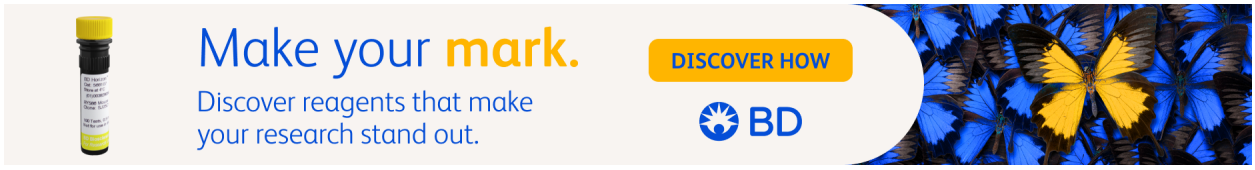

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J Immunol 2008; 180:4090-4097; ;
doi: 10.4049/jimmunol.180.6.4090
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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IL-10 and TGF-β Control the Establishment of Persistent and Transmissible Infections Produced by *Leishmania tropica* in C57BL/6 Mice

Charles F. Anderson, Rosalia Lira,[1] Shaden Kamhawi, Yasmine Belkaid, Thomas A. Wynn, and David Sacks[2]

Leishmania tropica **is the causative agent of Old World anthroponotic cutaneous leishmaniasis, which is characterized by lesions that take an extended period of time to heal, often resulting in disfiguring scars, and are more refractory to treatment than leishmaniasis caused by** ***Leishmania major*****. Immunologic studies involving experimental animal models of** ***L. tropica*** **infection are virtually nonexistent. In the current study, infectious-stage** ***L. tropica*** **were used to establish dermal infections in C57BL/6 and BALB/c mice. In both strains, the lesions were slow to develop and showed minimal pathology. They nonetheless contained a stable number of between 10^4 and 10^5 parasites for over 1 year, which were efficiently picked up by a natural sand fly vector,** ***Phlebotomus sergenti*****. Control of parasite growth depended on the development of a Th1 response, as C57BL/6 mice genetically deficient in Th1 cytokines and BALB/c mice treated with Abs to IFN-γ harbored significantly more parasites. By contrast, IL-10-deficient mice harbored significantly fewer parasites throughout the infection. To further study the immunologic mechanisms that may prevent efficient clearance of the parasites, IL-10 and TGF-β signaling were abrogated during the chronic phase of infection in wild-type C57BL/6 mice. Distinct from chronic** ***L. major*** **infection, IL-10 blockade alone had no effect on** ***L. tropica*****, but required simultaneous treatment with anti-TGF-β Abs to promote efficient parasite clearance from the infection site. Thus, chronic infection with** ***L. tropica*** **appears to be established via multiple suppressive factors, which together maintain the host as a long-term reservoir of infection for vector sand flies.** ***The Journal of Immunology,*** **2008, 180: 4090–4097.**

Leishmania tropica is the causative agent of anthroponotic cutaneous leishmaniasis (ACL),[3] which is endemic throughout the Middle East and in some areas of Africa. ACL has reached epidemic proportions in Afghanistan where the prevalence in 2003 was reported to exceed 200,000 active cases in Kabul alone (1). A recent outbreak of the disease has also been observed in the northwestern region of Pakistan, where the Afghan population has been displaced (2, 3). The skin lesion usually begins as an erythematous papule of the face or limbs, followed by a crusty nodule or an infiltrated ulcer. Cutaneous lesions caused by *L. tropica* tend to be drier, require longer periods of time to heal, and are more difficult to treat than lesions caused by *Leishmania major*. Resulting scars are often disfiguring, especially when found on the face. Following healing, papules can occur around the periphery of the original lesion, termed recidivans type (4). Additionally, *L. tropica* has been shown to cause viscerotropic leishmaniasis, described in veterans of Operation Desert Storm (5, 6).

Published data using animal models for leishmaniasis caused by *L. tropica* is lacking, in part because investigators have had difficulties in establishing infection in vivo (7). We previously obtained reproducible results in BALB/c mice by using parasites highly enriched for infectious, metacyclic promastigotes (8). These initial studies documented the early growth and stabilization of relatively high numbers of parasites in the footpad inoculation site associated with minimal pathology. The current studies extend the murine model of *L. tropica* infection to parasites inoculated in the ear dermis of C57BL/6 and BALB/c mice, and define the immunologic factors that are involved in establishing the long-term balance between host control and parasite survival in the skin.

Materials and Methods

Mice and reagents

The following mice were used in this study and were purchased from Taconic Farms: BALB/c, C57BL/6J, C57BL/10SgSnAi, C57BL/10Sg SnAi-(ko)IL-10 N10, C57BL/6NAi-(Ko)IL-4 N13, C57BL/6NAi-(Ko)IL-10-(Ko)IL-4, C57BL/6Tac-(KO)IFN-γ N12, C57BL/6NTac-(KO)IL-12p40 N10, and C57BL/6NTac-(KO)iNOS. All mice were maintained in the National Institute of Allergy and Infectious Diseases (NIAID) Animal Care Facility under specific pathogen-free conditions, and were used under a study protocol approved by the NIAID Animal Care and Use Committee. The following Abs used for in vivo treatments were purified by Harlan Bioproducts: anti-IL-10αR (1B1.3a), anti-IFN-γ (XMG1.2), anti-TGB-β1,2 (1D11.16.8), and rat IgG control (GL113). Mouse IgG1 control (MOPC 21) was purchased from Sigma-Aldrich.

Parasite preparation and intradermal inoculation

L. major clone V1 (MHOM/IL/80/Friedlin), *L. tropica* clone MHOM/AF/88/KK27 (8), and *L. tropica* clone L747 (ISER/IL/1998/LRC-L747) (9) were cultured in medium 199 with 20% heat-inactivated FCS (Gemini Bio-Products), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), 5 mg/ml hemin (in 50% triethanolamine), and 1 mg/ml 6-biotin (M199/S). Infective-stage metacyclic promastigotes were isolated from stationary cultures

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Received for publication July 17, 2007. Accepted for publication January 4, 2008.

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[3] Abbreviations used in this paper: ACL, anthroponotic cutaneous leishmaniasis; LN, lymph node; DLN, draining LN; BMDC, bone marrow-derived dendritic cell; iNOS, inducible NO synthase.

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by density gradient centrifugation as described previously (10). Metacyclic promastigotes (10^3 or 10^5) were inoculated into the ear dermis using a 30-gauge needle in a volume of ~5–10 μl. The development of pathology was monitored by measuring the diameter of the ear lesion with a direct-reading vernier caliper (Thomas Scientific).

Processing of ear tissue and estimation of parasite load

Parasite titrations were performed as previously described (11). The two sheets of infected ear dermis were separated, deposited in DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.2 mg/ml Liberase CI purified enzyme blend (Roche Diagnostics), and incubated for 2 h at 37°C. Digested tissue was placed in a grinder and processed in a Medimachine tissue homogenizer (BD Biosciences). Tissue homogenates were filtered through a 70-μm cell strainer (Falcon Products) and serially diluted in 96-well flat-bottom microtiter plates containing biphasic medium prepared using 50 μl of NNN medium containing 30% of defibrinated rabbit blood overlaid with 50 μl of M199/S. The number of viable parasites in each sample was determined from the highest dilution at which promastigotes could be detected after 15–30 days of incubation at 25°C.

Restimulation of leukocytes for cytokine analysis

To characterize leukocytes in the inoculation site, the ears were collected, and the ventral and dorsal dermal sheets were prepared as described above. Following preparation, cells were analyzed for surface phenotype by flow cytometry. For in vitro restimulation, unfractionated cervical draining lymph node (DLN) cells were incubated for 72 h at 37°C, 5% CO_2 at a concentration of 2×10^6 cells in 200 μl of RPMI 1640 containing 10% FCS, 10 mM HEPES, glutamine, penicillin, and streptomycin in round-bottom 96-well plates in the presence of 2×10^5 bone marrow-derived dendritic cells (BMDCs) (12) with or without freeze thaw *Leishmania* Ag prepared from *L. tropica* stationary-phase promastigotes. Supernatants were collected 72 h later for measurement of cytokines by ELISA. For restimulation of dermal lymphocytes, cells were cultured with BMDCs or *L. tropica*-infected BMDCs overnight, with monensin (Golgistop; BD Biosciences) added during the last 4 h. In some experiments, dermal cells were cultured with BMDCs and 10 or 100 μg/ml freeze thaw Ag overnight, with monensin added for the last 4 h. The cells were then sequentially stained for surface markers, followed by fixation/permeabilization and intracytoplasmic staining for cytokines.

Immunolabeling and flow cytometry

The following Abs used for immunophenotyping were purchased from BD Biosciences: allophycocyanin anti-mouse TCR-β-chain (H57-597), PE-Cy5 anti-mouse CD4(L3T4) (RM4–5), FITC anti-mouse CD103 (M290), FITC anti-mouse IFN-γ (XMG-1.2). The isotype controls used (all obtained from BD Pharmingen) were rat IgG2b (A95-1), rat IgG2a (R35-95), and hamster IgG, group 2 (Ha4/8). Before staining, LN or dermal cells were incubated with an anti-Fc III/II receptor mAb (2.4G2) in PBS containing 0.1% BSA and 0.01% NaN_3. The staining of surface and intracytoplasmic markers was performed sequentially: the cells were stained first for their surface markers, followed by a fixation/permeabilization step and staining for IFN-γ. For each sample, 250,000 cells were acquired for analysis. The data were collected and analyzed using CellQuest Pro software and a FACSCalibur flow cytometer (BD Biosciences). The lymphocytes from ear cells were identified by characteristic size (forward light scatter) and granularity (side light scatter), and by lymphocyte surface phenotype.

Real-time PCR

For analysis of gene expression, ears were removed and immediately placed in RNAlater (Qiagen). Ear tissue was then disrupted mechanically using liquid nitrogen and a mortar and pestle. Homogenates were then passed through Qiashredder columns and RNA was purified using the RNAeasy mini kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies). Real-time PCR was performed on an ABI Prism 7900 sequence detection system (Applied Biosystems). PCR primer probe sets were predeveloped gene expression assays designed by Applied Biosystems and the quantities of products were determined by the comparative threshold cycle (C_T) method using the equation $2^{-\Delta\Delta CT}$ to determine the fold increase in product. The gene of interest was normalized to the 18s rRNA endogenous control and the fold change in expression are displayed as relative to naive controls.

Transmissibility of parasites from infected ears to sand flies

At various months postinfection, the ability of the infected ears to provide a source of *L. tropica* to their natural sand fly vector, *Phlebotomus sergenti*, was investigated using a previously described method (13). Two- to 4-day-old emergent *P. sergenti* females were obtained from a colony initiated by field-caught specimens from the Jordan Valley and reared at the Department of Entomology (Walter Reed Army Institute of Research). Fifteen sand flies were placed in plastic vials covered at one end with a 0.25-mm nylon mesh. Individual ears of anesthetized mice were pressed flat against the mesh surface using specially designed clamps that held the vial and ear in place between support arms adapted at the ends with a flat rubber surface. The flies were allowed to feed in the dark for a period of 30 min to 1 h. Blood-fed females from each vial were separated, provided a 50% sucrose solution and water, and their midguts were dissected 48 h later and examined microscopically for the presence of promastigotes.

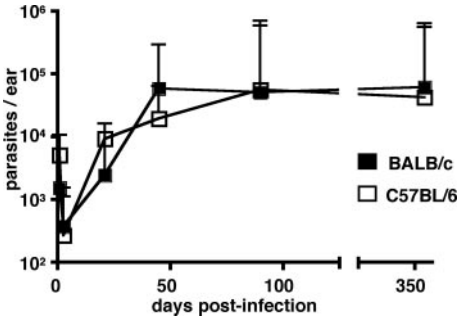

FIGURE 1. *L. tropica* grows with similar kinetics in BALB/c and C57BL/6 mice. A total of 10^5 infectious-stage metacyclic *L. tropica* KK27 parasites were injected intradermally into the ears of BALB/c and C57BL/6 mice. Parasite burdens were determined by limiting dilution analysis at various times postinfection up to 1 year. The data points are the mean and SD of six ears per time point.

Statistical analysis

Statistical significance between groups was determined by the unpaired, two-tailed Student *t* test.

Results

Course of L. tropica *infection in mice*

To follow the course of *L. tropica* infection in a murine model, C57BL/6 and BALB/c mice were infected in the ear dermis with 10^5 infectious stage, metacyclic promastigotes. Ear lesion development and parasite burdens were measured in both strains of mice at various times after infection. In C57BL/6 mice, parasites grew slowly and reached a peak burden of ~50,000–100,00 per ear, which was maintained over 1 year (Fig. 1). In BALB/c mice, which are hypersusceptible to *L. major*, *L. tropica* grew with similar kinetics as in C57BL/6 mice, with a peak parasite burden reached after 1 or 2 mo, and which was maintained for many months. Interestingly, the infection did not progress uncontrolled, but was similar in outcome as C57BL/6 mice. In both strains of mice, lesion development correlated with parasite burdens, with minimal, but persistent, pathology (data not shown).

These data reveal an infection course that is distinct from the typical course of cutaneous leishmaniasis caused by *L. major* in resistant mice in that, in the absence of a parasite clearance phase, the host-pathogen interaction reaches an apparent equilibrium and is maintained for a prolonged period with minimal pathology. Importantly, it reproduces some of the features of cutaneous leishmaniasis caused by *L. tropica* in humans, which is often marked by a sustained period of patency and small, nonulcerative lesions.

Immune mechanisms of protection

To determine the immunological mechanisms underlying chronic infection due to *L. tropica*, a variety of immune deficient mice on the C57BL/6 or C57BL/10 background were infected with either a low dose of 10^3 metacyclic promastigotes, approximating the dose

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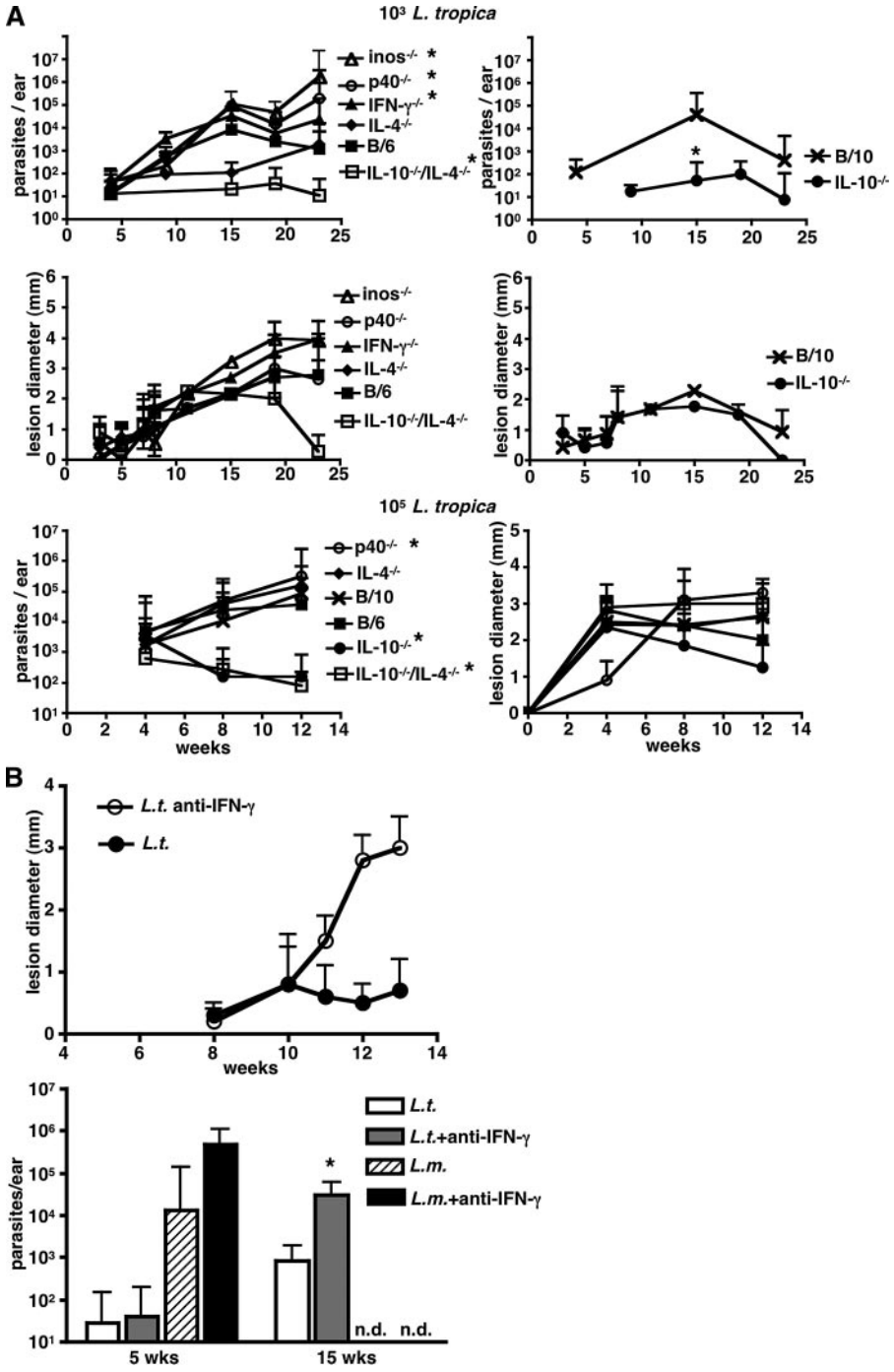

FIGURE 2. Outcome of *L. tropica* infection in immune deficient mice. *A*, Wild-type C57BL/6, C57BL/10, or gene-deficient mice were infected intradermally with 10^3 (*top* and *middle*) or 10^5 (*bottom*) metacyclic *L. tropica* KK27 parasites. At the indicated times, parasite burdens in the lesions were determined by limiting dilution analysis. For burdens, data points are the mean and SD of six ears per time point and the pathology score is the mean and SD of at least six ears per time point. ∗, $p < 0.05$ compared with wild-type control. C57BL/10 mice are the control for IL-$10^{-/-}$ mice; C57BL/6 mice are the control for all other gene-deficient mice. *B*, BALB/c mice were infected intradermally with 10^3 *L. tropica* KK27 or *L. major* FV1 metacyclic parasites and given weekly injections of anti-IFN-γ or isotype control Ab. Shown are the lesion scores for *L. tropica* and parasite burdens for both species, indicating the mean and SD of five mice per time point. ∗, $p < 0.05$.

of sand fly transmitted infections, or a higher dose of 10^5 (Fig. 2*A*). Wild-type mice again achieved peak burdens after 1 or 2 mo, which changed little over a prolonged period. In comparison, increased parasite growth was observed in mice deficient in IL-12p40, IFN-γ, and inducible NO synthase (iNOS), which correlated with increased lesion pathology, demonstrating the requirement for an intact Th1 response to prevent the development of progressive cutaneous disease. Next, IL-4 and IL-10 were investigated as possible counterregulatory cytokines responsible for the inability of C57BL/6 mice to more effectively clear *L. tropica* from the skin. Mice deficient in IL-4 did not exhibit an increase in parasite killing relative to wild-type mice, arguing against Th2-mediated susceptibility. In contrast, IL-10-deficient and IL-10/IL-4 double-deficient mice displayed increased control of parasites relative to wild-type mice, but did not result in sterile cure, which has been reported with *L. major* (14). These infection outcomes were similar in mice inoculated with 10^3 or 10^5 parasites. In BALB/c mice, for which mice genetically deficient in Th1 responses were not available, weekly treatment with anti-IFN-γ was administered from the time of inoculation. No difference was observed in early lesion development, which remained minimal throughout the course of infection in control treated mice, or in parasite burden at 5 wk postinfection. Continuation of treatment resulted in progressive lesion development after 10 wk, and a 100-fold increase in parasite burden when examined at 15 wk postinfection (Fig. 2*B*). In comparison, BALB/c mice were highly susceptible to *L. major* with or without Ab treatment, although anti-IFN-γ treatment promoted even faster parasite growth (Fig. 2*B*) and lesion progression,

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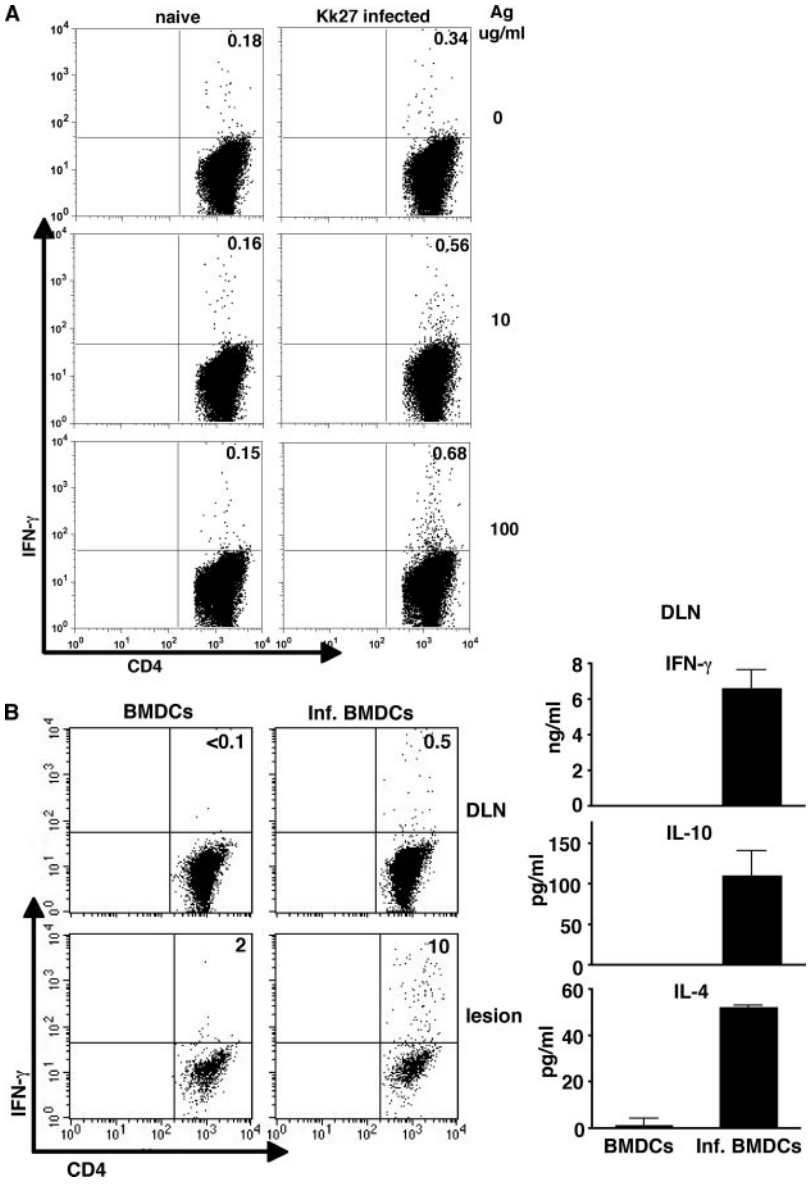

FIGURE 3. Analysis of the host immune response at the site of infection and DLNs. Two months after intradermal inoculation with 10^3 metacyclic *L. tropica* KK27 parasites, C57BL/6 mice were sacrificed for immune analysis in the ear lesion and DLN. *A*, DLN cells were pooled from three infected or naive mice, and restimulated in vitro with 10 or 100 μg/ml Ag prepared from *L. tropica* promastigotes. *B*, DLN and lesion cells were stimulated in vitro with uninfected or *L. tropica*-infected BMDCs. For intracellular staining, cells were incubated overnight, followed by the addition of monensin for the final 4 h. For ELISA measurements, supernatants were collected after 72 h. Data are representative of three independent experiments.

with lesion scores exceeding 4 mm in both groups at 5 wk postinfection (data not shown). In contrast to the high levels of Ag-specific IL-4 secreted by LN cells from *L. major*-infected BALB/c mice (300–425 pg/ml), IL-4 was only minimally produced in response to *L. tropica* infection (<50 pg/ml), and remained relatively low in the anti-IFN-γ-treated mice (<120 μg/ml; data not shown). Thus, despite inefficient clearance of parasites from the site, both C57BL/6 and BALB/c mice mount a Th1 response that exerts meaningful control over lesion development and parasite growth.

To directly measure the host immune response to *L. tropica* infection in C57BL/6 mice, DLN and lesional cells were analyzed 2 mo after inoculation with 10^3 metacyclic parasites. Accumulation of $CD4^+$ and $CD8^+$ lymphocytes at the infection site was detected by flow cytometry (data not shown). The *L. tropica* lesions contained approximately one-half the absolute number $CD4^+$ T cells compared with *L. major* ($(3.5 \pm 1.5) \times 10^4$ vs $(7.9 \pm 1.4) \times 10^4$, averaged from four experiments). For measurement of cytokine production, cells were restimulated in vitro with Ag prepared from *L. tropica* promastigotes, or with BMDCs uninfected or infected with *L. tropica* metacyclic promastigotes. $CD4^+$ cells staining for IFN-γ were detected in DLN cells from infected mice restimulated with 10 or 100 μg/ml Ag (Fig. 3*A*). No Ag-specific responses were observed in LN cells from uninfected mice. A higher frequency of $CD4^+$IFN-γ^+ T cells was observed when DLN cells from infected mice, and especially cells derived from the inoculation site, were restimulated in vitro using infected BMDC (Fig. 3*B*). IL-4 and IL-10 were below the limits of detection by intracellular staining (data not shown), but were detected in low amounts in supernatants of DLN cells by ELISA.

Because the *L. tropica* infections were so poorly established in the IL-10-deficient mice, these mice could not distinguish the contribution of IL-10 to the maintenance of infection during the chronic phase. For this purpose, C57BL/6 mice were treated with anti-IL-10R Ab for a period of 2 wk, starting at 12 wk postinfection. In contrast to the outcome with IL-10-deficient mice, for which IL-10 signaling was absent during the entire infection, a transient abatement during the chronic phase did not promote parasite clearance from the site (Fig. 4*A*). Additionally, analysis of the $CD4^+$ effector response in the lesion revealed no increase in $CD4^+$ cells producing IFN-γ in response to the treatment (Fig. 4*B*). In comparison, and as previously reported, *L. major*-infected mice given the same Ab regimen displayed a dramatic reduction in parasite load, which correlated with enhanced IFN-γ production from

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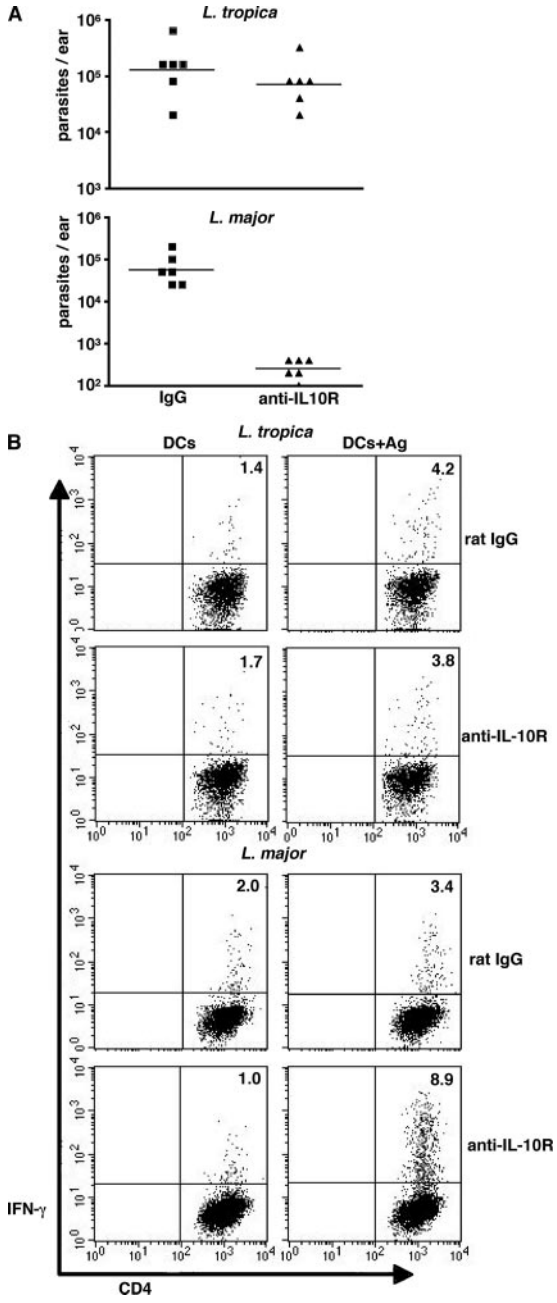

FIGURE 4. Infection outcome and immune response in anti-IL-10R-treated mice. Twelve weeks postinfection, mice were given biweekly injections of 200 μg of anti-IL-10R or control Ab over a period of 2 wk. *A*, Parasite burdens in individual ears, five to six mice per group. *B*, Lesional cells pooled from five to six mice were cultured overnight with BMDCs alone or with 100 μg/ml soluble *L. tropica* Ag, followed by the addition of monensin for the final 4 h before intracellular cytokine staining. Values in *upper right quadrants* are the percent of $CD4^+$ cells positive for IFN-γ. The data are representative of two independent experiments with similar results.

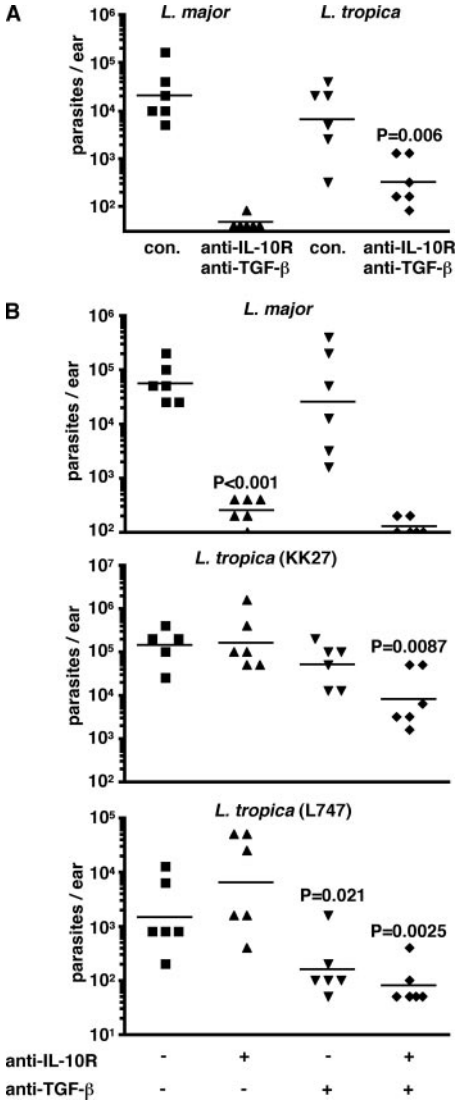

FIGURE 5. Combined treatment with anti-IL-10R and anti-TGF-β increases parasite killing in *L. tropica* infection. *A*, C57BL/6 mice infected with *L. major* FV1 or *L. tropica* KK27 were treated biweekly with 200 μg of anti-IL-10R and 200 μg of anti-TGF-β or control Abs for a period of 2 wk, beginning 12 wk postinfection. Each data point represents parasite burdens in individual ears, five to six mice per group. After the final treatment, mice were sacrificed and parasite burdens were determined by limiting dilution analysis. *B*, C57BL/6 mice infected with *L. major* FV1, *L. tropica* KK27, or *L. tropica* L747 were treated with anti-IL-10R, anti-TGF-β, or both Abs for a period of 2 wk, beginning 12 wk postinfection. Each data point represents parasite burdens in individual ears, five to six mice per group. The data are from one experiment and are consistent with three independent experiments.

$CD4^+$ cells in the lesion. Therefore, *L. tropica* infection, distinct from that of *L. major*, is unresponsive to IL-10-signaling blockade during the chronic phase of infection.

Combined treatment with anti-IL-10R and anti-TGF-β increases parasite killing

Because TGF-β has also been demonstrated to play a role in *Leishmania* infections (15), we considered the possibility that blocking its in vivo activity, either alone or in conjunction with IL-10, might be required to promote immune clearance of *L. tropica.* When mice were treated with anti-IL-10R in combination with anti-TGF-β Ab, a significant reduction in parasite load was achieved (Fig. 5*A*). In a repeat experiment in which the Abs were used alone or in combination, the anti-IL-10R treatment alone again had no effect on *L. tropica* parasite burdens, whereas treatment of *L. major*-infected mice produced a striking decrease (Fig. 5*B*). Anti-TGF-β treatment alone also had little effect, either in *L. major* or *L. tropica*. Treatment using anti-TGF-β in conjunction with anti-IL-10R Ab again resulted in a modest, but statistically significant, 10-fold reduction of *L. tropica* parasites in the skin. In *L. major*,

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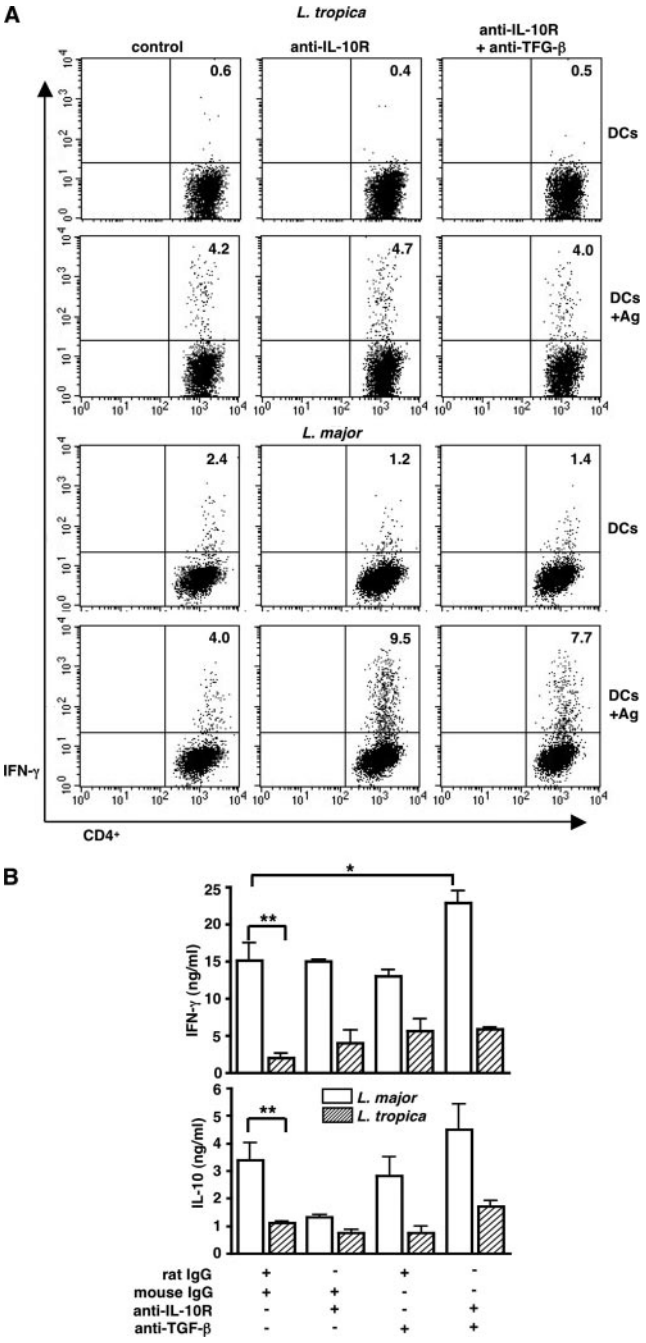

FIGURE 6. Immune response in anti-IL-10R- and anti-TGF-β-treated mice. *A*, Lesional cells from *L. tropica* KK27- or *L. major* FV1-infected mice treated as described in *B* were cultured overnight with BMDCs alone or with 100 μg/ml *L. tropica* Ag, with monensin added during the last 4 h. Cells shown are gated CD4$^+$ T cells, and the numbers shown are the percentage of CD4$^+$ cells positive for IFN-γ. *B*, DLN cells were cultured with BMDCs alone or with 100 μg/ml *L. tropica* Ag for 72 h, after which cytokines were measured by ELISA. The data are from one experiment and are consistent with three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

anti-TFG-β cooperated with anti-IL-10R treatment to produce an apparent sterile immunity in four of six mice. Although blocking TFG-β signaling during the chronic phase did not on its own achieve a therapeutic effect, we investigated whether treatment initiated at the time of infection might be sufficient to promote early resistance against *L. tropica*. C57BL/6 mice treated weekly from the time of challenge showed identical numbers of *L. tropica* in the

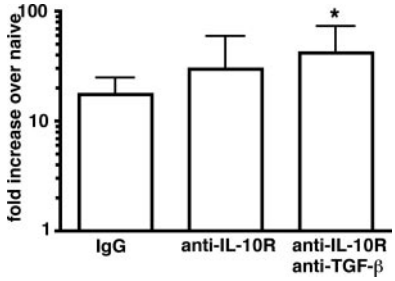

FIGURE 7. Combined treatment with anti-IL-10R and anti-TGF-β results in an increase in increases iNOS expression. *L. tropica* KK27-infected mice were treated as described in Fig. 6. Following treatment, mice were sacrificed and ear tissue was immediately processed for isolation of RNA. Relative gene expression was measured by real-rime PCR and is represented as the fold increase compared with naive ears. *, $p < 0.05$.

inoculation site 7 wk postinfection (data not shown). By contrast, and consistent with the results in the IL-10 knockout mice, treatment with anti-IL-10R Ab from the time of challenge significantly reduced the early establishment of *L. tropica* infection in the site (data not shown).

To corroborate the *L. tropica* results, and to address the possibility that the differences between *L. major* and *L. tropica* with respect to the immunologic conditions underlying chronic infection might be peculiar to the *L. tropica* strain from Afghanistan used in the analysis, an independent experiment was performed using a *L. tropica* strain, L747, originating from Israel. When initiated during the chronic stage of infection, anti-IL-10R treatment alone again failed to reduce parasite burdens in the site, while in combination with anti-TGF-β a significant decrease was observed. In this experiment, treatment with anti-TGF-β alone also produced a modest benefit.

Analysis of the effector response in the skin showed that an increase in IFN-γ-producing CD4$^+$ cells occurred following anti-IL-10R and combination therapy in the *L. major*-infected mice. In contrast, combination therapy with *L. tropica* infection did not result in a measurable increase in parasite specific IFN-γ production as measured by intracellular staining (Fig. 6*A*). There was also no increase in IFN-γ mRNA in the ear lesion, as measured by real-time PCR (data not shown). Measurement of cytokine production from DLN cells by ELISA was also performed (Fig. 6*B*). Ag-specific IFN-γ and IL-10 were detected in both infections, although each were secreted at significantly greater amounts in *L. major*-infected, isotype-treated control mice compared with *L. tropica*. A significant increase in both cytokines was observed in the *L. major*-infected mice treated simultaneously with anti-IL-10R and anti-TGF-β, while for *L. tropica* the increase was slight and not significant.

Although there was no increase in IFN-γ following combination therapy in the *L. tropica*-infected mice, the reduction in parasite load indicated that host macrophages responded with increased activation. A significant increase in iNOS mRNA expression was detected by real-time PCR in lesional cells from *L. tropica*-infected mice treated with combination therapy (Fig. 7), indicating that macrophage effector function was increased, despite the inability to detect an increase in IFN-γ at the site.

Parasites are transmissible to vector sand flies during the chronic phase of L. tropica *infection*

To determine whether the number of parasites persisting in the skin as a result of these regulatory processes was sufficiently high to be transmitted to vector sand flies, the ears of *L. tropica*-infected

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Table I. *L. tropica-infected ears transmit infection to a natural vector,* P. sergenti[a]

Time Postinfection (mo)	% of Blood-Fed Sand Flies Infected
1	0
2	0
4	0
6	7
10	16

[a] C57BL/6 mice were inoculated intradermally with 1000 *L. tropica* KK27 metacyclic promastigotes. Three mice (six ears) were used at each time point, and were exposed to 15 flies per ear. The total number of blood-engorged flies at each time point ranged from 43 to 63.

C57BL/6 mice were exposed to the bites of a natural vector, *P. sergenti* (15 flies per ear). Interestingly, no infected flies were observed until 6 mo postinfection, when 7% of blood-fed flies were found positive for parasites, and then again at 10 mo, when 16% of blood-fed flies were positive (Table I). Thus, the immunologic conditions that underlie persistent infection with *L. tropica* also establish this experimental host as a potential long-term reservoir of infection.

Discussion

Despite its widespread endemnicity, and the increased prevalence of ACL caused by *L. tropica* in Pakistan and Afghanistan, there is no data describing the host immune response to *L. tropica* infection in humans or in experimental animal models. This is largely due to a previously held consensus that the mouse is not a suitable host. By using *L. tropica* promastigotes highly enriched for metacyclic stage parasites, we previously demonstrated the growth and long-term persistence of these organisms in the footpad of BALB/c mice despite the absence of overt clinical pathology (8). The current work extends these findings to delineate the immunologic factors mediating the balance between host control and parasite survival in the skin.

The observation that treatment of chronically infected mice with IL-10R blockade did not result in enhanced parasite clearance is distinct from the effect of this treatment in *L. major*- and *Leishmania donovani*-infected mice (14, 16). The explanation may lie in the fact that the inflammatory response to *L. tropica* is low when compared with that of *L. major* in terms of the pathology and the number of effector cells present in the lesion, and the level of cytokines produced in the DLNs. To compensate for the lower IFN-γ, it appears that abrogation of multiple suppressive pathways is required to achieve a meaningful increase in effector function. As the frequency of cells producing IFN-γ in the lesion did not increase as a result of the functional inhibition of IL-10 and TGF-β, it is likely that these deactivating cytokines directly targeted infected macrophages to make them refractory to activation signals.

The disparity in infection outcome between the IL-10 knockout mice and wild-type mice treated with a blocking Ab to IL-10R during the chronic phase may likely be due to the presence of innate cell-derived IL-10 during the establishment of infection in wild-type mice, before the initiation of the adaptive immune response. In fact, parasites in IL-10-deficient mice never grew to beyond several hundred per lesion, and once established, persisted at relatively stable numbers. The source of the IL-10, either during the early or chronic stage in *L. tropica*-infected mice, is not clear from our studies. We have recently reported on the presence of IL-10/IFN-γ-producing $CD4^+$ cells as the source of IL-10-mediated suppression during infection with a nonhealing strain of *L. major* (17). In the present study, this population of effectors, or even IL-10 single producers, was not detectable by intracellular staining. It should be noted that while Ag-specific IL-10 from DLN cells was detectable by ELISA (Figs. 3 and 6*B*), the levels were substantially reduced compared with *L. major*, and may have been below the detection limits of the intracellular staining and flow analysis. With respect to IL-10 produced by natural regulatory T cells, which have been implicated in the persistence of *L. major* following healing in resistant mice, the *L. tropica* lesions were confirmed to contain an increased number $CD4^+CD25^+Foxp3^+$ cells (data not shown). Their depletion by anti-CD25 treatment during the chronic phase was incomplete, produced only a slight reduction parasite burden (data not shown), and in any case is difficult to interpret because $CD4^+$ effector cells producing IL-10/IFN-γ may be a source of suppression and may also express CD25.

A role for TGF-β in promoting susceptibility to various species of *Leishmania* has been known for some time (15). Experiments with *Leishmania chagasi* in C3H.HeJ mice, which produce a Th1 response and are resistant, have implicated TGF-β as the dominant Th2-independent suppressive pathway (18), and in IL-12-deficient mice, TGF-β is significantly elevated, suggesting that an intact Th1 response can actively suppress TGF-β production (19). In BALB/c mice infected systemically with *L. donovani*, anti-TGF-β treatment resulted in a modest inhibition of parasite growth, and exogenous TGF-β enhanced parasite growth (20). BALB/c mice, which are normally are refractory *Leishmania braziliensis*, developed lesions following treatment with TGF-β which were reminiscent of lesion reactivation that occurs in humans (21). More recent studies using *L. major*-infected BALB/c mice have demonstrated a role for FcR-mediated induction of TGF-β in parasitized cells (22). The results obtained with anti-TGF-β treatment in the current study have some similarities to previous experiments with *L. major*, in which in vivo treatment with the TGF-β-neutralizing Ab in CB6F1 mice, which have intermediate susceptibility to *L. major*, resulted in increased parasite killing that was associated not with increased IFN-γ, but with increased iNOS in the parasitized cells (23). In the current study, the therapeutic efficacy of TGF-β neutralization was potentiated by, and to some extent dependent on, simultaneous inhibition of IL-10. Again, this may be explained by the need to abrogate multiple deactivating signals for the relatively low levels of IFN-γ to achieve a leishmaniacidal threshold in the site. The finding that *L. tropica* induces a weaker Th1 response compared with *L. major* is consistent with prior studies indicating that compared with *L. major*, *L. tropica* has a reduced capacity to prime human dendritic cells for IL-12-p70 secretion following uptake of metacyclic promastigotes in vitro (24). The cellular source of TGF-β in the current study is unknown, as ELISA measurements of the activated form indicated that the concentration was below the limit of detection. It should be noted that in comparing two strains of *L. tropica*, while neither was responsive to anti-IL-10R treatment alone, one was responsive to anti-TGF-β treatment alone. This would point to the importance not only of interspecies differences, but also of intraspecies, strain differences in influencing the balance of activating and regulatory cytokines, and disease outcome.

As the analysis of the regulatory factors underlying chronic infection was confined to C57BL/6 mice, it is possible that the similar infection outcomes in BALB/c mice are controlled by distinct or additional suppressive cytokines, e.g., IL-4. Although BALB/c mice clearly have a Th2 bias that promotes uncontrolled infection with *L. major* and *Leishmania mexicana* complex parasites, Th2 polarization is not observed and does not explain the chronic but controlled infections in BALB/c mice involving *L. donovani, L. chagasi*, and some strains of *L. braziliensis* (19, 21, 25, 26). *L. tropica* infections in BALB/c mice would appear to have far more

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in common with the later strains than with *L. major* in producing a noncuring but nonprogressive infection, associated with Th1-mediated control and only low levels of IL-4. The current data regarding the persistence of *L. major* and *L. tropica* in C57BL/6 mice emphasize a similar point, that the conditions controlling chronicity can vary substantially between *Leishmania* species.

Finally, we have demonstrated that the chronic *L. tropica* lesions harbor sufficient numbers of parasites to be transmitted to their natural vector, *P. sergenti*. Interestingly, the transmission of parasites was most efficient at later time points, especially 10 mo postinfection. Although this aspect of the transmission data needs to be confirmed, the experiment formally demonstrates an especially well coadapted host-parasite relationship, in which the mammalian host, despite developing only minimal pathology, is established as a long-term reservoir of infection. The studies have revealed some of the key elements of the immune response that underlie this balance, and specify the immune-based therapies that might be used to improve the current treatment of ACL.

Disclosures

The authors have no financial conflict of interest.

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