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*J Immunol* published online 6 August 2014

http://www.jimmunol.org/content/early/2014/08/06/jimmunol.1400728

Supplementary Material

http://www.jimmunol.org/content/suppl/2014/08/06/jimmunol.1400728.DCSupplemental

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The Immunotherapeutic Role of Regulatory T Cells in Leishmania (Viannia) panamensis Infection

Allison Ehrlich,* Tiago Moreno Castilho,* Karen Goldsmith-Pestana, Wook-Jin Chae,† Alfred L. M. Bothwell,‡ Tim Sparwasser,‡ and Diane McMahon-Pratt* 

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 regulatory T cells (Tregs) in Leishmania (Viannia) disease pathogenesis is unclear. Using the mouse model of chronic L. (V.) panamensis infection, we examined the hypothesis that Treg functionality contributes to control of pathogenesis. Upon infection, Tregs (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. This indicated that an increased inflammatory response was commensurate with disease exacerbation and that the remaining impaired Tregs were important in regulation of disease pathology. Conversely, adoptive transfer of Tregs from naive mice halted disease progression, lowered parasite burden, and reduced cytokine production (IL-10, IL-13, IL-17, IFN-γ). Because Tregs appeared to be important for controlling infection, we hypothesized that their expansion could be used as an immunotherapeutic treatment approach. As a proof of principle, chronically infected mice were treated with rIL-2/anti-IL-2 Ab complex to expand Tregs. Treatment transitorily increased the numbers and percentage of Tregs (draining lymph node, spleen), which resulted in reduced cytokine responses, ameliorated lesions, and reduced parasite load (102-fold). Thus, immunotherapy targeting Tregs could provide an alternate treatment strategy for leishmaniasis caused by Leishmania (Viannia) parasites.

The Journal of Immunology, 2014, 193: 000–000.

T he members of the genus Leishmania, consisting 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 South and Central America. 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 also have been reported (4).

The immune response to Leishmania directly impacts the outcome of infection. However, the classic Th1/Th2 paradigm developed for L. major 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, as well as 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 L. (V.) 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 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 Ag-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-mediated (CTLA-4, CD39, CD73) and cytokine-mediated (IL-10, TGF-β) mechanisms (18, 19). Although Tregs were 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 Tregs 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 Tregs 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 used 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, Tregs in the draining lymph nodes (DLNs) display characteristics of dysregulation (express Tbet and IFN-γ) and have a reduced suppressive capacity in comparison with Tregs from naïve uninfected mice. Consequently, we sought to determine the impact of Tregs on infection. These studies 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, whereas temporal enhancement of Tregs (through cell transfer or rIL-2/anti–IL-2 Ab 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 (Frederick, MD) and housed at the Yale University School of Medicine facilities, which is 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. DEREG (Foxp3-eGFP/PDR) mice were used previously (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 μg/ml gentamicin. The infection protocol was described previously (9). Briefly, infective parasites were isolated from late stationary–phase promastigotes from the 45/60% Percoll gradient interface. Parasites (5 × 10^8) were injected intradermally into the top of a hind foot. Lesion development was monitored as indicated above. To determine the impact of Tregs on infection with L. (V.) panamensis, we used 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, Tregs in the draining lymph nodes (DLNs) display characteristics of dysregulation (express Tbet and IFN-γ) and have a reduced suppressive capacity in comparison with Tregs from naïve uninfected mice. Consequently, we sought to determine the impact of Tregs on infection. These studies 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, whereas temporal enhancement of Tregs (through cell transfer or rIL-2/anti–IL-2 Ab therapy) leads to disease amelioration. Taken together, these results indicate that Tregs can be beneficial in resolving infection with L. (V.) panamensis.

IDO inhibition and in vivo depletion of Tregs

1-Methyl-β-tryptophan (1-MT; Sigma-Aldrich) was formulated and administered to mice as previously described (30). Briefly, mice were treated with 2 mg/ml 1-MT in their drinking water starting 2 d postinfection and continuing for the duration of the experiment. Depletion of Foxp3 cells in DEREG mice was performed as previously described (31). Briefly, 3 wk postinfection, mice were administered 0.5 μg diphtheria toxin (DT; Enzo Life Sciences) i.p. on two consecutive days per week for 2 wk. PBMCs were isolated from mice 1 d following the last DT injection; flow cytometry was used to confirm Treg depletion.

Isolation of lymphocytes, cellular transfer, and suppression assays

CD4+ and CD4+CD25+ cells were isolated from the spleen or DLNs 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 × 10^6) were injected once intraleosionally in chronically infected mice (3–5 wk postinfection), and infections were monitored as indicated above.

For suppression assays, 5 × 10^6 isolated naïve CD4+CD25+ cells (effector T cells [Teffs]) were labeled with 5 μM CFSE (eBioscience) and cocultured with CD4+CD25– cells (Tregs) at various ratios using 2 × 10^6 T cell–depleted irradiated splenocytes as APCs. Cells were stimulated with 0.5 μg/ml anti-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 Teff+rIFNγ/g IFN-γ) / % proliferation Teff alone. The isolated CD4+ Tregs from both naïve and infected mice had comparable levels of CD25 and Foxp3 expression (CD4+CD25+ purity was >90%).

Flow cytometry and cytokine analyses

Single-cell suspensions were made from the DLNs and brought up to 5 × 10^6 cells/ml in RPMI 1640 supplemented with 10% FCS. Cells were cultured with PMA/ionomycin (BD Pharmingen) for 4 h, Fcs were blocked (anti-CD16/CD32; BD Pharmingen), and surface markers were stained with anti-CD3 (145-2C11; BD Pharmingen), anti-CD4 (RM4-5; BD Pharmingen), anti-CD8α (53–67; BD Pharmingen), anti-CD11b (M1/70; BD Pharmingen), anti-CD11c (N418; eBioscience), anti-CD91 (1D3; eBioscience), anti-Ly6G (RB6-8C5; eBioscience), anti-CD25 (PC61.5; eBioscience), anti-CD39 (24DSM1; eBioscience), anti-CD73 (TY/23; BD Pharmingen), and anti–CTLA-4 (UC10–4F10–11; BD Pharmingen) with corresponding isotype controls (eBioscience or BD Pharmingen). Cells were fixed and permeabilized (Cytolyt/Cytoperm; BD Pharmingen) for intracellular staining with anti-Foxp3 (FJK-16s; eBioscience), anti–IFN-γ (XMGl2.1, eBioscience), or anti-Tbet (4B10, eBioscience) Abs or the corresponding isotype control IgG2a, or IgGlk Abs (eBioscience). Alternatively, cells were cocultured for 72 h with soluble leishmania Ag [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).

IDO analyses

For the determination of IDO, RNA was isolated from infected and naïve lymph nodes (LN) 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 (Bio-Rad), according to the manufacturer’s instructions. IDO mRNA was normalized against GAPDH using the comparative C_Δ method (32). The following primers were used: IDO fwd, 5′-CGCAGCTGAGGAGGACAGTGTAAC-3′; IDO rev, 5′-ACACATCGCCATGTTGATGTAC-3′; GAPDH fwd, 5′-TGCAACCACACAGTCTTAG-3′; and GAPDH rev, 5′-GATGGCAGGATGATGGTCC-3′.

rIL-2/anti–IL-2 Ab complex treatment for Treg expansion

Anti-IL-2 Ab/BL2 complexes were formulated as previously described (33), using a 1:1 molar ratio. Briefly, mice with established lesions were injected i.p. injections of recombinant mouse IL-2 (PeproTech; 1 μg) in complex with anti–IL-2 mAb (JES6-1.5, 1 μg), every 3 d for a total of four doses. Because 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 Ab was not used. Two and eight 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 DLN and spleen. Cytokine analysis was conducted at the same time points and at the termination of the experiment. Lesion size was monitored as described above.

Statistical analyses

Statistical analyses were conducted using the Student t test or the Wilcoxon signed-rank test. The p values ≤ 0.05 were considered significant.

Results

Tregs are phenotypically altered in response to L. (V.) panamensis infection

Inflammation plays an important role in leishmaniasis caused by organisms of the Leishmania (Viannia) subgenus (10–12). Understanding the contribution of Tregs in modulating inflammation and, thus, disease may highlight areas for immunotherapeutic interventions. Tregs were found to play variable roles in studies of the diverse species of Leishmania; these range across a wide spectrum, from preventing strong antiparasitic Th1 responses and responses necessary for memory development that aid parasite
stimulation, 20.8 6 4.5% of Foxp3+ cells from the DLNs of L. (V.) panamensis–infected mice expressed IFN-γ (Fig. 1C). IFN-γ–producing Tregs were found in both human and mouse models of autoimmune (38, 45) and infectious (46) diseases and have been associated with Tregs taking on proinflammatory capabilities. Taken together, these data suggested functional dysregulation of Tregs during L. (V.) panamensis infection.

**Tregs 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 has 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 naive or infected mice to inhibit Teff proliferation was assessed. Overall, the CD4+CD25+ cells (Tregs) isolated from the DLNs of L. (V.) panamensis–infected mice were less effective than were Tregs isolated from pooled peripheral LNs of naive mice (despite having equivalent Foxp3 expression) in inhibiting CD4+CD25+ (Teff) proliferation in coculture with anti-CD3 and irradiated APCs (Fig. 2A). The percentage suppression of Teff proliferation when cocultured with Tregs from infected mice was 26.6, 11.0, and 3.7% compared with 40.9, 27.5, and 13.5% when cocultured with Tregs from naive mice at ratios of 2:1, 1:1, and 0.5:1 Treg/Teff, respectively (Fig. 2B). Thus Tregs from L. (V.) panamensis–infected mice had a reduced suppressive capacity (as little as 27% of that of naive Tregs) compared with Tregs from naive mice. Therefore, despite maintenance of certain features consistent with Treg function (CD25, Foxp3, CTLA-4, and the surface ectonucleases CD39 and CD73), the Tregs from L. (V.) panamensis–infected mice were significantly less effective in inhibiting Teff proliferation. These results are consistent with Treg function from human patients with active leishmaniasis caused by L. (V.) panamensis, in whom a reduced capacity of Teffs to inhibit IFN-γ production was observed (28). Thus, in both human infection and the mouse model, impaired Treg function is consistently

**FIGURE 1.** Characterization of Tregs in the DLN during L. (V.) panamensis infection. BALB/c mice were infected with 5 3 10⁵ parasites in the top of the rear foot. At various times postinfection, LN cell populations were removed, and Treg markers were evaluated; LN cells from naive mice were used as controls. (A) Cells from naive and chronically infected mice (>12 wk postinfection) were gated on CD4+Foxp3+ cells and analyzed for expression of CTLA-4, CD25, CD39, and CD73 (solid line, chronic infection; dashed line, naïve; shaded, isotype control). (B) SLA-stimulated DLN cells were gated on CD4+ cells and examined at the indicated times postinfection. Tbet and Foxp3 expression are shown. (C) LN 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 repeated independently at least two times.
observed, indicating that the inflammatory response to infection may persist as a result of reduced Treg function.

Reduction of Tregs during infection exacerbates disease caused by L. (V.) panamensis

To further examine the role of Tregs during infection, the effect of Treg reduction on infection was examined. Initially, the chemical inhibitor of IDO, 1-MT, was used. IDO is known to be critical for Treg differentiation, enhancing Treg numbers and function while suppressing Teff activation (47). It should be noted that the expression of IDO was reduced significantly in L. panamensis–infected mice (25-fold relative to expression in peripheral LNs of naive mice; Supplemental Fig. 1).

Therefore, IDO inhibition was used to further block the action of IDO and indirectly diminish Treg levels (48–50). Treatment of mice was initiated 2 d postinfection 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 ± 1.7%) reduction in the level of Tregs. Further, mice treated with 1-MT showed significantly larger lesions (Fig. 3A) and a >100-fold increase in parasite numbers (Fig. 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, including IFN-γ, IL-10, IL-13, and IL-17, in the DLN (Fig. 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 Tregs modulate both Th1 and Th2 responses (52). These results support the fact that a decrease in Tregs may be detrimental to disease control and that these cells modulate the mixed, hypercytokine response that is induced upon infection.

FIGURE 2. Tregs from L. (V.) panamensis–infected mice have reduced suppressive capacity. (A) CFSE-labeled CD4+CD25− cells (Teffs) from naive mice were cocultured with irradiated APCs, anti-CD3, and CD4+CD25+ cells (Treg) from chronically infected or naive mice at the indicated ratios. Unstimulated CFSE-labeled CD4+CD25− cells (shaded graph) are shown; CFSE levels are plotted, and the percentage of proliferated cells is indicated. (B) Suppression was calculated using the following equation: (% proliferation Teff − % proliferation Treg+Teff)/% proliferation 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 with 5 × 10⁵ parasites and then treated with 1-MT (2 mg/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 wk) parasite load in the foot was determined using the limiting dilution method (6) (B), and DLN cells were stimulated with SLA for 72 h (C). Supernatants were collected and analyzed in duplicate by ELISA. Data represent two independent experiments. *p ≤ 0.05.
Although inhibition of IDO resulted in decreased levels of Tregs and exacerbated disease, IDO can directly suppress the proliferation and differentiation of Teffs, in part through tryptophan catabolism metabolites. Reducing IDO was shown to promote the development of Teffs in various systems (53–55). Therefore, a significant reduction in IDO is consistent with both Teff expansion and the findings of reduced functionality of Tregs in the *L. V. panamensis*-infected mice. Further, the inhibitor 1-MT might impact parasite survival through the buildup of tryptophan. Such a nutrient-rich environment could aid in the survival of the parasite, and it was reported that increased tryptophan levels improve trypanosome survival (a related kinetoplastid) (56). Tryptophan was 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 depending on the origin (human versus mouse) of the macrophage (58). Consequently, an alternate approach was used: DEREG mice, in which eGFP and the DT receptor are under the control of the Foxp3 promoter and allows the specific ablation of Foxp3+ cells (31).

As expected, Tregs from infected DEREG mice were completely depleted in response to DT treatment in vivo (data not shown). In response to DT treatment, the Foxp3 cell-depleted mice developed exacerbated lesions (Fig. 4A) and a 19-fold increase in parasite levels in comparison with control mice (Fig. 4B). It should be mentioned that, as previously reported (59, 60), infected BALB/c mice treated with DT developed less severe disease than did either control infected mice or DT-treated DEREG mice (Supplemental Fig. 2). An effect of DT toxin in vivo (although well below the known LD50 for mice (61)) was noted recently (59, 60). This effect undoubtedly masks the level of the exacerbatory response to DT treatment, the Foxp3+ cell–depleted mice developed exacerbated lesions (Fig. 4A) and a 19-fold increase in parasite levels in comparison with control mice (Fig. 4B). It was shown (9) that IL-10 and IL-13 were no significant change in the level of IL-13 or IL-10 at 9 wk postinfection (Fig. 4C). It was shown (9) that 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; rather, they exacerbated disease.

**FIGURE 4.** Depletion of Tregs in DEREG mice results in disease exacerbation. DEREG (DTR and eGFP under Foxp3 locus) mice were used to specifically deplete Foxp3+ cells. Three weeks postinfection (after lesions developed), mice were given 0.5 μg DT i.p. Lesion size was monitored over the course of infection (A), and parasite burden was calculated as described (6) (B). (C) At the termination of the experiment, the DLN cells were isolated from spleens of naive mice and adoptively transferred into infected mice with established lesions. Two days posttransfer, a modest and comparable increase (15–17%) in Tregs was found at both the lesion site and at the DLN. The transfer of Tregs impaired lesion progression, with significant lesion reduction evident 5 d posttransfer in comparison with control mice receiving CD4+CD25+ T cells (Fig. 5A). This impact upon lesion development was accompanied by a reduction in parasite burden (Fig. 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 (Fig. 5C). Consistent with experiments demonstrating that depletion of Tregs resulted in disease exacerbation, the reconstitution of Tregs during established infection led to significant reductions in pathology and parasite levels. Overall, these results demonstrate the importance of Tregs in controlling the immunopathologic state.

**Treatment with rIL-2/anti–IL-2 Ab complexes induces Tregs and ameliorates disease**

Because the transfer of CD4+CD25+ Tregs 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 used IL-2/anti–IL-2 Ab complex treatment [rIL-2/anti–IL-2 (JES6)] to target and promote the expansion of CD25+ Tregs. Such treatment was shown to...
preferentially amplify Tregs by blocking the medium-affinity IL-2Rβγ receptor (expressed on CD8+ T cells and NK cells) binding site, whereas the high-affinity IL-2R, CD25 (IL-2Rαβγ), binding site on Tregs remained exposed (33).

Following lesion development and establishment of chronic infection at 5 wk postinfection, mice were systemically treated i.p. four times over a 9-d period (every 3 d) with IL-2/anti–IL-2 complex. As expected (33, 62, 63), an expansion in total cells (spleen and DLNs) was observed in response to treatment (Fig. 6) at days 1 and 8 posttreatment. CD4+ and CD8+ T cells, as well as CD4+Foxp3+ T cells, responded to IL-2/anti–IL-2 complex treatment (Fig. 6B–D). However, the overall fold increase in CD4+Foxp3+ cells in the DLNs and spleen was higher than that found for total CD4+ or CD8+ T cells, with a 3-fold Treg increase and a 1.5-fold CD4+ and CD8+ increase in the DLNs and a 5-fold increase in Tregs and a 1.3- or 2.3-fold increase in CD4+ or CD8+, respectively, in the spleen at day 2 posttreatment. These results indicate a change in the overall balance of these cell populations. However, whether this translates into a modulation in the level of Tregs at the site of infection remains to be determined. Concomitantly, markers

**FIGURE 5.** Transfer of Tregs reduces leishmaniasis severity. CD4+CD25+ and CD4+CD25− cells were isolated from the spleens of naive mice, as described in Materials and Methods. Three weeks postinfection, 3 × 10⁵ cells (CD4+CD25+ or CD4+CD25−) were injected intraleonally. Lesions were measured throughout the course of infection (A) and parasite burden was analyzed as described (6) at the termination of the experiment (B). (C) DLN cells were stimulated with SLA for 72 h. Supernatants were collected and analyzed in duplicate by ELISA. Data represent three independent experiments. *p ≤ 0.05.

**FIGURE 6.** IL-2 Ab complex treatment expands Tregs in vivo. Following the development of lesions (3–4 wk postinfection), mice were given IL-2/anti–IL-2 in complex, as described in Materials and Methods. (A) Representative FACS analysis (gated on CD4+ cells) comparing CD4+Foxp3+ cell populations between control and IL-2 complex–treated mice 2 d posttreatment. At 2 d (left panels) and 8 d (right panels) posttreatment, DLNs and spleens were excised, and cell numbers (B) and cell populations (CD4+, CD8+, and CD4+Foxp3+) (C) were assessed. (D) DLN and spleen cells from control and treated mice (2 d posttreatment) were gated on CD4+Foxp3+ cells and analyzed for expression of CTLA-4, CD25, CD39, and CD73. *p ≤ 0.05.
of Treg capacity increased: the mean fluorescence intensity of CD73, CTLA4, CD39, and CD25 increased significantly 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 Tregs. The reduced level of Tregs at 8 d posttreatment is consistent with the known half-life (24 h) of the IL-2 Ab complex (62), as well as 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, the IL-2/anti–IL-2 complex treatment regimen led to lesion resolution (Fig. 7A), with a significant reduction (100,000-fold) in parasite burden (Fig. 7B). This level of parasite reduction through rIL-2/anti–IL-2 Ab immunomodulation appears more effective than that for direct Treg transfer; however, this could be due to a larger, more sustained increase in Treg numbers. Interestingly, even given the short temporal enhancement of Tregs, pronounced changes in parasite levels and pathology were found.

The successful expansion and enhancement of Tregs led to a significant decrease in proinflammatory IL-17 cytokine production at day 2 posttreatment in both the DLNs and spleen (Fig. 7C). Previous studies showed that IL-2/anti–IL-2 complexes led to increased levels of p-STAT5 and a reduction in p-STAT3, which are related to the development of Tregs 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 DLNs; this may reflect, in part, the higher induction of CTLA4, CD39, CD25, and CD79 of Tregs observed in the spleen, as well as the i.p. delivery route used.

The successful expansion of Tregs led to a significant reduction in IFN-γ, IL-10, IL-13, and IL-17 at the termination of the experiment (Fig. 7). Consequently, there ultimately was a downregulation of inflammatory and anti-inflammatory responses. These results are consistent with those found for the transfer of Tregs from naive mice (Fig. 5A, 5B) and may reflect, in part, the lower number of parasites found in the treated mice. Taken together, these experiments demonstrate that increasing Treg 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 that targeting these cells may provide a novel approach to disease treatment.

**Discussion**

Tregs 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 that these cells had a negative impact on host defense, allowing pathogens to thrive by dampening immune system activation (66), further investigations revealed a more complex dynamic that is dependent upon the disease state and pathogen (65, 66). Recent work using Foxp3-DTR (DEREG) mice 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 on infection (68–70) and indicate that evaluation of Tregs should be assessed by multiple approaches during infection to fully understand the ongoing dynamic and consequent potential as targets for therapy/treatment.

The role of Tregs in the spectrum of diseases caused by leishmanial parasites appears to vary. This is not surprising, because 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 are dependent upon the genetic background and, thus, the susceptibility of the mouse. Although a Th1 response can lead to healing, a Th2 response, as well as multiple responses that downregulate 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.

![FIGURE 7](http://www.jimmunol.org/) In vivo induction of Tregs improves immunopathology. Following the development of lesions (3–4 wk postinfection), mice were given IL-2/anti–IL-2 in complex, as described in Materials and Methods. Lesions were measured throughout the course of infection (A), and parasite burden was analyzed at the termination of the experiment (B). Two days following the final treatment (C) and at the termination of the experiment (D), LN cells (C and D) and splenocytes (C) were stimulated with SLA for 72 h. Supernatants were collected and analyzed in duplicate by ELISA. Data represent two independent experiments *p ≤ 0.05.
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 2-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, Tregs ameliorate disease; the reconstitution of RAG1-deficient C57BL/6 mice with naive spleen cells depleted of CD4+CD25+ T cells before infection results in disease exacerbation in comparison with 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 deficient, CITA deficient, and MHCII deficient). Hence, a dampening of the immune response and cellular recruitment provides for parasite control.

However, within the Leishmania (Viannia) subgenus, the role of Tregs 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 DLNs in the mouse model of L. (Viannia) braziliensis, Tregs do not interfere with the development of protective immunity (27). In the nonhuman primate macaque model, lesion resolution was ascribed to the recruitment of Tregs and suppression of an Teff-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 nonfunctional properties) have been identified in lesions of patients infected with L. (Viannia) braziliensis (24, 25). For L. (Viannia) guyanensis (35), in contrast, although Foxp3 and IDO expression in lesions decreases during disease, higher levels are found in patients with chronic disease, suggesting a role for Tregs in pathogenesis. In this study, we show evidence that Treg responses are impaired during infection with L. (Viannia) panamensis and that restoration of these cells (through cell transfer or therapeutically through the use of rIL-2/anti–IL-2 Ab complexes) or their depletion has a beneficial or negative effect, respectively, in disease healing.

IDO is important for the development of Tregs and functions through both its signaling and enzymatic activities (47, 79, 80); tryptophan catabolism products are also known to suppress Teff function. One of the striking results found in response to L. (Viannia) panamensis infection is the reduction in IDO expression in the DLN in chronically infected mice. This is in contrast to findings in vitro or in vivo for L. major infection (dendritic cells; C57BL/6 mice), in which parasites induce the upregulation 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, down-regulation of IL-17 was an early response (2 d) to treatment in the healing animals. These findings are consistent with other studies using IL-2 or IL-2/anti–IL-2 complexes (64, 82) that showed decreased Th1/IL-17 responses. The role of IL-17 in Leishmania (Viannia) infection (72) is not well understood, but it warrants further investigation. The contrasting relationship between L. (Viannia) panamensis and L. major and IDO (and consequent Tregs) may be a result of evolutionary advantages/disadvantages associated with an inflammatory response. Although IDO inhibition enhanced local inflammation in both infectious states, a heightened inflammatory response counteracts L. major survival but appears to be advantageous for L. (Viannia) panamensis persistence. Overall, Leishmania (Viannia) parasites are notable for inflammation associated with disease pathology (9, 11, 35, 77, 78, 83). Thus, increased levels of Tregs in L. (Viannia) panamensis infection lead to control and counteract this species’ survival strategy.

In addition, a portion of CD4+Foxp3+ Tregs 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). Tregs capable of producing both proinflammatory and anti-inflammatory cytokines have been observed (85); these cells were hypothesized to be targeted to distinct immune environments during diverse types of inflammatory responses. Notably, the ability of Foxp3+ cells to coexpress 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 CXCXR3, without impacting regulatory function (87). Conversely, following lethal Toxoplasma infection, the cytokine storm induces Foxp3+ cells that have equivalent Tbet expression levels to non-Treg effector cells. The Foxp3+Tbethi cells acquired the ability to produce IFN-γ, allowing them to contribute to the proinflammatory 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 Treg proliferation (38). Moreover, recent studies of Mycobacterium 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. (Viannia) panamensis model, there is a sustained and elevated level of IFN-γ (9) that might induce Tregs; 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 Foxp3hi 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 showed that BALB/c mice deficient in either IL-10 or IL-13 are resistant to L. (Viannia) panamensis infection (9), suggesting that immune responses counteracting the IFN-γ response prevent disease resolution. Interestingly, however, in DEREG 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. (Viannia) braziliensis parasites are less susceptible to IFN-γ–mediated NO-induced macrophage killing compared with L. major parasites (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 with wild-type mice. Persistence and thriving within an inflammatory environment is not unique to L. (Viannia) 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 Tregs (5, 72, 75, 76). The absence of Teffs 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 among the T cell response, macrophage activation, and cellular recruitment is important for parasite containment. In the case of L. (Viannia) panamensis infection, Tregs...
ameliorate disease through the downregulation 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 [relationship between Treg function and cellular recruitment in L. (V.) panamensis infection] needs to be explored further.

Together, these studies demonstrated the beneficial role of Tregs 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 Tregs would have potential therapeutic value. Indeed, we showed that the in vivo induction of CD4+Foxp3+ Tregs can lead to resolution of active disease. Thus, our data provide support for future research to develop localized treatment methods that expand Tregs as an immunotherapeutic approach for L. (V.) panamensis infection.

Acknowledgments

We thank Drs. Nancy Saravia and Daniel Rodriguez for helpful discussions and insight concerning the human disease caused by L. (V.) panamensis.

Disclosures

The authors have no financial conflicts of interest.

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