The *Leishmania major* LACK Antigen with an Immunodominant Epitope at Amino Acids 156 to 173 Is Not Required for Early Th2 Development in BALB/c Mice

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The *Leishmania major* LACK antigen contains an immunodominant epitope at amino acids 156 to 173 (LACK\textsubscript{156,173}) that is believed to nucleate the pathological Th2 immune response in susceptible BALB/c mice. To test this hypothesis, we generated *L. major* parasites that express a mutated LACK that fails to activate \( V_{B4}/V_{a8} \) T-cell receptor transgenic T cells specific for this epitope. Although mutant parasites attenuated the expansion of endogenous LACK-specific, interleukin-4 (IL-4)-expressing, CD4 T cells compared to wild-type parasites in vivo, the overall frequency of IL-4 and gamma interferon-secreting lymphocytes was similar to that elicited by wild-type *L. major*. Mutant parasites demonstrated diminished amastigote viability and delayed lesion development in mice, although parasites could be recovered over 200 days after infection. Complementation with a wild-type lack fusion construct partially rescued these defects, indicating a role for endogenous LACK in parasitism. Mice inoculated with mutant parasites were not protected against subsequent infection with wild-type *L. major*.

*Leishmania major* is a clinically important intracellular protozoan pathogen that has been used extensively in inbred strains of mice to examine the relationship between T helper (Th) cell subset differentiation and disease susceptibility. Immunologic control of the intracellular amastigotes requires activation of phagocytic cells, particularly macrophages, in which the organisms live and divide in the vertebrate host. The protective response is dependent on gamma interferon (IFN-\( \gamma \)) elaborated by CD4 Th1 cells, which orchestrate immunity in resistant strains, such as C57BL/6 and B.10.D2 mice. Conversely, susceptible BALB/c mice develop an aberrant Th2 response to the parasite, which results in failure to restrict the growth of intracellular amastigotes with fatal dissemination (1, 15).

A striking observation remains the ability of interleukin-4 (IL-4) antibody, when administered at the time of infection, to reverse the phenotype in BALB/c mice by a process associated with the differentiation of protective Th1 cells (19). Expression cloning was used to identify the immunodominant focus of the early BALB/c CD4 Th2 cell response as LACK, an *L. major* RACK-like homolog of the WD protein family (12), and peptide mapping was used to establish a dominant epitope comprising amino acids 156 to 173 as the major LACK peptide (LACK\textsubscript{156,173}) presented by I-A\textsuperscript{d} major histocompatibility complex (MHC) molecules (9). Corroboration of these findings included the generation of resistant BALB/c mice by expression of LACK in the thymus, thus leading to the deletion of the LACK-reactive repertoire (6), and the use of antagonist LACK peptides to attenuate the course of disease in BALB/c mice (13). Furthermore, deletion of the few CD4 T cells identified by using LACK\textsubscript{156,173}/I-A\textsuperscript{d} tetramers from the endogenous BALB/c T-cell repertoire resulted in attenuation, although not cure, when transferred into immunodeficient recipients (23). Moreover, deletion of the LACK-specific repertoire in resistant B10.D2 mice further facilitated parasite clearance, suggesting that this immune response is deleterious in I-A\textsuperscript{d}-expressing mice, even on a resistant genotype (5).

The LACK\textsubscript{156,173} epitope is highly conserved among all *Leishmania* species (24). In recent studies, we provided evidence that at least one lack copy is required for parasite viability and that at least two lack copies appear to be required to maintain virulence in the vertebrate host (7). We mutated the LACK\textsubscript{156,173} epitope to create a peptide that did not activate transgenic T cells specific for the wild-type sequence. Although mutant promastigotes were generally comparable to wild-type parasites, amastigote forms grew poorly within macrophages and were less virulent in BALB/c mice. Immunologic studies, however, showed that the overall numbers of CD4 T cells that secreted IL-4 and gamma interferon (IFN-\( \gamma \)) were unchanged early after infection with the mutant parasites and further that prior infection with mutant parasites did not protect from subsequent challenge with wild-type organisms.

**MATERIALS AND METHODS**

**Construction of plasmids for LACK gene targeting and complementation.** To replace *LACK* with the mutated LACK coding region *LACKH164N* (amino acid residue His 164 substituted for Asn), designated LACK-N164, the construct pL1SD-LKN-L1 (Fig. 1B) was made by ligating the 1.3-kb stop codon (fragment \( \alpha \), Fig. 1) 5' to the SAT– DHFRTS drug selection cassette (7), followed by LACK-N164 (13) ligated downstream of SAT– DHFRTS. A 3.3-kb fragment (fragment \( \beta \)) from immediately downstream of the *LACK* stop codon was then ligated 3' to the LACK-N164 sequence (Fig. 1).

*L. major* LACK-N164 mutants were complemented with pXGLACK. This plasmid consisted of the LACK coding region downstream of sequence encoding a hemagglutinin epitope, six histidine residues, and a factor X cleavage site (13) inserted into pXG (3; S. Pingel, unpublished data).

**Transfection and selection of LACK-deficient *L. major* clones.** Replacement of the remaining *LACK* gene of *lack*\textsuperscript{−/−} parasites (7) with LACK-N164 was
performed by electroporation with 20 μg of purified pL1SD-LKN-L1 insert, followed by selection with nourseothricin as described previously (7) to give rise to L. major LACK-N164 mutant lines. L. major LACK-N164 mutants were complemented by transfection with 40 μg of pXGLACK followed by selection in G418.

Targeting and transfections were verified by sequencing and Southern and Western blotting for LACK protein as described previously (7).

Parasites and mice. Parasites were cultured in medium 199 (M199) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics as described previously (7). BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, Maine). BALB/c T-cell receptor (TCR)-Co−/− mice (7), BALB/c 4get mice (10), and LACK TCR transgenic ABLE mice (14) were each backcrossed at least 9 generations onto BALB/c. Prior to inoculation into mice, infectious metacyclic forms were purified from stationary-phase promastigote cultures by using peanut agglutinin (PNA), which specifically binds terminal β-linked galactose moieties on surface lipophosphoglycan side chains from noninfective promastigotes (18), as previously described (13). For infections, designated naïve T cells were purified from ABLE × TCR-Co−/− mice. These cells were labeled with CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester) as described previously (2), and 10⁶ cells were transferred into recipient BALB/c mice. After infection with L. major, popliteal lymph node cells were collected at 24, 72, and 96 h to assess the early CD4 T-cell response. Cells from ABLE × TCR-Co−/− BALB/c mice were resuspended at 2 × 10⁶/ml in 5% FCS in phosphate-buffered saline (PBS), and 15-μl aliquots were stained for 30 min on ice by mixing 1:1 with the following antibody cocktail: 1/50 fluorescein isothiocyanate (FITC)–anti-CD69; 1/50 phycoerythrin (PE)–anti-TCR Vβ4; 1/50 Tricolor (TC)–anti-CD8; 1/50 TC–anti-B220 and 1/50 antigen-presenting cell (APC)–anti-CD4 (Pharmingen, San Diego, Calif.; Caltag, Burlingame, Calif.) in 5% FCS–PBS. The cells were washed with 5% FCS–PBS and resuspended at 10⁷/ml in 5% FCS–PBS prior to analysis with a FacsCalibur flow cytometer (Becton Dickinson). Acquired data were analyzed with FlowJo software (Tree Star, San Carlos, Calif.).

Staining with recombinant MHC II I-A^d/LACK tetramers was used to assess endogenous LACK-specific T cells in infected wild-type and 4get BALB/c mice with a MoFlo cytometer (DakoCytomation, Fort Collins, Colo.) as described previously (23), except that staining for CD4 was performed with APC-Cy7-anti-CD4. To control for nonspecific binding of the tetramer reagent to activated CD4 T cells, activated CD4 T cells were isolated from the popliteal lymph nodes of ovalbumin-specific D011.10 BALB/c TCR transgenic mice that had been injected in the footpad 4 days previously with 250 μg of ovalbumin in incomplete Freund’s adjuvant. These cells were stained with I-A^d/LACK tetramers and used as a negative control to set gates defining LACK-specific CD4 T-cell staining.

FIG. 1. Targeting the L. major lack genes. (A) Physical map of the L. major lack genes with restriction enzyme sites indicated: B, BamHI; H, HindIII; K, KpnI; N, NsiI; S, StuI; Sp, SphI. The gray box denotes the LACK1 coding region. Fragments a and b (heavy lines) were used to make the targeting construct. (B) Diagram of the targeting construct. DHFR, dihydrofolate reductase; LACKN, LACK-N164. (C) Southern blot hybridizations of L. major transfected genomic DNAs. Genomic DNAs (10 μg/lane) from L. major lack−/− parasites (lane 1), L. major lack−/− parasites transfected with pL1SD-LKN-L1 (L. major LACK-N164; lane 2), and L. major LACK-N164 parasites transfected with pXGLACK (L. major LACK-N164/pXGLACK; lanes 3 and 4) were triple digested with StuI, BamHI, and SpeI. Genomic DNAs were size fractionated, blotted, and hybridized with 32P-labeled lack coding sequence (lined bar). (D) Lysates from 2 × 10⁶ LACK-deficient L. major promastigotes were loaded as follows: lane a, lack−/−/−; lane b, LACK-N164; lane c, LACK-N164/pXGLACK. After size fractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins were blotted and incubated with rabbit polyclonal antisera against eukaryotic 40S ribosomal protein S6 (lower panel).
**Immunologic analyses.** Lymph node suspensions from 4-day-infected mice were cultured as described earlier (21) and restimulated overnight with 50 μg of soluble *Leishmania* antigen extracts per ml (20) prepared by sonication of stationary-phase, wild-type *L. major* parasites in PBS at 4°C. The number of IL-4- and IFN-γ-producing cells per 10⁶ lymphocytes was determined as described previously (21).

**Parasite enumeration.** Viable parasites were enumerated by culturing dispersed popliteal lymph nodes from infected mice in 5 ml of M199 plus 10% FCS. Suspen­sions were serially diluted in microtiter plates. After 7 days of culture at 27°C, dilutions containing 1 or more viable parasites were scored as positive.

**Macrophage infections.** Bone marrow-derived macrophages from BALB/c mice were cultured on glass chamber slides as described previously (7). Promas­tigotes were added at a ratio of approximately 10 per macrophage and incubated for 16 h before repeated washing with PBS to remove extracellular organisms. Monolayers were stained after 16 and 96 h with Diff-Quik (DADE Behring, Dudingen, Switzerland), and the numbers of amastigotes per cell were determined microscopically.

**RESULTS**

**Generation of LACK-N164-deficient *L. major* by gene targeting.** Transfection of *L. major* lacking parasites with a pL1SD-LKN-L1 insert (Fig. 1A and B) resulted in a 3-kb StuI/BamHI LACK-N164 fragment and the SpeI/BamHI yielded digestion of genomic DNA from the transfectants with StuI, which is thus consistent with replacement of the remaining wild-type LACK coding region with LACK-N164. The intensity of this band was approximately threefold greater than that of the lack-hybridizing band observed in *L. major* lacking parasites (Fig. 1C, lane 1), indicating that insertion of several copies of LACK-N164 had occurred. DNA sequenc­ing of PCR products amplified from these parasite clones, designated *L. major* LACK-N164, using lack-specific primers, confirmed replacement of the wild-type His 164 codon for Asn (B.K., data not shown).

*L. major* LACK-N164 parasites were complemented by transfection of the *Leishmania* expression construct pXGLACK that expresses a LACK fusion protein previously shown to activate LACK-specific TCR transgenic T cells (13) (Fig. 1C, lanes 3 and 4). As predicted by successful transfection, triple digestion of genomic DNA from the transfectants with StuI, BamHI, and SpeI yielded lack-hybridizing genomic DNA fragments of 3 and 1 kb, corresponding to the integrated StuI/BamHI LACK-N164 fragment and the SpeI/BamHI lack coding fragment from pXGLACK, respectively. These complemented parasite lines were designated *L. major* LACK-N164/pXGLACK.

LACK protein levels in *L. major* LACK-N164 promastigotes were determined by Western blotting using anti-LACK antisera (Fig. 1D) as described previously (7). Taking into account the S6 ribosomal protein loading control (Fig. 1D, lower panel), the *L. major* LACK-N164 promastigotes showed moderately reduced levels of LACK protein (Fig. 1D, upper panel, lane b) compared to *L. major* lacking parasites (Fig. 1D, upper panel, lane a), despite possessing several copies of LACK-N164. Unexpectedly, *L. major* LACK-N164/pXG LACK organisms showed markedly reduced levels of LACK-N164 protein compared to *L. major* LACK-N164 (Fig. 1D, upper panel, lane c, lower band) and high levels of the wild-type LACK fusion protein expressed from pXG (Fig. 1D, upper panel, lane c, upper band).

Fluorescence-activated cell sorting analysis of the mutant parasites after incubation with labeled PNA indicated that the ability of these mutant parasites to display determinants consistent with metacyclogenesis was comparable to that of wild-type organisms (B.K., data not shown).

*L. major* LACK-N164 parasites do not activate LACK TCR transgenic T cells. ABLE mice express a Vβ4/Vα8 TCR specific for the immunodominant LACK peptide in I-A² (9, 14). Following inoculation of *L. major* LACK-N164 into the hind footpads of ABLE × TCR-Cα⁻/⁻ BALB/c mice, the frequency of Vβ4⁺ LACK-specific T cells that became activated (Fig. 2A), as indicated by enhanced CD69 expression at 20 h postinfection, was comparable to that of naive controls, ranging from 1.2 to 1.25%. In contrast, inoculation of *L. major* lacking parasites resulted in robust T-cell activation that was comparable to inoculation of wild-type *L. major* and *L. major* lacking parasites (7). Inoculation of *L. major* LACK-N164/pXGLACK also caused readily apparent T-cell activation (23.8% CD69-positive cells). The lesser response in LACK-N164/pXGLACK as compared to lack⁺⁺⁻⁻ parasites may reflect partial antagonism by the LACK-N165 epitope, but also the loss of the pXGLACK plasmid from parasites as they escape the selecting drug pressure in vivo.

The ability of *L. major* LACK-N164 parasites to elicit proliferation of LACK-specific T cells was also tested. As shown in Fig. 2B, when mice that had been previously injected with CFSE-labeled, ABLE T cells were challenged with parasites, cell division of I-A²/LACK-positive T cells, as shown by CFSE dilution at 72 h postinfection, occurred only in mice that received wild-type (lack⁺⁺/+⁺) or lack⁺⁻⁻⁻ parasites. Robust ABLE T-cell division was also observed with *L. major* LACK-N164/pXGLACK (B.K., data not shown). In contrast, no significant proliferation was observed in mice that received an equal dose of *L. major* LACK-N164. Thus, the mutant LACK epitope has abrogated T-cell recognition by these LACK-spe­cific transgenic T cells.

*L. major* LACK-N164 parasites elicit reduced expansion of endogenous LACK-specific T cells. Having established that *L. major* LACK-N164 cells do not activate LACK-specific TCR transgenic T cells, activation of endogenous LACK-specific CD4 T cells was investigated with recombinant MHC class II/LACK peptide tetramers and IL-4 reporter mice (4get), as described in Materials and Methods (Fig. 3A). Consistent with the diminished LACK TCR-transgenic T-cell response to LACK-N164 parasites, these organisms elicited reduced expansion compared to LACK wild-type-expressing *L. major*, with averages of 38, 77, and 132 tetramer-positive cells per lymph node for *L. major* LACK-N164, LACK-N164/pXGLACK, and lack⁺⁺⁻⁻⁻⁻ parasites, respectively, at the peak of the response. The numbers of tetramer-positive cells that were also expressing IL-4 (i.e., green fluorescent protein positive) were 19, 34, and 75 cells per lymph node for *L. major* LACK-N164, LACK-N164/pXGLACK, and lack⁺⁺⁻⁻⁻⁻, respectively. These represent fourfold- and twofold-higher numbers of IL-4-expressing LACK-specific cells for *L. major* lacking and LACK-N164/pXGLACK, respectively, as compared to the *L. major* LACK-N164 mutant.

*L. major* LACK-N164 parasites elicit Th2 cells and fail to protect from challenge with wild-type *L. major*. To determine whether or not the reduced number of IL-4-expressing LACK-specific CD4 T cells observed following inoculation of *L. major* LACK-N164 influenced Th cytokine protein expression pro-
files in the draining lymph nodes, enzyme-linked immunospot assays were performed on popliteal lymphocytes from BALB/c mice infected 96 h previously with either *L. major* lack/H11001/H11001/H11002/H11002, *L. major* LACK-N164, or *L. major* LACK-N164/pXGLACK parasites (Fig. 3B). Unexpectedly, after restimulation in vitro with soluble *Leishmania* extract, both *L. major* LACK-N164 and LACK-N164/pXGLACK parasites induced numbers of IL-4-producing and IFN-γ-producing cells that were similar to *L. major* lack+/− parasites, with 120 to 145 IL-4-producing cells and 40 to 55 IFN-γ-producing cells per 10^6 lymphocytes. Since *L. major* LACK-N164 elicited T-helper cytokine profiles in draining lymph nodes that were similar to wild-type *L. major*, despite the reduced expansion of IL-4-expressing, tetramer-specific cells in 4get mice, the host response to these mutants was further clarified by determining whether or not *L. major* LACK-N164 could mediate protection in BALB/c mice against subsequent challenge with virulent wild-type *L. major* (Fig. 3C). The mutant parasites were injected into the left-hind footpad, and 30 days later, the mice were challenged with virulent wild-type *L. major* in the right footpad, which was
subsequently monitored for lesion development. None of the mutant *L. major* strains conferred protection on subsequent wild-type parasite infection in BALB/c mice. Taken together, these data indicate that the BALB/c Th2 response remains unperturbed in response to infection with parasites lacking the LACK156-173 epitope.

**BALB/c mice fail to resolve infection with LACK-N164 and LACK-N164/pXGLACK parasites despite delayed lesion development.** The ultimate course of infection of BALB/c mice with *L. major lack*+/+ mutants, LACK-N164, or LACK-N164/pXGLACK parasites was also determined (Fig. 4). Infection with *L. major lack*−/− parasites resulted in progressive infection that necessitated sacrificing the mice after 80 days. In contrast, infections by both *L. major* LACK-N164 and LACK-N164/pXGLACK parasites were significantly attenuated and comparable to previous observations of single-copy lack+/− mutants (7). Despite the attenuated course of disease, small ulcerations were present on the primary lesions on most of the mice, consistent with parasite persistence. This was confirmed by terminal cultures of the footpads that revealed low numbers of the LACK-N164 mutants that were somewhat compensated for by the presence of the pXGLACK rescue plasmid (Fig. 4B).

The attenuated growth of these mutant LACK parasites likely reflects parasite-intrinsic, rather than immunologic, differences, as lesion development was similarly attenuated in BALB/b mice (B.K., data not shown), which do not express I-Ad and do not present the LACK 156-173 epitope. To assess this in vitro, bone marrow-derived macrophages demonstrated comparable uptake of each of the mutant organisms, but completely constrained replication of the LACK-N164 mutants at 96 h as compared to either lack++/− or LACK-N164/pXGLACK parasites (Fig. 5).

**DISCUSSION**

Earlier findings suggested a critical role for LACK156-173−specific Vβ4+ CD4 T cells in the pathological Th2 response to *L. major* (6, 9, 13). These observations were based on modifications of the host immune response. To clarify these findings, we sought to modify the parasite in a complementary fashion by removing the LACK156-173 epitope from the parasite genome as a stringent test for its requirement in the BALB/c immune response. We used gene targeting to generate *L. major* cells that express a variant of the LACK protein, LACK-N164, which does not activate these T cells (13). As predicted, the LACK-N164 mutant parasites failed to activate transgenic LACK156-173−specific Vβ4+ T cells and attenuated the expansion and secretion of IL-4 and IFN-γ from these T cells (13).
sion of endogenous LACK-specific CD4 T cells in vivo. Despite the clear demonstration that these parasites elicited activation of the dominant epitope-specific response to LACK, the overall activation of IL-4-producing and IFN-γ-producing cells in the draining lymph nodes remained unaltered as compared to wild-type L. major. These parasites, although highly attenuated for virulence in vivo, failed to protect against subsequent infection with wild-type organisms, consistent with their failure to activate a protective anti-Leishmania immune response.

A major conclusion of this study is that the aberrant BALB/c response to L. major proceeds independent from the presence or absence of the dominant LACK epitope.

In spite of this finding, while the LACK-N164 peptide is clearly antagonistic in vitro and in vivo (13), the failure of LACK-N164 to antagonize the response to wild-type LACK in the context of live parasites is intriguing. One possible explanation is that the LACK-N164 peptide may not be processed and presented efficiently from the mutant parasites. Indeed, the mutant LACK protein was clearly regulated differently from the wild-type copy, consistent with our previous studies identifying LACK as critical to the intracellular survival of amastigotes (7). It seems unlikely that the response was driven solely by replacement with LACK-N164-specific CD4 T cells that nucleated the pathology, however, since restimulation with wild-type L. major extracts lacking this mutant epitope revealed comparable numbers of IL-4-producing CD4 T cells from mice infected with LACK-N164 parasites (Fig. 3B). Rather, these data corroborate multiple reports that the levels of induction of IL-4-producing CD4 T cells in the draining lymph nodes early after infection (0.5 to 1 week) are comparable in both resistant and susceptible infected animals (11, 16, 22, 23), and thus induction is not linked to the eventual outgrowth of protective Th1 or nonprotective Th2 cells. Furthermore, multiple nonoverlapping approaches have clearly demonstrated that ablation of the LACK-specific CD4 T-cell response prior to infection enhances resistance to the parasite (6, 9, 13, 23). Taken together, the data suggest that ablating a
restricted set of epitope-specific T cells achieves a different outcome from ablating the epitope of those T cells in the pathogen. Although it remains unclear why the outcomes of these two experimental approaches differ, several variables, including aberrant expression of alternate parasite antigens, abnormal trafficking of mutant parasites in host cells, and effects on T-cell responses after manipulation of the host repertoire, may affect variables in the host response in unknown ways. Indeed, administration of anti-IL-4 or deletion of the LACK-specific T-cell response was associated with a decrease in the overall numbers of early IL-4-producing cells, whereas mutation of the epitope, as shown here, did not decrease the magnitude of the early IL-4 response.

Despite the ability of LACK-N164 and LACK-N164/pXGLACK L. major parasites to elicit a robust Th2 response in BALB/c mice, the attenuation of amastigote growth in vivo suggested parasite-intrinsic defects in these mutants. Parasite recovery at the termination of the experiments suggested that pXGLACK did rescue some of the phenotype, although this was partial (Fig. 4B). The deficiency in these parasites undoubtedly reflects the crucial role for LACK protein in amastigote growth in vivo. Indeed, inoculation of 10-fold-higher parasite doses of LACK-N164 could not overcome the growth attenuation in vivo (B.K., data not shown). Although the ability of LACK-N164 and LACK-N164/pXGLACK parasites to gain entry into macrophages was comparable to that of wild-type parasites, their subsequent proliferation as intracellular amastigotes was impaired (Fig. 5). Similar defects were seen in single-copy lack−/− parasites (7), which suggests that LACK-N164 does not function as efficiently as the wild-type protein. Indeed, His 164 is conserved across all -N164 does not function as efficiently as the wild-type protein.

To more fully understand the molecular mechanisms underlying this phenotype, we created an attenuated, transgenic parasite strain containing a glucuronidase reporter gene in the 5′-untranslated region of the LACK-N164 gene. Integration of the transgenic construct into the genome of a wild-type LACK parasite line, VYD, was achieved by a PCR-based method (167). The resulting transgenic parasite, VYD/LACK-N164/pXGLACK, was selected by tetracycline resistance and confirmed by PCR and Southern blot analysis, as described above. Our transgenic parasite line expresses detectable levels of functional LACK-N164 and is therefore called LACK-N164/pXGLACK. In mammalian cells, the ability of the LACK homolog, RACK1, to suppress certain signaling pathways by diverting and/or sequestering IIIPKC has been suggested (8), implying that overexpression of these proteins may have deleterious physiological consequences. Furthermore, in fission yeast, overexpression of the WD repeat adaptor protein, Sum 1, inhibits cell cycle responses to stress (4).

Taken together, these data suggest that stringent qualitative and quantitative restrictions may have been placed upon LACK during the evolution of macrophage parasitization by these trypanosomatids. Our observations support further elucidation of the biochemical and physiologic roles of LACK in these parasites as a means to the development of potential drug targeting strategies. Although it is clear that robust Th1 and Th2 responses lead to resistance and susceptibility to L. major infection, respectively, the precise mechanisms by which these disease outcomes occur remain complex (17). These findings emphasize the need for further study of the interplay between host and parasite determinants in this disease.

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