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CD4\(^+\)CD25\(^+\) Regulatory T Cells Restrain Pathogenic Responses during *Leishmania amazonensis* Infection\(^1\)

**Jiaxiang Ji,\(^2\)* Joseph Masterson,\(^*\) Jiaren Sun,\(^*\) and Lynn Soong\(^2,3*\)†

Although activation of CD4\(^+\) T cells mediates pathogenesis in *Leishmania amazonensis* (La)-infected mice, these susceptible mice do not develop a polarized Th2 response, suggesting a unique mechanism of disease susceptibility. To understand how Th cell activities are regulated, we examined the frequency and phenotypes of regulatory T (Treg) cells. At 1–3 wk of infection, relatively high percentages of CD4\(^+\)CD25\(^+\)CD86\(^+\) T cells, as well as high levels of FoxP3, TGF-\(\beta\), and IL-10RI transcripts, were detected in the skin and draining lymph nodes, indicating local accumulation of Treg cells. Lesion-derived, IL-10-producing CD4\(^+\)CD25\(^+\) cells effectively suppressed proliferation and cytokine (IL-2 and IFN-\(\gamma\)) production of CD4\(^+\)CD25\(^-\) effector cells. Adoptive transfer of lesion-derived CD4\(^+\)CD25\(^+\) cells to syngeneic, naive C57BL/6 mice before infection significantly reduced disease development. To further validate the beneficial role of Treg cells in La infection, we adoptively transferred CD25\(^+\) T cell-depleted splenocytes (derived from naive mice) into RAG1\(^−/−\) mice. This transfer rendered RAG1\(^−/−\) mice more susceptible to La infection than the mice receiving control splenocytes. The beneficial effect of Treg cells was transitory and correlated with decreased activation of IFN-\(\gamma\)-producing effector T cells. This study uncovers an intriguing role of Treg cells in restraining pathogenic responses during nonhealing *Leishmania* infection and emphasizes a balance between Treg and Th1-like effector cells in determining the outcome of New World cutaneous leishmaniasis. *The Journal of Immunology*, 2005, 174: 7147–7153.

*Leishmania* infections cause a wide spectrum of clinical manifestations, with the outcome of disease determined by complex host-parasite interactions (1). *Leishmania amazonensis* (La), a member of the *Leishmania mexicana* complex, has been identified from South American patients presenting with varied disease forms, ranging from leishmaniasis that is of the relatively benign cutaneous, diffuse cutaneous form to the life-threatening visceral type (2). This parasite species is described as the unique etiologic agent of diffuse cutaneous leishmaniasis, a condition that is associated with specific impairment of the cell-mediated immune response at an early stage of infection. At present, the mechanisms responsible for this specific suppression of cell-mediated immune responses following La infection remain unclear. Different from *Leishmania major* infection that mainly causes self-healing cutaneous lesions, La infection shows variable abilities to induce disease pathogenesis and tendencies to self-cure (3).

New evidence revealed in mouse systems has highlighted unique aspects of immune responses to *La* in comparison to *L. major* infection. Most inbred strains of mice, including BALB/c, C57BL/6 (B6), and C3H, are all susceptible to *La* and inevitably develop lesions with a significant accumulation of heavily infected macrophages as well as CD4\(^+\) and CD8\(^+\) T cells (4). However, MHCI\(^+/−\), RAG2\(^+/−\), and SCID mice are refractory to La infection, showing no gross lesions and minimal cellular infiltrates, with low parasite burdens in tissues (4). In these models, host immune responses, especially CD4\(^+\) T cell responses, are responsible for tissue parasite loads and the pathogenesis associated with it (5–7). In addition, the presence of circulating Ab is linked to lesion development following infection with parasites in the *L. mexicana* complex (8), suggesting a role for B cells as well as for various costimulatory interactions between T and B cells in disease pathogenesis. The parasite-specific Ab may facilitate parasite uptake by macrophages and dendritic cells and thus modulate the fate of intracellular parasites and the function of APCs (9–12). Unlike *L. major* infection, T cells derived from La-infected mice usually display a Th1-like or Th1/Th2 mixed phenotype, producing IFN-\(\gamma\) and TNF-\(\alpha\), but little or no IL-4 and IL-10 (7, 13–15). Thus, a novel, yet undefined mechanism is responsible for susceptibility to La infection in mice. At present, it remains unclear which CD4\(^+\) T subset contributes to pathogenesis and which immune components restrain disease progression.

A greater heterogeneity of CD4\(^+\) T cells beyond the classical Th1/Th2 categorization can influence immune responses to *Leishmania* infection (16, 17). Much attention has been given to a specialized subset of CD4\(^+\)CD25\(^+\) regulatory T (Treg) cells. These Treg cells have been grossly grouped into natural and adaptive based on their development, antigenic specificity, mechanisms of action, and dependence on TCR and costimulatory signaling (18, 19). Treg cells are capable of recognizing self-Ags in autoimmune disorders and non-self Ags in infectious diseases and down-regulate both Th1 and Th2 immune responses, respectively (20, 21). Phenotypically, Treg cells have been further characterized as expressing CTLA-4 (22), glucocorticoid-induced TNF receptor (GITR) (23), CD86 (24), and FoxP3 (a transcription factor) (25, 26). However, it is less clear which molecules are faithful markers for natural vs infection-induced Treg cells and what the precise roles are of these molecules in Treg cell function (21).

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4 Abbreviations used in this paper: La, *Leishmania amazonensis*; L.N, lymph node; Treg, regulatory T GITR, glucocorticoid-induced TNF receptor; FOR, forward REV, reverse.

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Much Treg research has been focused on these cells’ role in the regulation of immunity to human pathogens. In *Helicobacter pylori*-infected mice, the absence of CD4+ CD25+ Treg cells is associated with a loss of suppression of IFN-γ-producing CD4+ T cells, leading to increased pathology (27, 28). Treg cells from *H. pylori*-immunized mice have a decreased ability to suppress proliferation of CD4+ CD25+ T cells when compared with naive mice (27). In *Pneumocystis carinii*-infected RAG2−/− mice, cotransferred CD4+ CD25+ Treg cells can reduce the *P. carinii* load and prevent lethal pneumonia triggered by CD4+ CD25+ T cells (29).

More relevant to this study are the recent findings derived from murine infection with *L. major*. Belkaid et al. (30) have demonstrated that CD4+CD25+ Treg cells not only suppress dominant Th1 cells and prevent the parasites from being completely eliminated in genetically resistant B6 mice, but also suppress dominant Th2 cells and disease development in susceptible BALB/c mice (31). Therefore, Treg cells can down-regulate both protective Th1 cells and disease development in susceptible BALB/c mice (31). Since non-healing disease caused by *L. major* infection is mediated by a unique mechanism that does not involve a polarized Th1/Th2 dichotomy, it is important to understand whether and how Treg cells influence *L. major* infection and disease progression. We speculate that the degree of susceptibility to *L. major* infection is regulated by the dynamics between Treg and effector T cells. In this study, we found an increase of CD4+ CD25+ CD68− Treg cells, along with an increased expression of Foxp3, TGF-β, and IL-10, in local tissues and draining lymph nodes (LN) following *L. major* infection. The adoptive transfer of lesion-derived Treg cells restrained disease development, whereas transfer of naive splenocytes devoid of CD4+ CD25+ Treg cells rendered RAG1−/− mice more susceptible to infection than mice reconstituted with intact splenocytes. These results suggest that Treg cells can limit immunopathogenesis caused by *Leishmania* infection. This beneficial effect, however, is transitory and gradually overridden by the expansion of pathogenic effector T cells.

**Materials and Methods**

**Mice**

Female C57BL/6 (B6) and BALB/c mice were purchased from Harlan Sprague Dawley. Female RAG1−/− mice (generated on the B6 background) and wild-type controls were purchased from The Jackson Laboratory. All mice were maintained under specific pathogen-free conditions and used for experimentation at 6–8 wk of age according to protocols approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, TX).

**Isolation of infective-stage promastigotes and mouse infection**

Infected of *La* (MHOM/BR/77/LTB0016) was maintained by regular passage through BALB/c mice. Promastigotes were cultured at 23°C in Schneider’s *Drosophila* medium, pH 7.0 (Invitrogen Life Technologies) supplemented with 20% FBS (Sigma-Aldrich), 2 mM t-glutamine, and 50 μg/ml gentamicin. For *La* preparation, promastigotes were subjected to three cycles of freeze/thaw and sonication. Infective-stage promastigotes were isolated by negative selection (34). Briefly, stationary-phase promastigotes were resuspended at 5 × 10^6/ml in PBS containing 1,500 diluted 3A1 ascites fluid (a gift from Dr. D. Sacks, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Bethesda, MD). After incubation for 30 min at room temperature, the suspension was centrifuged at 250 × g for 5 min, and the agglutinated, nonmetacyclic parasites were removed. The purified metacyclics were used for infection at either the foot or ear (2 × 10^5 parasites/site).

**Purification of T cell subsets**

Lesion-derived CD4+ T cells were purified from tissues of infected mice by positive selection using magnetic beads, as in our previous report (7). Briefly, B6 mice were infected in both hind feet with 2 × 10^5 metacyclics. At 4–6 wk of infection, the soft foot tissues were harvested, cut into small pieces, and incubated for 1 h at 37°C in complete IMDM containing 1 mg/ml collagenase. Following the treatment, the tissues were transferred into a 40-μm cell strainer (BD Falcon) and disrupted with a 5-ml syringe pestle. After washing, CD4+ T cells were purified by anti-CD4 mAb (L3T4)-coated Dynal beads (Dynal). To further separate CD4+ T cells into CD25+ and CD25− populations, CD4+ T cells were incubated with biotin-conjugated anti-CD25 mAb (7D4; BD Biosciences) for 30 min at 4°C. After washing, cells were incubated with streptavidin-coated microbeads (Miltenyi Biotec) for 30 min at 4°C. Magnetic separation was performed using a MACS separation column according to the manufacturer’s suggested protocol. The flow-through was collected and used as CD4+ CD25− T cells. The retained cells were eluted from the column as purified CD4+ CD25+ T cells. The cells were stained with anti-CD25 mAb, and the purity of cell preparations was determined by FACS analysis (FACSCalibur; BD Labware). The purities of CD4+ CD25+ and CD4+ CD25− T cells were routinely >90 and 99%, respectively. Additional analyses of T cell phenotypes were also performed using FACS. Cells were stained using 1 μg Ab per 1 × 10^6 cells and either run immediately or fixed (1% paraformaldehyde in PBS) for later usage. The panel of Abs used for T cell phenotyping included: CD4 (L3T4; Caltag Laboratories), CD25, GITR (108619 R&D Systems), CD86 (GL1), CD69 (H1.2F3), and CTLA-4 (UC10-4F10-11). Unless indicated otherwise, Abs were purchased from eBioscience.

**Purification, and characterization of denph lymphocytes**

At the indicated time points, infected and contralateral uninfected ears were excised and rinsed in 75% ethanol for 10 min and allowed to air dry. Dermal sheets were separated, and the resulting layers were placed on PBS containing 15% FBS and 1 mg/ml collagenase (disperse [Boehringer Mannheim] and incubated at 37°C for 60 min. The treated ears were then passed through 40-μm nylon strainers. The resulting cell suspensions were washed with ice-cold PBS, passed through a 40-μm strainer, and analyzed by flow cytometry for cellular composition (see above) or RT-PCR for gene expression (see below).

**Proliferation assays and cytokine ELISAs**

Lesion-derived CD4+ CD25− cells (5 × 10^5) and T-depleted, mitomycin C-treated syngeneic splenocytes (5 × 10^5) were cultured in the absence or presence of increasing numbers of lesion-derived CD4+ CD25+ cells for 4 days in 96-well, round-bottom plates. Anti-CD3 (2c11, 0.125 μg/ml) or parasite Ag (equivalent to 5 × 10^5 parasites/well) were added to the culture for stimulation in the presence or absence of anti-IL-10 (JES-516E3, 25 μg/ml; BD Biosciences) or anti-TGF-β (1D11, 25 μg/ml; R&D Systems). In some experiments, recombinant murine IL-2 was added to the cocultures of CD4+ CD25− cells and CD4+ CD25+ cells. One μg of [3H]thymidine was added 18 h before harvest and incorporated radioactivity was determined on a beta emission reader. Supernatants were collected from the culture of CD4+ CD25+ cells and/or CD4+ CD25− cells at 24 h (for IL-2) or 72 h (for other cytokines). In some cases, draining LN cells were prepared (1 × 10^5 cells/ml per well) and stimulated with parasite Ag (equivalent to 5 × 10^5 parasites/well) for 24 h (for IL-2) or 72 h. The levels of cytokines in supernatants were determined by specific ELISAs using paired mAbs for IL-2, IL-4, IL-10, and IFN-γ, along with the appropriate mouse cytokine controls (BD Biosciences).

**Adoptive cell transfer and evaluation of disease outcome**

To prepare CD25− cell-depleted cells, splenocytes of naive B6 mice were incubated with anti-CD25 mAb for 30 min at 4°C. After washing, the cells were incubated with sheep anti-rat, IgG-coated Dynal beads (Dynal) for 20 min at 4°C under gentle shaking. The cells were collected as CD25− cell-depleted splenocytes. The adherent cell layer was resuspended by using a magnetic separator. The CD25− cell-depleted and undepleted splenocytes were transferred i.v. into RAG1−/− mice (5 × 10^5 cells/mouse). Where indicated, lesion-derived CD4+ CD25− cells or CD4+ CD25+ cells were transferred i.v. into wild-type B6 mice (1 × 10^5 cells/mouse). Mice receiving no cells were included as controls. One day posttransfer, mice were infected s.c. in the hind foot with 2 × 10^5 metacyclic promastigotes. Lesion size was monitored with a digital micrometer (Control) and expressed as the difference in thickness between infected and uninfected contralateral tissues. Tissue parasite burdens were measured at indicated time points via a limiting dilution assay as previously described (4).

**RT-PCR primers and cycling conditions**

Total RNA from spleen, draining LN, foot, or ear tissues was isolated using RNA-STAT60 (Tel-Test), and 100 ng of RNA was used for each reaction.
In some cases, skin-derived RNA failed to be amplified via RT-PCR, possibly due to contaminating sugars or abundant ribonucleases found on the skin; however, this inhibitory effect was ameliorated by the addition of high concentrations of BSA (2.5 μg/ml). Annealing temperatures were determined empirically and were at 58°C for β-actin and FoxP3 and at 60°C for TGF-β1 and IL-10RI. Primer sequences (listed as 5′ to 3′) were as follows: TGF-β1 forward (FOR): tgaagcctacggtgtaacgcg, reverse (REV): ggttagcagctggttaggtac FoxP3 FOR: cagctgctacggccgcttcagcag, REV: catttgcagcagcctgctgc; IL-10RI FOR: ggcagagagcagccggccagctgactgtgcaggtgc; IL-10RII FOR: ggcagctacacaggtgtcacttgagtagtctg, REV: aagttctcttcgctacagacβ-actin FOR: ccacgctcctctctcctgttgga, REV: ctagagccttggtgcga. Statistical analysis The difference between experimental groups was determined by the Student’s t test. A difference in mean values was deemed significant when p ≤ 0.05 or very significant when p ≤ 0.01. Results

The frequency and phenotypes of Treg cells in infected sites at initial stages of La infection

The progression of nonhealing disease in La-infected B6 mice requires activation of Th1-like cells (4, 7, 14); however, it is unclear whether Treg cells are involved in this process and, if so, how they regulate disease pathogenesis. To address these questions, we infected B6 mice s.c. with 2 × 10⁵ metacyclic promastigotes in the hind foot and analyzed the frequencies of CD4⁺CD25⁺ T cells in infected sites at various time points. In foot tissues of naive B6 mice, the frequency of CD4⁺CD25⁺ cells was ~6.5 ± 1.7% (among a total of 60 ± 20 cells recovered per foot), representing the steady-state Treg cells that are circulating in uninfected foot tissues. At 1 wk of infection, however, ~32% of tissue-infiltrating cells were found to be CD4⁺CD25⁺ cells (Fig. 1A) (among a total of 1.6 ± 0.3 × 10⁵ cells recovered per foot), suggesting a rapid accumulation of Treg cells in situ. As parasite loads increased during the infection (data not shown), the percentages of tissue Treg cells gradually dropped to ~11% by 8 wk. Similar to this trend of expansion and contraction of Treg frequencies, the absolute numbers of these cells were ~1.8 ± 0.3, 2.5 ± 0.2, and 1.5 ± 0.2 × 10⁵ per foot at 2, 4, and 8 wk of infection, respectively. Compared with these in foot tissues, the percentages of CD4⁺CD25⁺ T cells in the draining LN and spleen fluctuated considerably less during the course of infection (Fig. 1A). For instance, the frequencies of Treg cells in LN started with 6.6% before infection (data not shown), reached 12% at 1 wk, and dropped to 7% at 3 wk (p < 0.05, Fig. 1B). Of note, most, if not all, LN-derived Treg cells were also positive for CD86 (Fig. 1C), suggesting a possible involvement of B7 molecules in Treg function. In addition, CTLA-4 and GITR were detectable on the surface of Treg cells (data not shown).

To further characterize Treg cells in La-infected mice, we took the ear infection approach, because it is easier in that model to recover infiltrating cells with far less contamination of tissue-derived cells than in the foot model. We performed RT-PCR assays to estimate the abundance of TGF-β1, FoxP3, IL-10RI, and IL-10RII transcripts in the ear, draining LN, and spleen tissues. The transcripts for TGF-β1, FoxP3, IL-10RII, and IL-10RII were readily detectable in ear-derived cells at week 1, but were mostly undetectable in the spleen or LN (Fig. 1D). As the infection progressed to week 3, TGF-β1, FoxP3, IL-10RIII, and IL-10RIII transcripts became detectable in the peripheral lymphoid organs (Fig. 1D). The cell type that expressed FoxP3 mRNA was CD4⁺CD25⁺ but not CD4⁻ T cells (Fig. 1F). These results were consistent with the finding of high percentages of CD4⁺CD25⁺ Treg cells in local tissues at early stages of infection (Fig. 1, A and B). Despite the relatively stable percentages of CD4⁺CD25⁺ cells in the draining LN,

FIGURE 1. Frequency and phenotype of Treg cells in draining LN, spleen, and inflamed skin tissues during La infection. B6 mice (five per group) were infected with 2 × 10⁵ metacyclic La promastigotes in the hind foot (A) or in the ear (B–E). A. At the indicated time points, cells were collected from draining LN, spleen, and infected foot tissues, stained, and analyzed by FACS for surface expression of CD4 and CD25. Results are representative of at least two independent experiments. B. Draining LN cells were harvested from infected mice at 1 wk (■) or 3 wk (□) to assess CD86 expression by FACS. Live, CD4⁺ T cells were gated, followed by additional gating of CD25 and CD86. Results are shown as percentages of cells positive for CD4, CD25, and CD86. Data are presented as mean ± SD for each group. * (p < 0.05) indicates statistically significant differences. C. The staining pattern for CD86 (solid arrows) was highly repeatable and much above that seen in the isotype controls (arrows). Shown is a representative result from two independent experiments, indicating the percentages of triple-positive cells at 1 wk (dashed lines) and 3 wk (solid lines). D. Tissues from infected ear, draining LN, and spleen (Sp) were harvested at 0, 1, and 3 wk for RNA isolation and RT-PCR analyses for indicated genes. The gel shown is representative of three separate experiments. Except for low levels of TGF-β1 and FoxP3 in draining LN of naive mice, transcripts for other genes of interests were undetectable in tissues of naive mice (data not shown). E. Band intensity from D was determined for ear-infiltrating cells at 1 wk (■) and 3 wk (□), respectively. F. RT-PCR analysis of FoxP3 expression in naive draining LN (lane 1) as well as spleen-derived CD4⁺, CD4⁻, CD25⁺, and CD25⁻ T cell preparations (lanes 2–5).
and spleen throughout the infection (Fig. 1A), the RT-PCR data suggested that Treg cells induced by La parasites migrated from the infection sites to draining LN and the spleen, exerting their regulatory functions systemically after the first week of infection.

Lesion-derived CD4⁺CD25⁺ Treg cells suppress the function of CD4⁺CD25⁻ effector T cells in vitro

Following La infection, B6 mice usually show measurable lesions around 4 wk, and some of the lesions become ulcerated around 8 wk (4, 7, 14). To examine whether the lesion-derived Treg cells can modulate the function of effector T cells, we purified CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells from foot tissues of 4- to 6-wk infected mice. The proliferation of CD4⁺CD25⁻ responder T cells was measured via anti-CD3 stimulation in the presence of T-depleted, mitomycin C-treated, syngeneic splenocytes. Lesion-derived CD4⁺CD25⁺ Treg cells efficiently suppressed the proliferation of CD4⁺CD25⁻ T cells in a dose-dependent manner (Fig. 2A). Addition of Treg cells also resulted in gross suppression of IL-2 secretion by CD4⁺CD25⁻ T cells in vitro (Fig. 2B).

To determine whether CD4⁺CD25⁺ Treg cells inhibit parasite-specific T cell responses, we stimulated lesion-derived CD4⁺CD25⁻ T responders and CD4⁺CD25⁺ Treg cells alone or in a mixture with syngeneic spleenocytes and parasite lysates. The suppression of Ag-specific responses was determined by the quantity of effector cytokine IFN-γ secreted by T responders, because these cells exhibited very low levels of Ag-specific proliferation (data not shown). In the absence of Treg cells, CD4⁺CD25⁻ effector T cells (dotted bar) produced high levels of IFN-γ in supernatants (~5.5 ng/ml) in response to La Ag stimulation, whereas CD25⁺ T cells produced negligible levels of IFN-γ (solid bar, Fig. 2C). The addition of Treg cells significantly reduced IFN-γ production by CD4⁺CD25⁻ effector T cells (striped bar, p < 0.01). A similar pattern was observed for IL-2 production (data not shown). Interestingly, Treg cells produced significantly higher amounts of IL-10 (~500 pg/ml) than did CD4⁺CD25⁺ cells (20 pg/ml) (p < 0.01, Fig. 2D). In the absence of parasite Ag, neither IFN-γ (Fig. 2C) nor IL-10 (Fig. 2D) was detectable. Therefore, lesion-derived CD4⁺CD25⁺ T responders mainly produced IFN-γ and IL-2 in response to La Ag stimulation, whereas Treg cells were the major source of IL-10.

Adoptively transferred CD4⁺CD25⁺ T cells inhibit La infection and delay disease onset

The above results indicated that La-induced Treg cells secreted IL-10 and suppressed proliferation and cytokine production of effector T cells (Fig. 2). Given the profound impairment in Th1 responses in La-infected mice (7), we speculated that Treg cells would hamper the full activation of Th1 cells and exacerbate the disease. To test this possibility, we isolated CD4⁺CD25⁺ T cells and CD4⁺CD25⁺ Treg cells from foot tissues of 4- to 6-wk infected mice and adoptively transferred these cells (1 × 10⁵ cells/mouse) into naive B6 mice followed by La infection a day later. Surprisingly, mice receiving Treg cells showed a delayed onset of disease and developed significantly smaller lesions (Fig. 3A) that contained considerably fewer parasites than did the infection controls and mice receiving CD4⁺CD25⁺ T cells (p < 0.01, Fig. 3B). To further analyze the cytokines involved in these protective effects, we examined cytokine production at 2 and 10 wk of infection via stimulating draining LN cells with parasite Ag in vitro. Consistent with their regulatory functions, LN cells from mice receiving Treg cells produced significantly lower levels of IFN-γ and IL-4 at 2 wk, as well as lower levels of IFN-γ at 10 wk, than did the infection controls (Fig. 3, C and D). IL-4 was undetectable at 10 wk.
10 wk (data not shown). Taken together, these results indicate that in this nonhealing model of cutaneous leishmaniasis caused by *La* infection, Treg cells disrupt the usual interaction between parasites and effector T cells through an as yet undefined mechanism and thus restrain disease progression.

**Transfer of Treg-depleted splenocytes exacerbated lesion development following *La* infection**

The above studies were conducted in animals that contained the mixed parasite-induced and natural Treg cells (Figs. 2 and 3), and these two populations are known to have several unique features that may influence Treg functions in response to infection (18, 19). To focus on the role of natural Treg cells in *La* infection, we took an alternative approach using RAG-deficient mice, which are known to be resistant to *La* infection due to their profound defects in cellular responses (4). We predicted that RAG-1−/− mice receiving splenocytes with or without Treg cells would develop different responses to *La* infection, if Treg cells can truly modulate the *La*-specific T cell functions in vivo. To ensure the quality of cell preparations, we separated CD4+CD25− Treg cells out of the spleen of naive B6 mice using magnetic beads and monitored their mRNA and surface protein expression by RT-PCR and FACS. FoxP3 mRNA was detected in purified CD4+ and CD25+ T cells, but not in the CD4− and CD25− compartments (Fig. 1F), confirming the efficient extraction of Treg cells. We then transferred 5 × 10^6 intact splenocytes or CD25+ T cell-depleted splenocytes into RAG-1−/− mice, followed by infection with *La* metacyclics a day later. Wild-type mice and RAG-1−/− mice were also infected as controls. As seen in our previous report (4), RAG-1−/− mice receiving intact splenocytes developed larger lesions (Fig. 4A) with higher parasite burdens than did those receiving no cell transfer (p < 0.05, Fig. 4B). Interestingly, RAG-1−/− mice receiving splenocytes devoid of Treg cells developed more progressive lesions that contained ~3-log more parasites than found in those receiving no cell transfer (p < 0.01, Fig. 4B). LN cells derived from RAG-1−/− mice receiving splenocytes devoid of Treg cells produced more IFN-γ than did those from RAG-1−/− recipients receiving intact splenocytes (Fig. 4C). Consistent with the findings described in Fig. 3, these results indicated a beneficial role of Treg cells in *La* infection. They also confirmed our previous observations (35), in which *La* parasites took advantage of the host IFN-γ response for their intracellular survival. By suppressing T cell proliferation and IFN-γ production, Treg cells act to disrupt this cycle and alleviate disease progression.

**Discussion**

Several groups of T cells, including CD4+CD25+ Treg cells, Tr1, Th3, CD4+CD8−, and NKT cells, display regulatory functions and play important roles in autoimmune disorders and host immune responses to microbial pathogens and tumor Ag (19, 21, 36). In this study, we focused on the prototypical CD4+CD25+ Treg cells (which represent 5–10% of the CD4+ T cell population in general) and asked the question of whether they influence the disease outcome in *La* infection. We found that the frequency and total number of CD4+CD25+ T cells increased rapidly shortly after parasite infection and then contracted gradually (Fig. 1). Phenotypically, these CD4+CD25+ cells resemble those previously described Treg cells, based on the expression of their surface and intracellular markers (CD86, FoxP3, TGF-β, and IL-10R) (Fig. 1). Lesion-derived CD4+CD25+ Treg cells were capable of suppressing proliferation, as well as IL-2 and IFN-γ production of lesion-derived effector T cells (Fig. 2), and were the major sources of TGF-β (Fig. 1C) and IL-10 (Fig. 2D). The rapid accumulation and retention of Treg cells locally imply an early involvement of these cells in regulating host immune responses to the parasite.

Using two different cell transfer models, we confirmed the beneficial role of Treg cells in *La*-infected mice. First, adoptive transfer of lesion-derived CD4+CD25+ Treg cells into susceptible B6 mice before infection markedly delayed disease progression, and this protective effect was accompanied by a reduced production of IFN-γ and IL-4 by parasite-specific effector T cells (Fig. 3). Given the kinetics of Treg cells (Fig. 1) and their responsiveness to *La* Ag (Fig. 2D), it is conceivable that some of these lesion-derived Treg cells are infection induced; however, additional studies with in vitro-propagated Treg cells in the presence of *La* Ag would further clarify this issue. Second, adoptive transfer of naive splenocytes devoid of CD4+CD25+ Treg cells into RAG-1−/− mice before infection markedly exacerbated disease progression, and this effect was associated with a marked production of IFN-γ by the effector T cells (Fig. 4). Therefore, regardless of the source (naturally existing vs infection induced) or mode of action of Treg cells (cell contact dependent vs cytokine mediated) in *La*-infected mice, enrichment of Treg cells reduces the pathogenic activities of effector CD4+ T cells and tissue injuries associated with them (4). In this regard, the role of Treg in our model is comparable to those described in herpetic keratitis and other models of infection-triggered, immune-mediated inflammatory lesions, including *L. major* infection in BALB/c mice (31, 36). More importantly, this study extends our current view on the role of Treg in cutaneous leishmaniasis: instead of promoting infection and preventing recovery, as demonstrated in *L. major*-infected resistant B6 mice (30, 37), Treg cells restrain disease progression in a non-cure model of leishmaniasis in the same mouse strain. A better understanding of how Treg cells fulfill their protective role in *La* infection and other immune-mediated diseases would be of great therapeutic value.
The benevolent role of Treg cells during La infection is transitory, since mice receiving Treg cells eventually developed non-healing lesions (Fig. 3A). This is likely attributable to differential recruitment and/or expansion of Treg and effector T cells at the infection site. For example, Treg cells may replicate much more slowly in local tissues than do effector T cells and are gradually outgrown by the latter cells. It is known that Treg cells are relatively quiescent in the steady state and have a life span of a few months (38) and that IL-2 is required for the generation and maintenance of Treg cells (21). During the course of La infection, the percentages of IL-2-producing CD4$^{+}$ T cells in draining LN increased to $\sim$10% at 2 wk and maintained $\sim$17% at 6 wk and thereafter (6). Although CD4$^{+}$CD25$^{-}$ T cells are the major producer of IL-2 (2 Fig. 2B), CD4$^{+}$CD25$^{+}$ Treg cells are incapable of producing IL-2 (data not shown) (39, 40). If Treg cells and effector T cells indeed compete for IL-2 and homeostatic factors for their expansion in vivo, the replication of Treg cells would be less favored. Under the circumstance of low parasite loads (the “silent” phase of parasite amplification in the skin (41)) and high CD25$^{-}$ to CD25$^{+}$ T cell ratios at the first few days of infection, parasite-specific effector T cells may undergo cell cycle arrest with limited proliferation (40). As parasite replication increases at later times, effector T cells may replicate exponentially and override the suppression of Treg cells. This shift in equilibrium between Treg and effector T cells, rather than between Th1 and Th2 cells (42, 43), correlates with the onset of lesions and disease progression in La-infected mice. Therefore, the host susceptibility to La infection is regulated by the dynamics of Treg and effector T cells. Conceivably, it is commendable to explore therapeutic potential of Treg cells and other immune interventions for the control of an established cutaneous leishmaniasis (31, 44).

The several mechanisms such as intrinsically poor innate (7) and Th1 responses (5, 6, 13, 15) in the face of Ab production (8, 45) account for the susceptibility of different mouse strains to La infection. Paradoxically, pathogenic T cells in La-infected mice (especially B6 mice) are Th1-like cells, and elimination of functional CD4$^{+}$ T cells prevents disease progression due to restrained cellular recruitment and local expansion of effector T cells (4). The present study has significantly extended our previous studies on gene-targeted knockout mice by demonstrating Treg-mediated suppression of IFN-\(\gamma\)-producing effector cells. The role of IFN-\(\gamma\) in human leishmaniasis has been investigated by several groups. Given locally in sufficient amounts, this cytokine can promote healing of cutaneous leishmaniasis caused by Leishmania tropica and L. b. guyanensis (46). However, IFN-\(\gamma\) may act as a double-edged sword (47). In the absence of a secondary signal derived from other molecules such as TNF-\(\alpha\) and LPS, IFN-\(\gamma\) alone is inefficient to activate parasite-killing mechanisms in macrophages. Our previous studies have indicated that insufficient levels of IFN-\(\gamma\) not only fail to activate macrophages to eliminate La amastigotes, but rather promote parasite survival and replication within infected macrophages (35), suggesting that this parasite may have evolved unique strategies to subvert the host immune responses.

Consistent with this hypothesis, our results further demonstrated that IFN-\(\gamma\) production correlated with parasite replication and disease progression in vivo (Fig. 4A). During Leishmania panamensis and L. mexicana infection, induction of early IFN-\(\gamma\), but not IL-4, is also associated with the development of cutaneous lesions (48). Although IFN-\(\gamma\) is undoubtedly not a suitable indicator for protection in the study of New World cutaneous leishmaniasis (48), it is not a sole mediator for disease progression in mice and patients either. We previously observed that adoptive transfer of in vitro-generated, parasite-specific Th1 cells provided protective immunity to infection initiated by La promastigotes but not amastigotes (35), and that deletion of the IFN-\(\gamma\) gene in the B6 or BALB/c background before infection with La amastigotes did not significantly alter the course of infection (49). Based on these studies, we therefore speculate that the combined effects of low levels of IFN-\(\gamma\) (35) and several other mediators of inflammation (5–7), as well as parasite-specific Ab (8), promote the rapid recruitment of immature or insufficiently activated macrophages, which creates a microenvironment that favors the replication of La amastigotes in the “safe targets” and progression of the disease. We further speculate that modulating IFN-\(\gamma\)-producing effector T cells by Treg cells markedly slows down this pathogenic process and provides short-term protection to the host, and that additional interventions for full activation of macrophages are necessary for the control of this infection. It appears that chemokines such as CXCL10/IFN-\(\gamma\)-inducible protein 10 are excellent accessory molecules to fulfill this request (R. Vasquez and L. Soong, manuscript in preparation).

It is generally agreed that IL-10 has multilateral effects in animal models of cutaneous and visceral leishmaniasis: suppressing NO production and leishmaniacidal activities in macrophages, suppressing Th1 responses, and consequently preventing parasite clearance in susceptible mice and the generation of sterile immunity in genetically resistant mice (45). In the case of La infection in mice, IL-10 contributes partially to deficient immune responses (5, 7) and is a main factor for enhanced susceptibility in mice coinjected with sand fly saliva (50). As in the findings from studies of L. major infection (32), we also found that Treg cells are a major source of IL-10 in La-infected mice (Fig. 2). Although IL-10-producing Treg cells appear to be utilized by L. major as a part of its immune evasion and persistence strategies in mice, the precise role of Treg-produced IL-10 in La infection is less clear. Although we found that the addition of neutralizing mAb against TGF-\(\beta\) and IL-10 did not eradicate a suppressive effect of Treg cells in vitro (data not shown), we do not know whether or not IL-10 is responsible for down-regulating effector T cells in vivo. In this study, we also observed the increased expression of TGF-\(\beta\) and FoxP3 transcripts and surface expression of CD86 on Treg cells at 1–3 wk of infection, which was concurrent with the high frequency of CD4$^{+}$CD25$^{+}$ T cells (Figs. 1 and 2). It is known that the expression of CD86/B7.2 and CD80/B7.1, typical markers of APCs, on Treg cells can enhance their suppressive function (24, 41). Treg cells therefore may be an excellent accessory population to fulfill this request.
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Disclosures

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