studies of *L. V. panamensis*, Treg functionality was diminished during infection but was restored following successful treatment (28). Tregs with varying functionality (with suppressive or non-functional properties) have been identified in lesions of patients infected with *L. (V.) braziliensis* (24,

25). In contrast for *L. (V.) guyanensis* (35), although FoxP3 and IDO expression in lesions decrease during disease, higher levels are found for patients with chronic disease, suggesting a role for Tregs in pathogenesis. Herein, we show evidence that regulatory T cell responses are impaired during infection with *Leishmania (Viannia) panamensis* and that restoration of these cells (through cell transfer or therapeutically through the use of rIL-2/anti-IL-2 antibody complexes) or their depletion has either a beneficial or negative effect, respectively in disease healing.

Indoleamine 2,3-dioxygenase (IDO) is important for the development of T regulatory cells and functions through both its signaling and enzymatic activities (47, 79, 80); tryptophan catabolism products are also known to suppress T effector cell function. One of the striking results found in response to *L. (V.) panamensis* infection is the reduction in IDO expression in the draining lymph node in chronically infected mice. This is in contrast to findings *in vitro* or *in vivo* for *L. major* infection (DCs; C57Bl/6 mice), where parasites induce the up-regulation of this enzyme (74, 81). Chemical inhibition of IDO by 1-MT enhanced expression of IL-17 in *L. major* infected mice; this corresponded with reduced parasite burden and lesion size (74). Notably, in IL-2/anti-IL-2 complex treated *L. V. panamensis* infected mice, an early response (2 days) to treatment in the healing animals was the down-regulation of IL-17. These findings are consistent with other studies employing IL-2 or IL-2/anti-IL-2 complexes (64, 82), showing decreased Th17/IL-17 responses. The role of IL-17 in *Leishmania (Viannia)* infection (72) is not well understood, but warrants further investigation. The contrasting relationship between *L. (V.) panamensis* and *L. major* with IDO (and consequent T regulatory cells) may be a result of evolutionary advantages/disadvantages associated with an inflammatory response. While in both infectious states IDO inhibition enhanced local inflammation, a heightened inflammatory response counteracts *L. major* survival but it appears to be advantageous for *L. (V.) panamensis* persistence. Overall, *Leishmania (Viannia)* parasites are notable for inflammation associated with disease pathology (9, 11, 35, 77, 78, 83). Thus, increased levels of T regulatory cells in *L. (V.) panamensis* infection lead to control and counteract this species survival strategy.

In addition a portion of CD4⁺Foxp3⁺ T regulatory cells expressed Tbet⁺ following infection. Treg plasticity in response to changes in the inflammatory microenvironment has been widely described in patients and in disease model systems (84). T regulatory cells capable of producing both pro-inflammatory and anti-inflammatory cytokines have been observed (85); these cells have been hypothesized to be targeted to distinct immune environments during diverse types of inflammatory responses. Notably, the ability for Foxp3⁺ cells to co-express Tbet, in the presence of IFN- γ and/or IL-12, redirects the migratory and functional properties of this regulatory subset (84, 86). A low level expression of Tbet aids in the cellular recruitment of Foxp3⁺ Tregs to sites of Th1 inflammation, through the upregulation of CXCR3, without impacting regulatory function (87). Conversely, following lethal *Toxoplasma* infection, the cytokine storm induces Foxp3⁺ cells with equivalent Tbet expression levels to non-Treg effector cells. The Foxp3⁺Tbet^{hi} cells acquired the ability to produce IFN- γ , allowing these cells to contribute to the pro-inflammatory phenotype (46). This finding is consistent with *in vitro* studies showing that Foxp3⁺ cells skewed toward a Th1-like phenotype had reduced ability to suppress T effector cell proliferation (38).

Moreover, recent studies of *M. tuberculosis* found that Tbet⁺Tregs expanded but subsequently underwent selective culling and elimination in response to IL-12. The mechanisms sustaining Treg populations during an inflammatory response are not fully understood; there are many factors that can impact Treg proliferation and abundance (84). In the chronic *L. (V.) panamensis* model, there is a sustained and elevated level of IFN- γ (9), which potentially could induce T regulatory cells; however, this occurs with the concomitant expression of IL-10 and IL-13. How this might impact on Treg development is unclear. However, the population of Foxp3⁺ cells in infected mice has high expression of Tbet, produces IFN- γ and exhibits a reduction in regulatory capacity. This together with the suppression of IDO expression appears to lead to an overall reduction in the regulation of the ongoing response to infection which is deleterious to the host.

Previous work has shown that BALB/c mice deficient in either IL-10 or IL-13 are resistant to *L. (V.) panamensis* infection (9), suggesting that immune responses counteracting the IFN- γ response prevent disease resolution. However interestingly in DEREK mouse experiments, following Foxp3⁺ cell depletion, disease exacerbation was found in the absence of a change in either IL-10 or IL-13. Instead, increases in the IFN- γ and IL-17 responses were observed. IFN- γ is typically associated with intracellular pathogen killing; however, in this case increased numbers of parasites were found, which corresponded with the elevated cytokine levels. *L. (V.) braziliensis* parasites are less susceptible to IFN- γ mediated NO induced macrophage killing compared to *L. major* (65). Additionally, and consistent with our findings, this study showed that TLR9 deficient mice had increased parasite loads and increased IFN- γ expression in comparison to wild type mice. Persistence and thriving within the presence of an inflammatory environment is not unique to *L. (V.) panamensis*. Previous work with *L. amazonensis* revealed parasite control (lesion size and parasite numbers) in immunodeficient (nude or RAG-2) mice and control of infection by Treg cells (5, 72, 75, 76). The absence of T effector cells in these immunodeficient models correlates with a reduction in monocytes/macrophages at the lesion site, and thus reduces the number of available cellular hosts for the parasite. Therefore, the balance between the T cell response, macrophage activation and cellular recruitment is important for parasite containment. In the case of *L. (V.) panamensis* infection, T regulatory cells ameliorate disease through the down regulation of inflammatory cytokines (Th1/Th2/Th17) and appear to limit the recruitment of target host cells (as observed by the reduction in lesion size). However, this hypothesis (relation of Treg function and cellular recruitment in *L. V. panamensis* infection) needs to be further explored.

Together, these studies have demonstrated the beneficial role of regulatory T cells in controlling excessive immunopathology and parasite numbers following infection with *L. (V.) panamensis*. These experiments were focused on the modulation of an established infection, with the goal of determining whether an intervention manipulating T regulatory cells would have potential therapeutic value. Indeed, we have shown that the *in vivo* induction of CD4⁺Foxp3⁺ T regulatory cells can lead to resolution of active disease. Thus, our data provide support for future research to focus on treatment (local delivery) expanding regulatory T cells as an immunotherapeutic approach for *L. (V.) panamensis* infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

L	Leishmania
V	<i>Viannia</i>
Treg	regulatory T cell
SLA	soluble leishmanial antigen
DT	diphtheria toxin
IDO	indoleamine 2,3-dioxygenase
1-MT	1-methyl tryptophan

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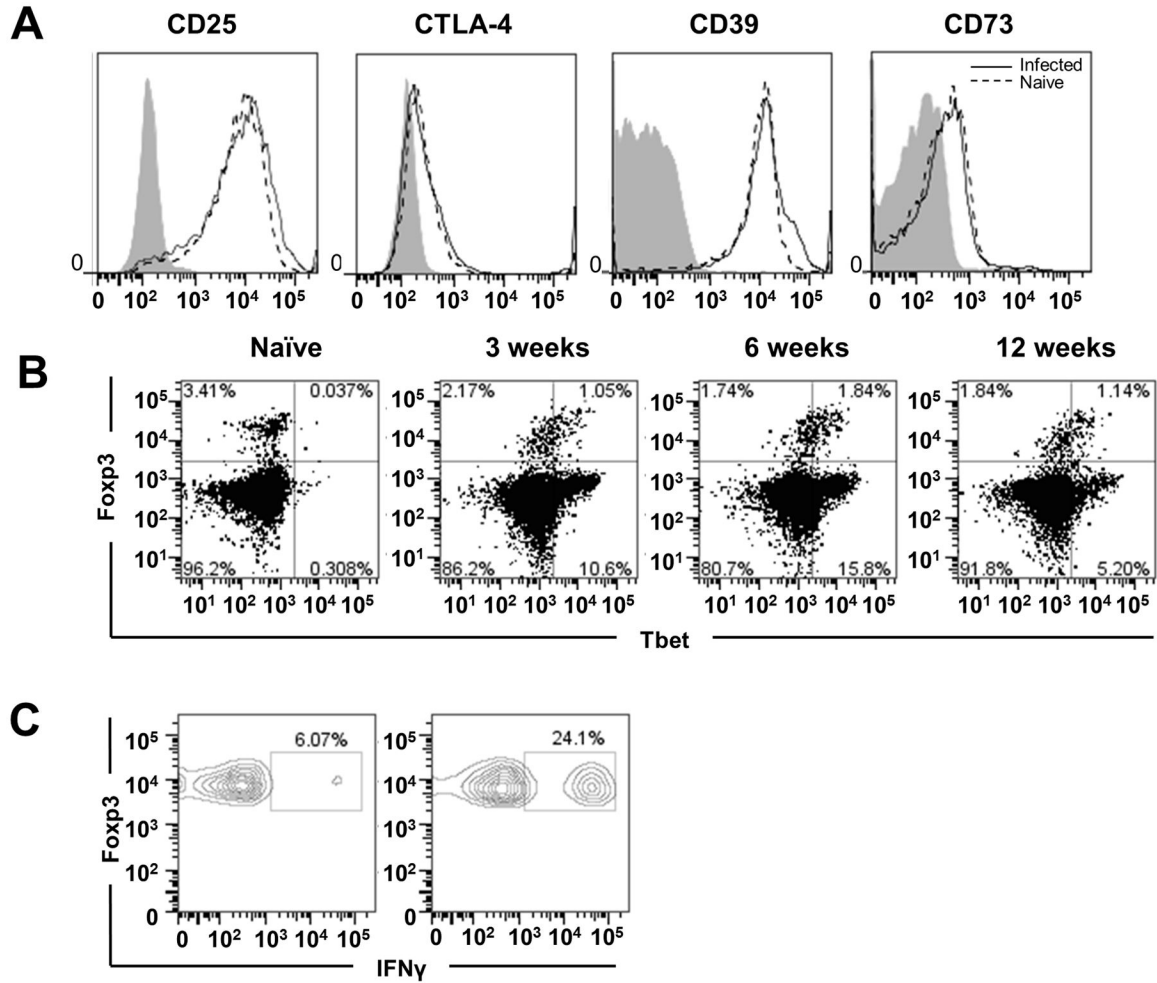


Figure 1. Characterization of T regulatory cells in the draining lymph node during *L. (V.) panamensis* infection

BALB/c mice were infected with 5×10^4 parasites in the top of the rear foot. At various times post-infection, LN cell populations were removed and T regulatory cell markers were evaluated; LN cells from naïve mice were used as controls. (A) Cells from naïve and chronically infected mice (>12 weeks post-infection) were gated on CD4⁺Foxp3⁺ cells and analyzed for expression of CTLA-4, CD25, CD39, and CD73 (solid line, naïve; dashed, chronic infection; shaded, isotype control). (B) SLA-stimulated draining lymph node cells were gated on CD4⁺ cells and examined at the indicated times post-infection. Tbet and Foxp3 expression are shown. (C) Lymph node cells from chronically infected mice were directly cultured *ex vivo* with PMA/ionomycin and examined for Foxp3 and IFN- γ . Cells were gated on CD4⁺Foxp3⁺ cells. Experiments were independently repeated at least 2 times. *, p 0.05.

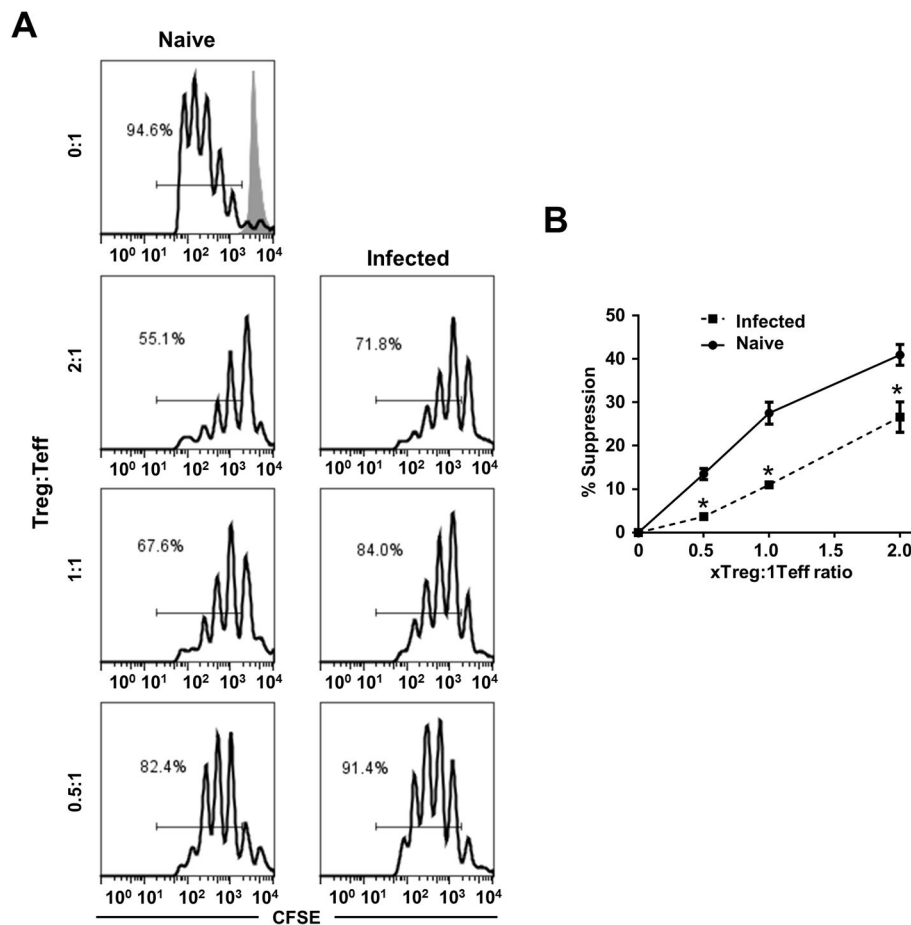


Figure 2. Tregs from *L. (V.) panamensis* infected mice have reduced suppressive capacity
(A) CFSE labeled CD4+CD25⁻ cells (Teff) from naïve mice were cocultured with irradiated APCs, α CD3, and CD4+CD25⁺ cells (Treg) from chronically infected or naïve mice at the indicated ratios. Unstimulated CFSE labeled CD4+CD25⁻ cells (shaded line) are shown; CFSE levels are plotted and % proliferated cell indicated. **(B)** Suppression was calculated using the following equation, $(\% \text{proliferation}_{\text{Teff}} - \% \text{proliferation}_{\text{Treg+Teff}}) / \% \text{proliferation}_{\text{Teff}}$. Samples were analyzed in triplicate and data are representative of two independent experiments. *, p 0.05.

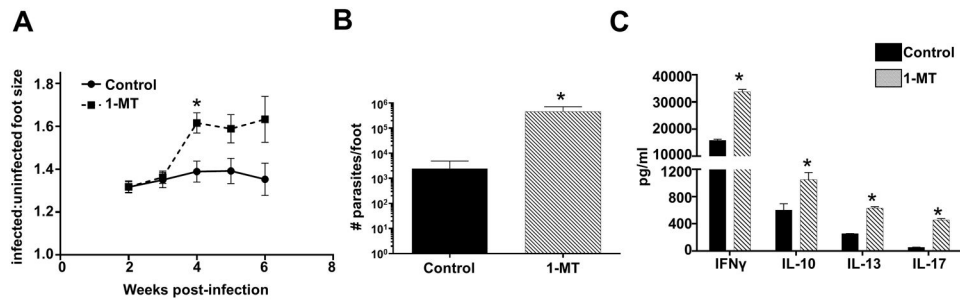


Figure 3. Inhibition of IDO exacerbates disease

Mice were infected 5×10^4 parasites then treated with 1-methyl tryptophan (2mg/ml) ad libitum in their drinking water throughout the course of infection. (A) Lesion size was evaluated throughout the course of infection. At the termination of the experiment (6 weeks) (B) parasite load in the foot was determined using the limiting dilution method and (C) draining lymph node cells were stimulated with SLA for 72 hours. Supernatants were collected and analyzed in duplicate by ELISA. Data represent two independent experiments. *, p 0.05.

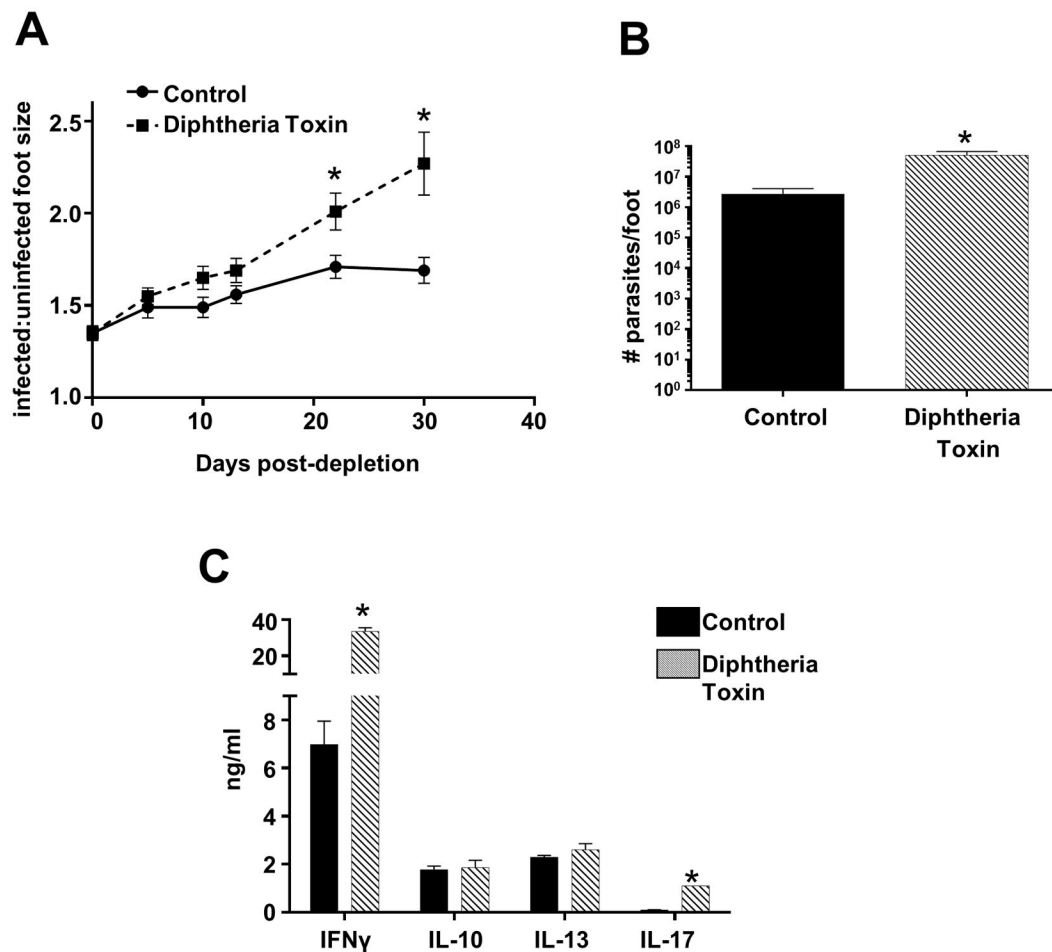


Figure 4. Depletion of T regulatory cells in DEREg mice results in disease exacerbation
 DEREg (DTR and eGFP under Foxp3 locus) mice were utilized to specifically deplete Foxp3+ cells. Three weeks post infection (after lesions developed), mice were given 0.5ug diphtheria toxin, i.p. (A) Lesion size was monitored over the course of infection and (B) parasite burden was calculated as described above. (C) At the termination of the experiment, the draining lymph node cells were stimulated with SLA for 72 hours and supernatants were collected and analyzed in duplicate by ELISA. Data represent three independent experiments. *, p 0.05.

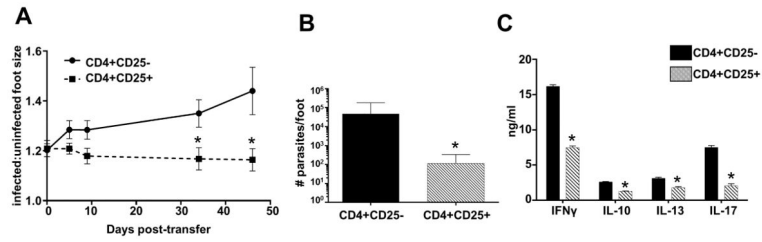


Figure 5. Transfer of regulatory T cells reduces leishmaniasis severity

CD4+CD25+ and CD4+CD25- cells were isolated from the spleens of naïve mice, as described in Materials and Methods section. Three weeks post-infection, 3×10^5 cells (CD4+CD25+ or CD4+CD25-) were injected intralesionally. (A) Lesions were measured throughout the course of infection and (B) parasite burden was analyzed as described above, at the termination of the experiment. (C) Draining lymph node cells were stimulated with SLA for 72 hours. Supernatants were collected and analyzed in duplicate by ELISA. Data represent three independent experiments. *, p 0.05.

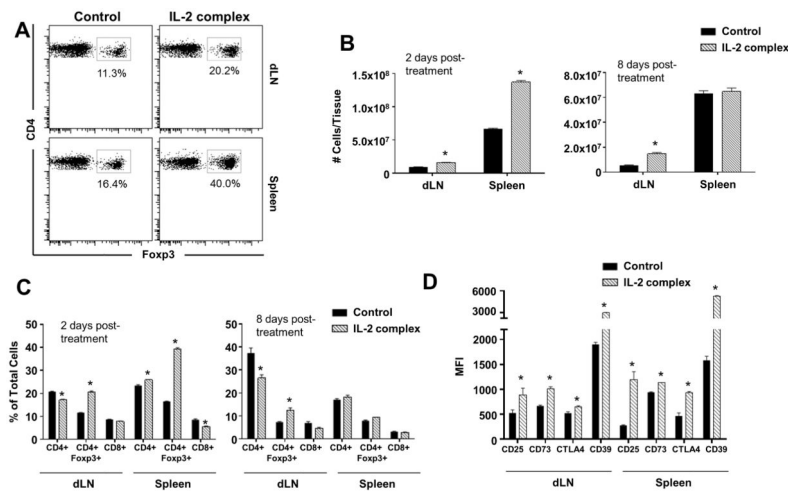


Figure 6. IL-2 antibody complex treatment expands regulatory T cells *in vivo*
 Following the development of lesions (3–4 weeks post-infection), mice were given IL-2/anti-IL-2 in complex as described in Materials and Method section. **(A)** Representative FACS analysis (gated on CD4⁺ cells) comparing CD4⁺Foxp3⁺ cell populations between control and IL-2 complex treated mice two days post-treatment. **(B, C)** At two (left) and eight (right) days post-treatment, draining lymph node and spleens were excised and **(B)** cell numbers and **(C)** cell populations (CD4⁺, CD8⁺ and CD4⁺FoxP3⁺) were assessed. **(D)** Draining lymph node cells from control and treated mice (2 days post-treatment) were gated on CD4⁺Foxp3⁺ cells and analyzed for expression of CTLA-4, CD25, CD39, and CD73.

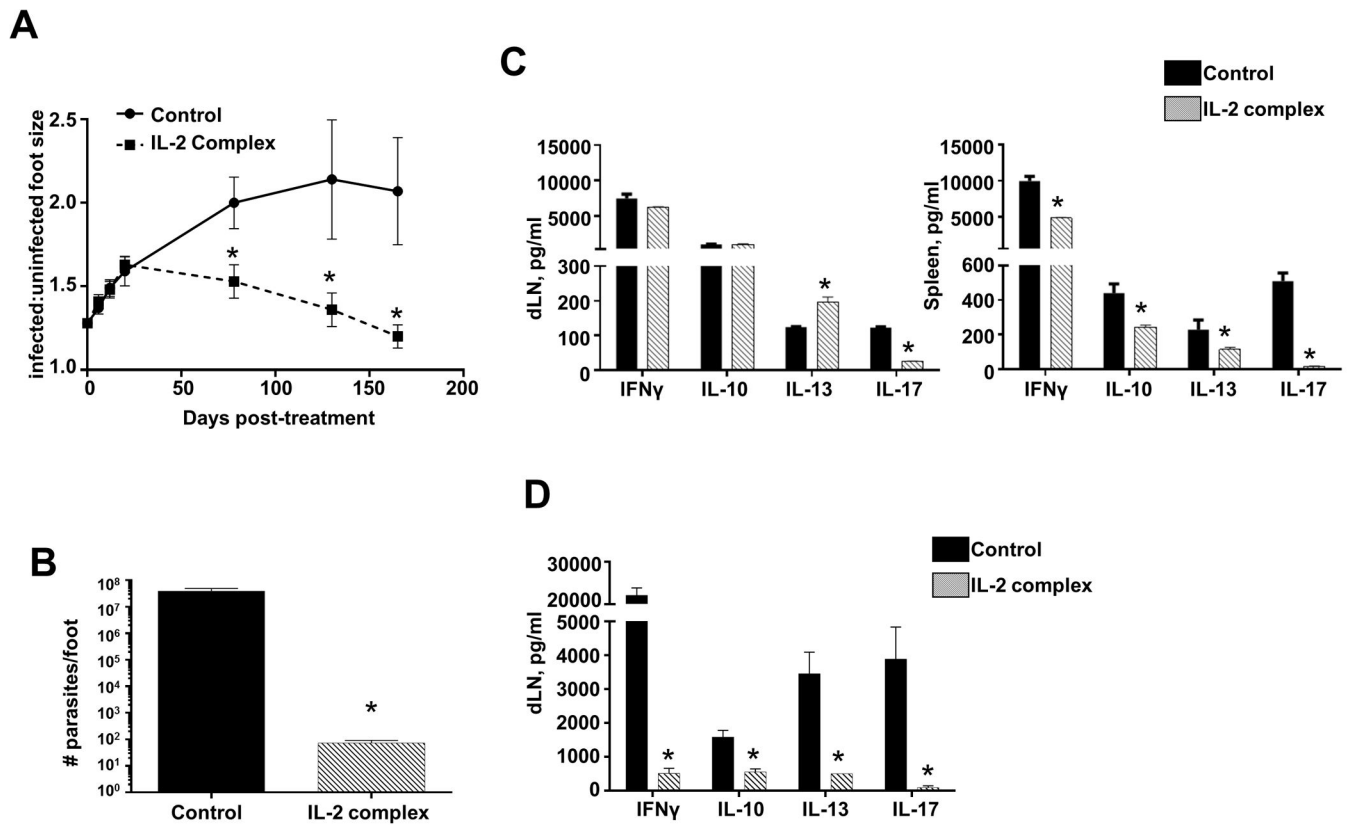


Figure 7. *In vivo* induction of regulatory T cells improves immunopathology

Following the development of lesions (3–4 weeks post-infection), mice were given IL-2/anti-IL-2 in complex as described in Materials and Method section. (A) Lesions were measured throughout the course of infection and (B) parasite burden was analyzed at the termination of the experiment. (C) Two days following the final treatment and (D) at the termination of the experiment, lymph node cells (C, D) and splenocytes (C) were stimulated with SLA for 72 hours. Supernatants were collected and analyzed in duplicate by ELISA. Data represent two independent experiments *, p 0.05.