ANALYSIS OF T HELPER CELL RESPONSES DURING INFECTION WITH LEISHMANIA AMAZONENSIS

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Abstract. Most inbred strains of mice are susceptible to Leishmania amazonensis infection and develop progressive cutaneous lesions. However, the role of Th subsets in the disease and the molecular basis of pathogenesis are unclear. To address this issue, we examined the frequency of cytokine-producing CD4+ T cells in the potential contribution of early cytokine production in other inbred mouse strains, and have been used to examine those seen in cutaneous leishmaniasis caused by L. major. BALB/c mice, on the other hand, are highly susceptible to the infection, and develop a strong Th2 response that interferes with the activation of macrophages to a leishmanicidal state.2–4 In contrast, most inbred strains of mice examined to date are susceptible to L. amazonensis infection, developing chronic lesions that mimic those seen in cutaneous leishmaniasis patients.1 BALB/c mice are more susceptible to L. amazonensis and L. mexicana than are other inbred mouse strains, and have been used to examine the potential contribution of early cytokine production in the outcome of the infection. Using this mouse strain, Guera-Mendeoza and others5 have shown a rapid increase of interferon-γ (IFN-γ) transcripts in draining lymph nodes (LN) at 24 hr of infection with L. mexicana. This infection, however, does not result in any elevation of interleukin-4 (IL-4) mRNA even at day 7. In accordance with this, treatment with either anti-IL-4 or anti-IFN-γ does not show a major effect on the course of L. amazonensis infection.5–9 These studies collectively suggest that multiple mechanisms may contribute to susceptibility of mice to L. amazonensis. Consistent with this view, Barral-Netto and others10 have demonstrated an increased production of transforming growth factor-β (TGF-β) by peritoneal macrophages and in foot tissues of L. amazonensis-infected mice, as well as an enhancement of lesion development in mice xenografted with TGF-β. It has also been suggested that an impaired expression of IL-12 receptor β2 chain, and, consequently, the insufficient activation of Th1 responses, is in part responsible for the susceptibility of mice to this parasite species.11

Although CD4+ T cells are shown to be more important in the pathogenesis of L. amazonensis infection than are CD8+ T cells,12 there is little information on the nature and kinetic activation of Th subsets during the course of the disease. In this study, we analyzed the frequency of cytokine-producing CD4+ T cells and the amounts of cytokine production by isolated CD4+ LN T cells. To further test if a particular CD4+ T cell subset is preferentially activated during the infection, we also examined the T cell receptor (TCR) usage in CD4+ LN T cells, as well as in Th2-type cell lines generated via in vitro stimulation with amastigote antigens. The functional role of these Th2 cells in the disease outcome was examined by an adoptive cell transfer.

INTRODUCTION

Leishmania amazonensis is a member of the L. mexicana complex. In South American countries, it can cause a broad spectrum of clinical manifestations, ranging from single cutaneous lesions to multiple, disfiguring nodules and even visceral complications.1 This parasite species is described as the unique etiologic agent of anergic diffuse cutaneous leishmaniasis in Brazil, a condition that is associated with specific immune response at an early stage of infection. Diseases caused by this parasite display variable abilities or no tendency to self-cure.2 At present, the molecular and genetic basis for the development of different clinical diseases following infection with L. amazonensis is undefined.

The contribution of host genetic backgrounds to the outcome of Leishmania infection is well described in murine cutaneous leishmaniasis caused by L. major. In this system, most inbred strains of mice (e.g., C57BL/6 and C3H) are genetically resistant to the infection and capable of mounting a Th1-dominated response. BALB/c mice, on the other hand, are highly susceptible to the infection, and develop a strong Th2 response that interferes with the activation of macrophages to a leishmanicidal state.2–4 In contrast, most inbred strains of mice examined to date are susceptible to L. amazonensis infection, developing chronic lesions that mimic those seen in cutaneous leishmaniasis patients.1 BALB/c mice are more susceptible to L. amazonensis and L. mexicana than are other inbred mouse strains, and have been used to examine the potential contribution of early cytokine production in the outcome of the infection. Using this mouse strain, Guera-Mendeoza and others5 have shown a rapid increase of interferon-γ (IFN-γ) transcripts in draining lymph nodes (LN) at 24 hr of infection with L. mexicana. This infection, however, does not result in any elevation of interleukin-4 (IL-4) mRNA even at day 7. In accordance with this, treatment with either anti-IL-4 or anti-IFN-γ does not show a major effect on the course of L. amazonensis infection.5–9 These studies collectively suggest that multiple mechanisms may contribute to susceptibility of mice to L. amazonensis. Consistent with this view, Barral-Netto and others10 have demonstrated an increased production of transforming growth factor-β (TGF-β) by peritoneal macrophages and in foot tissues of L. amazonensis-infected mice, as well as an enhancement of lesion development in mice xenografted with TGF-β. It has also been suggested that an impaired expression of IL-12 receptor β2 chain, and, consequently, the insufficient activation of Th1 responses, is in part responsible for the susceptibility of mice to this parasite species.11

Although CD4+ T cells are shown to be more important in the pathogenesis of L. amazonensis infection than are CD8+ T cells,12 there is little information on the nature and kinetic activation of Th subsets during the course of the disease. In this study, we analyzed the frequency of cytokine-producing CD4+ T cells and the amounts of cytokine production by isolated CD4+ LN T cells. To further test if a particular CD4+ T cell subset is preferentially activated during the infection, we also examined the T cell receptor (TCR) usage in CD4+ LN T cells, as well as in Th2-type cell lines generated via in vitro stimulation with amastigote antigens. The functional role of these Th2 cells in the disease outcome was examined by an adoptive cell transfer.

MATERIALS AND METHODS

Mice. Female BALB/c and C57BL/6 mice were obtained from Harlan Sprague Dawley (Indianapolis, IN) and were used when they were between six and eight weeks old. For infection, mice were inoculated subcutaneously 2 x 105 or 2 x 106 stationary-phase promastigotes of L. amazonensis in 10 μl of phosphate-buffered saline (PBS) in the hind foot.

Parasite culture and antigen preparation. Promastigotes of L. amazonensis (MHOM/BR/77/LTB0016) were cultured at 23°C in Schneider’s Drosophila medium, pH 7.3 (Gibco, Grand Island, NY), supplemented with 20% fetal bovine serum and 50 μg/ml of gentamicin. Lesion-derived amastigotes were cultured in the same medium at 32°C, except that the pH was adjusted to 5.0 and gentamicin concentration was reduced to 25 μg/ml.13 Promastigotes of L. major (MHOM/IS/79/strain Neal), L. chagasi (MHOM/BR/00/1669, provided by Dr. M. Wilson, University of Iowa, Iowa City, IA), and L. donovani (MHOM/IN/54/strain WR352, provided by Dr. R. Tesh, University of Texas Medical Branch, Galveston, TX) were cultured in complete Schneider’s medium. Frozen stocks of ookinetes of Plasmodium gallinaceum (8A strain) were...
obtained from Dr. J. Vinetz (University of Texas Medical Branch, Galveston, TX). For antigen preparation, parasites (1 × 10⁶/ml in PBS) were subjected to three rounds of freeze-thaw, followed by sonication for 45 min.

**Antibodies and recombinant cytokines.** Unless specified, all monoclonal antibodies (MAbs) specific for mouse cell surface molecules, cytokines, and recombinant cytokines were obtained from BD PharMingen (San Diego, CA). For fluorescence-activated cell sorting (FACS) analysis of TCR usage, fluorescein isothiocyanate (FITC)-conjugated MAbs specific for Vβ2, 3, 4, 5.1/5.2, 6, 7, 8.1/8.2, 8.3, 9, 10, 11, 12, 13, 14, and 17, as well as biotinylated MAbs specific for Vα2, 3, 2, 8, and 11.1/11.2 were used. Phycoerythrin (PE)-labeled MAbs specific for IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, tumor necrosis factor-α (TNF-α), and granulocyte-macrophage colony-stimulating factor (GM-CSF) were used in intracellular cytokine staining of T cells. FITC-, PE-, or Tricolor-labeled anti-CD4 MAbs, as well as labeled rat IgG1 (R3-34), IgG2a (R35–95), and IgG2b (R35–38) isotype controls were applied as needed. For detection of cytokine secretion by enzyme-linked immunosorbent assay (ELISA), pairs of MAbs (purified and biotinylated) specific for IL-2, IL-4, IL-10, IFN-γ, GM-CSF, and TGF-β and the corresponding recombinant cytokines were used. Rat MAb M5/114.15.2 (specific for mouse I-A^d^ and I-E^b^; TIB 120; American Type Culture Collection, Gaithersburg, MD) was used for blocking T cell proliferation in vitro.

**Isolation of CD4^+^ LN T cells.** At indicated time points, CD4^+^ T cells were purified from draining popliteal LN by positive selection using magnetic beads (Dynal, Lake Success, NY). Briefly, LN cells were incubated with the appropriate amount of anti-CD4 MAbs-coated beads for 20 min at 4°C under gentle shaking. The rosetted cells were collected using a magnetic separator, and CD4^+^ cells were detached from the beads after treatment with a detachment enzyme (Dynal). Purified cell populations usually contained > 97% of CD4^+^ T cells as analyzed by FACS. This purification method was used because negative selection used anti-CD8, and anti-MHC class II MAbs failed to provide a reasonable yield of CD4^+^ T cells, partially due to the expression of MHC class II molecules, cytokines, and recombinant cytokines by these cells.

**Stimulation of CD4^+^ T cells.** CD4^+^ T cells were cultured with 5 × 10⁵ DCs and lysates of *L. amazonensis* amastigotes (equivalent to 2 × 10⁶ parasites) in complete IMDM for seven days in 24-well plates. After centrifugation on Lympholyte-M separation media (Cedarlane, Westbury, NY) at 2,000 rpm for 20 min, viable cells enriched at the interface were collected, adjusted to a concentration of 2 × 10⁵ cells/ml, and rested in the absence of antigen for seven days. Viable cells (2 × 10⁵) were restimulated with amastigote lysates in the presence of 5 × 10⁶ syngeneic splenocytes. Two CD4^+^ T cell lines, designated as S1A and S2A, were generated after several cycles of stimulation and rest.

**FACS analyses for intracellular cytokines and TCR usage.** Intracellular cytokines were measured according to protocols provided by PharMingen (San Diego, CA). For mitogenic stimulation, cells were cultured with phorbol myristate acetate (PMA, 50 ng/ml) and ionomycin (500 ng/ml) for 5 hr in complete IMDM containing GolgiStop (BD PharMingen, San Diego, CA). For antigenic stimulation, cells (2.5 × 10⁶) were cultured with 5 × 10⁵ DCs and lysates of *L. amazonensis* amastigotes (equivalent to 2.5 × 10⁶ parasites) for 12 hr in 24-well plates. GolgiStop was added during the last 5 hr of stimulation. Cells were incubated with normal rat IgG for 15 min to block non-specific binding sites. Cells were first stained with FITC-labeled anti-CD4 MAb, fixed/permeabilized, and then incubated with PE-labeled anti-cytokine MAbs. For double staining of IFN-γ and IL-4-producing cells, cells were stained with a Tricolor-labeled anti-CD4 MAb and then with PE-labeled anti-IL-4 and FITC-labeled anti-IFN-γ MAbs. The TCR usage was assessed following protocols provided by PharMingen. After the Fc receptors were blocked, cells were stained with PE-conjugated, anti-CD4 MAb, and then with either FITC-labeled, Vβ-specific MAbs or biotin-conjugated, Vα-specific MAbs. Streptavidin-tricolor (PerCP) was used as a second-step label for biotinylated MAbs. Cell staining was detected on a FACScan and analyzed using CellQuest software (Becton Dickinson, San Jose, CA). Among the Vβ family analyzed, naive and infected mice showed background levels of Vβ3, 5, 11, and 12, which were consistent with clonal deletion of these Vβ-bearing T cells in BALB/c mice. **Adoptive cell transfer and infection.** Viable cells harvested seven days after rest were injected intravenously into the tail vein at a concentration of 5 × 10⁶ or 10⁵ cells per mouse. One day later, mice were infected subcutaneously in the hind foot with 2 × 10⁴ stationary promastigotes of *L. amazonensis*. The increase in foot thickness was measured biweekly using a micrometer. Mice were killed at 16 weeks post-infection (wpi) to
collect draining LNs and the infected foot. Purified CD4+ LN T cells (1 × 10^6) were cultured with 5 × 10^5 irradiated, syngeneic splenocytes in the presence of parasite antigens (equivalent to 1 × 10^5 parasites). The levels of IL-4, IL-10, and IFN-γ in culture supernatants were determined by ELISA. Parasite loads in the infected foot were estimated by limiting dilution, as in our previous report. Briefly, tissue homogenates of individual mice were filtered through 40-μm cell strainers and subjected to serial 10-fold dilutions in complete Schneider’s medium in 96-well plates. After cultivation for 10 days, live parasites were scored under an inverted microscope. Results were expressed as -log parasite titer.

**Statistical analysis.** The significance of the differences between groups was determined by the Student’s t-test.

**RESULTS**

Persistence of a mixed Th1 and Th2 response throughout the course of infection with *L. amazonensis*. It has been well described in the *L. major* model that although both IFN-γ and IL-4 can be detected in LN cells within the first few days of infection, cytokine production in BALB/c mice becomes strongly polarized toward a Th2 phenotype at the onset of the disease. Failure to down-modulate IL-4 production in the host is responsible for the extremely high susceptibility of BALB/c mice to *L. major*. The molecular basis of susceptibility of most mouse strains to *L. amazonensis* remains largely unclear. To evaluate patterns of T cell differentiation during infection, we first examined cytokine profiles of CD4+ T cells by FACS analysis. Mice were infected with 2 × 10^6 promastigotes of *L. amazonensis*. Draining LNs from five mice per group were pooled and lymphocytes were stained for intracellular cytokines. At 2 wpi, CD4+ T cells produced IL-2, IFN-γ, and TNF-α (Figure 1A), but also IL-4, IL-5, IL-6, and IL-10 (Figure 1B). The percentages of CD4+ T LN cells that expressed Th1- or Th2-type cytokines were both increased at 6 wpi (the onset of measurable lesions). A similar profile of cytokine expression was maintained at 12-18 wpi, during which large lesions had been established (refer to Figure 6 for lesion development). Similar analysis using CD4+ T cells isolated from foot lesions of 6- or 18-week-infected mice also revealed a mixed profile of Th1 and Th2 responses. It is also noteworthy that in draining LN, the non-CD4+ compartment contained about 3- to 4-fold higher numbers of IFN-γ-producing cells than did the CD4+ compartment (Figure 1C), but it contained minimal IL-4-producing cells (Figure 1D). Few IL-4- and IFN-γ-double positive CD4+ T cells (< 0.6%) could be attributed to the background of intracellular staining (Figure 1E).

To examine the levels of cytokine production by parasite-specific CD4+ T cells, we isolated CD4+ LN T cells at 0, 2, 6, and 18 wpi, stimulated the cells for 72 hr with different concentrations of amastigote antigens in the presence of irradiated syngeneic splenocytes, and measured the secretion of IL-4 and IFN-γ in culture supernatants by ELISA. As shown in Figure 2, antigen-stimulated CD4+ T cells produced comparable levels of IL-4 and IFN-γ, which reached peaks at 6 wpi and were sustained to 18 wpi. The levels of cytokine production correlated with elevated concentrations of antigens in the culture (10^4–10^6 amastigote equivalent/10^6 T cells) and were specific to parasite stimulation. Together, these *ex vivo* and *in vitro* studies indicated that a mixed Th1/Th2 response was maintained in BALB/c mice throughout the course of *L. amazonensis* infection.

**Analysis of T cell phenotypes following in vitro stimulation with *L. amazonensis* antigens.** To further examine the antigenic specificity as well as *in vivo* function of CD4+ T cells, we analyzed cytokine profiles of LN cells stimulated for several rounds with amastigote antigens. As determined by ELISA, there was a strong increase in the production of IL-4 and IL-10, and a correspondent decrease in the production of IL-2 and IFN-γ, within 2–3 cycles of restimulation (Figure 3). Two stable cell lines, S1A and S2A, were generated from independent cultures. These cell lines were Th2 in nature because they produced high levels of IL-4 and IL-10, but no or minimal levels of IL-2 and IFN-γ, when stimulated with either PMA/ionomycin or antigen-pulsed DCs (Table 1). Measurable levels of GM-CSF (Table 1) and TGF-β (mean ± SD = 175 ± 4 pg/ml) were detected. To determine the antigenic
specificity of these T cell lines, we compared the levels of T-cell proliferation in responses to stimulation with different concentrations of promastigote lysates of *L. amazonensis*, *L. major*, *L. chagasi*, and *L. donovani*. While two cell lines could respond to antigens of all tested leishmanial species in a dose-dependent fashion (Figure 4), they did not respond to lysates of an irrelevant protozoan parasite (*P. gallinaceum*). These cell lines proliferated to a similar extent in response to lysates of *L. amazonensis* amastigotes and promastigotes, but not to parasite antigens when splenocytes of C57BL/6 mice were used. The proliferative activities of both cell lines to parasite antigens were completely blocked by an MAb specific to MHC class II molecules (used at 10 ng/ml). Thus, both cell lines could recognize, in a class II-restricted fashion, some common antigens expressed in different developmental stages and species of *Leishmania*.

It has been reported that a selective expansion of T cells can occur in *L. major*-infected mice. No information is currently available on TCR usage among CD4+ cells of *L. amazonensis*-infected mice. To test if certain populations of CD4+ T cells were preferentially expanded during *L. amazonensis* infection, we examined TCR usage among CD4+ T cells using a panel of MAbs specific to the variable regions of α and β chains. When compared with cells of naive mice, CD4+ T cells collected at 2 and 18 wpi showed no significant increase in the usage of examined Vα and Vβ chains (Figure 5), indicating no selective bias on TCR usage among CD4+ T cells in *L. ama-

zonensis*-infected mice. On the other hand, line S1A showed a strong bias toward Vβ4 and Vβ8.1/8.2 (a total of approximately 45%), whereas approximately 50% of the cells in line S2A predominantly expressed Vα2 (Figure 5). Double staining with available MAbs did not reveal any evident paring of Vα2 with the other tested Vβ chains.

The biological activity of *L. amazonensis*-reactive Th2 cell lines. To determine the role of Th2 cell lines that had perpetuated and expanded upon prolonged antigenic stimulation, we transferred 5 × 10^6 or 1 × 10^7 cells (collected at or after five cycles) into naive BALB/c mice one day prior to parasite inoculation. Usually, at week 6 of infection with 2 × 10^6 parasites, lesions were visible and petuated and expanded upon prolonged antigenic stimulation. For the rest of the cycles, 2 × 10^7 blast cells were stimulated with lysates in the presence of 5 × 10^5 irradiated syngeneic splenocytes. Supernatants were collected at 24 or 72 hr for the measurement of interleukin-2 (IL-2) and interferon-γ (IFN-γ) (A), as well as IL-4 and IL-10 (B) by enzyme-linked immunosorbent assay. Values are the means of duplicate determinations. Results are representative of two separate experiments.

FIGURE 2. Interleukin-4 (IL-4) and interferon-γ (IFN-γ) production by CD4+ T cells during infection with *Leishmania amazonensis*. BALB/c mice were infected as in Figure 1. CD4+ lymph node T cells were isolated by positive selection using magnetic beads. T cells (1 × 10^6) were stimulated with amastigote lysates (equivalent to 10^5-10^6 parasites), or without antigens as a control, in the presence of 5 × 10^5 irradiated syngeneic splenocytes for 72 hr in 24-well plates. Levels of IL-4 (A) and IFN-γ (B) in the supernatants were measured by enzyme-linked immunosorbent assay. Data are the mean ± SD of triplicate determinations. Results are representative of three separate experiments. Ag = antigen.

FIGURE 3. Kinetic analysis of cytokine secretion during *in vitro* expansion of CD4+ T cells. Draining lymph node cells were collected from BALB/c mice at 18 weeks post-infection and were cultured (5 × 10^6 cells/well) with amastigote lysates of *Leishmania amazonensis* (equivalent to 2 × 10^6 parasites) in 24-well plates for the first cycle of restimulation. For the rest of the cycles, 2 × 10^7 blast cells were stimulated with lysates in the presence of 5 × 10^5 irradiated syngeneic splenocytes. Supernatants were collected at 24 or 72 hr for the measurement of interleukin-2 (IL-2) and interferon-γ (IFN-γ) (A), as well as IL-4 and IL-10 (B) by enzyme-linked immunosorbent assay. Values are the means of duplicate determinations. Results are representative of two separate experiments.
control mice. Thus, these Th2 cell lines could exacerbate the disease in BALB/c mice.

**DISCUSSION**

Previous studies from our group and others have indicated coexistence of IFN-γ and IL-4 during *L. amazonensis* infection in susceptible BALB/c, C57BL/6, and C57BL/10 mice. However, these studies were centered on ELISA analyses of culture supernatants derived from splenocytes or LN cells stimulated with parasite antigens, and could not define the specific role of CD4+ T cells in disease because several different lymphocyte populations can contribute to IFN-γ production. In this study, we examined in detail the responses of CD4+ T cells to *L. amazonensis* infection and found comparable kinetics and percentages of CD4+ T cells expressing type-1 (IFN-γ and TNF-α) and type-2 (IL-4, -5, -6, and -10) cytokines. To address the potential concern that PMA/ionomycin may enhance cytokine production in primed T cells, irrespective of their antigenic specificity, we also analyzed CD4+ LN cells that were restimulated with parasite lysates in vitro. The frequencies of parasite-reactive, cytokine-producing CD4+ T cells were ranged from 0.1% to 1.5% in cells stimulated with antigen-pulsed splenocytes or DCs, respectively, and were consistently higher than that of isotype controls (0–0.04%). Importantly, antigen-stimulated CD4+ T cells displayed a comparable expression profile for type-1 and type-2 cytokines. To provide an independent measure of CD4+produced cytokines, we measured the secretion of IL-4 and IFN-γ by purified CD4+ LN cells in response to parasite stimulation (Figure 2). These studies indicate that there is a balanced expansion of Th1 and Th2 cells throughout the course of *L. amazonensis* infection. In contrast, Sommer and

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**TABLE 1**

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<th>ELISA (pg/ml)</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-6</th>
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<th>GM-CSF</th>
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<tr>
<td>S1A</td>
<td>5,418 ± 421</td>
<td>ND</td>
<td>ND</td>
<td>4,667 ± 364</td>
<td>261 ± 36</td>
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<tr>
<td>S2A</td>
<td>5,965 ± 458</td>
<td>ND</td>
<td>ND</td>
<td>4,274 ± 419</td>
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Intracellular staining (%)

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<tr>
<td>S1A</td>
<td>84.4 ± 6.4</td>
<td>5.2 ± 0.6</td>
<td>5.9 ± 0.5</td>
<td>88.3 ± 8.1</td>
<td>21.3 ± 2.0</td>
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<td>S2A</td>
<td>78.4 ± 7.3</td>
<td>7.1 ± 0.7</td>
<td>6.1 ± 0.7</td>
<td>75.3 ± 7.6</td>
<td>26.9 ± 2.0</td>
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Antigen-pulsed DC

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<tr>
<td>S1A</td>
<td>79.7 ± 6.6</td>
<td>22.1 ± 1.9</td>
<td>5.1 ± 0.3</td>
<td>62.0 ± 5.1</td>
<td>11.8 ± 1.9</td>
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<tr>
<td>S2A</td>
<td>71.7 ± 7.1</td>
<td>23.9 ± 1.7</td>
<td>5.6 ± 0.5</td>
<td>47.8 ± 5.2</td>
<td>19.9 ± 1.7</td>
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* CD4+ T cell lines (S1A and S2A) were generated using a protocol of seven-day restimulation with antigens of *L. amazonensis* and seven-day rest. At the fourth cycle, 2 x 10^6 T cells were stimulated with parasite lysates (equivalent to 2 x 10^6 amastigotes) in the presence of 5 x 10^6 irradiated syngeneic splenocytes. Supernatants collected at 24 or 72 hr were tested for indicated cytokines by a specific enzyme-linked immunosorbent assay (ELISA). Results are representative of three experiments. The production levels of interleukin-2 (IL-2) and interferon (IFN)-γ were measured using specific monoclonal antibodies (MAbs) and biotinylated isotype controls (0–0.04%). Importantly, antigen-stimulated CD4+ T cells displayed a comparable expression profile for type-1 and type-2 cytokines. To provide an independent measure of CD4+produced cytokines, we measured the secretion of IL-4 and IFN-γ by purified CD4+ LN cells in response to parasite stimulation (Figure 2). These studies indicate that there is a balanced expansion of Th1 and Th2 cells throughout the course of *L. amazonensis* infection. In contrast, Sommer and

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**Figure 4**. Antigen-specific proliferation of Th2 cells. S1A cells were rested for 10 days and were cultured (10^4) with 5 x 10^5 irradiated syngeneic splenocytes in the presence of indicated concentrations of promastigote lysates of *Leishmania amazonensis*, *L. major*, *L. chagasi*, and *L. donovani* for four days in 96-well plates. One μCi of [3H]-thymidine was added 18 hr before harvest, and the radioactivity was counted. Data represent the mean ± SD of triplicate determinations, and are representative of at least three separate experiments. CPM = counts per minute.

**Figure 5**. Analysis of T cell response (TCR) usage by CD4+ lymph node (LN) cells during infection and by *Leishmania amazonensis*-reactive Th2 cell lines. BALB/c mice were infected as in Figure 1. Th2 cell lines (S1A and S2A) were generated using a protocol of seven-day restimulation with amastigote lysates and seven-day rest as described in the Materials and Methods. The LN cells derived from naive or infected mice as well as Th2 cell lines, were stained with phycoerythrin-conjugated anti-CD4 monoclonal antibody (MAb) in conjunction with either the biotinylated MAbs specific for TCR Vα and fluorescein isothiocyanate-conjugated anti-TCR Vβ MAb. Streptavidin-tricolor (Per-CP) was used as a second-step label for biotinylated antibodies. The results represent three independent experiments. Bars show the mean ± SD. Asterisks indicate statistically significant differences between cell lines and the uninfected control (*P* < 0.05, **P* < 0.01). wk = weeks.
others detected 4−5% of IL-4- and IFN-γ-producing cells in BALB/c mice during the first few weeks of infection with *L. major*. However, the frequency of IFN-γ-producing cells started to decline at 4 wpi, whereas that of IL-4-producing cells remained at a high level. This switch to a Th2-dominant response during *L. major* infection has been noted that cells from genetically susceptible BALB/c mice. Interestingly, it has been noted that cells from genetically susceptible BALB/c (H-2d) mice or resistant B10.D2 (H-2b), C57BL/6 (H-2b), and C3H/HeN (H-2k) mice all show a similar expansion of Vα8+/Vβ4+CD4+ cells following infection with *L. major*. To better understand the antigenic specificity of CD4+ T cells in *L. amazonensis* infection, we tested whether there was an expansion of a particular CD4+ cell subset. We did not observe a significantly biased expansion of any TCR-bearing CD4+ cells following infection with *L. amazonensis* in BALB/c (Figure 5) as well as in C57BL/6 mice (Ji J and others, unpublished data). Therefore, it appears that selective T cell expansion is more related to *Leishmania* species than to the host genetic backgrounds (MHC class II haplotypes).

The S1A line shows a strong bias toward Vβ4 and Vβ8.1/8.2, whereas the S2A line predominantly expresses Vα2 (Figure 5). This might be due to minor differences in culture conditions, as rIL-2 was included in generation of S1A line, but was excluded in that of the S2A line. It is possible that these lines are selected for the cells that grow best under the restimulation regimen, and, therefore, they may not represent the cells that are preferentially activated in animals. When defined parasite antigens are available, this issue could be addressed by examining the kinetic expansion of antigen-specific CD4+ T cells, using approaches including FACS analysis of tetramer, or immunospot assays. Given that LACK is a soluble antigen and is recognized by the host immune system when intracellular parasites are killed, we examined whether our Th2 cell lines are reactive to this antigen. When tested at a concentration of 10 μg/ml, both cell lines display relatively weak responses to *L. major* LACK protein (mean ± SD cpm = 628 ± 148) or its dominant I-A4 peptide (cpm = 862 ± 192) compared with parasite lysates (cpm = 203 ± 889). These results are consistent with recent reports, suggesting a minimal role for LACK in susceptibility of BALB/c mice to parasites in the *L. mexicana* complex and in protective responses to *L. donovani* infection in mice. Based upon accumulated evidence, we speculate that
pathogenic CD4+ T cells in L. amazonensis-infected mice may recognize different sets of antigens from those of L. major-infected mice. Our current research effort is directed toward identifying the antigens recognized by Th2 cells. Given that these Th2 cell lines are reactive to lysates prepared from other Leishmania species (Figure 4), identification of relevant antigens would help us to define the molecular basis of leishmanial pathogenesis.

In summary, this study demonstrates that unlike the L. major model, there is no evident skewing of TCR usage among CD4+ T cells, nor a polarized Th2 response, following infection with L. amazonensis in mice. Investigation into why host immune responses to these two parasite species are so different would help us to understand the biology of the parasite and the pathogenesis associated with the infection.

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