Protective Immunity Against the Protozoan *Leishmania chagasi* Is Induced by Subclinical Cutaneous Infection with Virulent But Not Avirulent Organisms[1]

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Protective immunity against *Leishmania major* is provided by s.c. immunization with a low dose of *L. major* promastigotes or with dihydrofolate-thymidylate synthase gene locus (*DHFR-TS*) gene knockout *L. major* organisms. Whether these vaccine strategies will protect against infection with other *Leishmania* species that elicit distinct immune responses and clinical syndromes is not known. Therefore, we investigated protective immunity to *Leishmania chagasi*, a cause of visceral leishmaniasis. In contrast to *L. major*, a high dose s.c. inoculum of *L. chagasi* promastigotes was required to elicit protective immunity. Splenocytes from mice immunized with a high dose produced significantly greater amounts of IFN-γ and lower TGF-β than mice immunized with a low dose of promastigotes. The development of protective immunity did not require the presence of NK cells. Protection was not afforded by s.c. immunization with either attenuated *L. chagasi* or with *L. major* promastigotes, and s.c. *L. chagasi* did not protect against infection with *L. major*. Subcutaneous immunization with *DHFR-TS* gene knockouts derived from *L. chagasi*, *L. donovani*, or *L. major* did not protect against *L. chagasi* infection. We conclude that s.c. inoculation of high doses of live *L. chagasi* causes a subclinical infection that elicits protective immune responses in susceptible mice. However, *L. chagasi* that have been attenuated either by long-term passage or during the raising of recombinant gene knockout organisms do not elicit protective immunity, either because they fail to establish a subclinical infection or because they no longer express critical antigenic epitopes. *The Journal of Immunology*, **2001, 166: 1921–1929.**

Human infection with *Leishmania chagasi*, the protozoan causing South American visceral leishmaniasis, causes diverse sequelae ranging from subclinical infection to progressive fatal disease (1). Subclinical infection results in the development of cellular immune responses detected by a positive delayed-type hypersensitivity skin test. These immune responses are presumed to protect against subsequent disease (2). One would assume that "immunization" by inducing a subclinical infection with parasites that are rendered avirulent by virtue of attenuation or knockout of a conditionally essential gene might mimic this naturally acquired protective immunity. The purpose of this study was to determine whether subclinical infection with virulent or avirulent strains of *L. chagasi* also induces protective immunity in a susceptible mouse model.

Murine models of infection with an agent of cutaneous leishmaniasis, *Leishmania major*, have provided important information on the development of protective vs permissive immune responses toward this pathogen. Expansion of a Th1 subset of $CD4^+$ lymphocytes that secretes IFN-γ and IL-2 is associated with resistance to infection (3–5). IL-12, likely secreted by APCs, enhances expansion of Th1 cells through a pathway involving NK cells (6–8). Susceptible mice instead expand $CD4^+$ lymphocytes belonging to the Th2 subset that secretes IL-4, IL-10, and IL-13 (3–5). Early IL-4 secretion by an as yet undefined population of cells plays a role in differentiation of T cells toward the Th2 phenotype and suppression of Th1 subset development (9, 10). Several laboratories have shown that, in contrast to murine *L. major* infection, permissive murine *Leishmania donovani* or *L. chagasi* infection results from failure to expand an Ag-specific Th1 subset of $CD4^+$ cells in the absence of a detectable Th2 response (11, 12).

Genetically susceptible strains of mice can mount a protective immune response against *L. major* infection after a variety of experimental manipulations that deplete the animal of disease-exacerbating $CD4^+$ cells. Protective immunity can also be induced by immunization of mice with parasite Ags combined with IL-12 (13), by cure of prior active infection (14), or by immunization with *Leishmania* Ags in the form of recombinant proteins (15–19) or expressed in DNA vaccines (20, 21). In addition, expression of *Leishmania* Ags in recombinant organisms, such as bacillus Calmette-Guérin, *Salmonella*, and vaccinia virus, can lead to partial protection against infectious challenge (22–24). Although the use of attenuated *Leishmania* for immunization runs the risk of inoculating infectious organisms into the host, a novel means of immunization uses *Leishmania* that are rendered avirulent by deletion of genes encoding enzymes essential for the metabolism of the parasite (e.g., dihydrofolate-thymidylate synthase gene locus (*DHFR-TS*)[3]). Such "genetically attenuated" parasites were

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[3] Abbreviations used in this paper: *DHFR-TS*, dihydrofolate-thymidylate synthase gene locus; *dhfr-ts*$^-$, gene knockout organism lacking both alleles of *DHFR-TS*; *Lcdhfr-ts*$^-$, *L. chagasi* line of *dhfr-ts*$^-$; *Lddhfr-ts*$^-$, *L. donovani* line of *dhfr-ts*$^-$ organisms; VFMS, virus-free BALB/c mouse serum; HOMEM, hemoflagellate minimal essential medium.

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found to induce protection against *L. major* infection of susceptible mice (25).

Another successful means of immunizing with live *L. major* parasites entails footpad infection of BALB/c mice with small numbers (10^2) of virulent parasites. This results in the expansion of a Th1-type T cell subset and protection against reinfection. In contrast, large numbers of s.c. parasites lead to Th2 expansion and progressive disease (26, 27). Because of these studies, low doses of vaccine inocula have been proposed to enhance the efficacy of immunization against organisms that are controlled via cellular immunity (26). Whether s.c. inoculation of live parasite Ag will also induce protective immunity to *Leishmania* species causing visceralizing infection (*L. chagasi, L. donovani*), similar to the well-documented immunity to *L. major*, is not known. During this study, we determined that s.c. immunization with a high dose but not a low dose of *L. chagasi* promastigotes induces a protective immune response, similar to the immunity induced by subclinical *L. chagasi* infection of humans. We applied these findings to a comparison of immunization strategies using *L. chagasi* promastigotes that were rendered avirulent either by long-term cultivation (attenuation) or by deletion of both alleles of a conditionally essential gene locus, *DHFR-TS*.

Materials and Methods

Parasites

A strain of *L. chagasi* (MHOM/BR/00/1669), originally isolated from a patient with visceral leishmaniasis in northeast Brazil, was maintained by serial intracardiac injection in hamsters. Parasites were isolated from infected hamster spleens, cultured as promastigotes in liquid HOMEM medium (28), and used within 3 wk of isolation. The attenuated strain of *L. chagasi*, L5, was raised from the same parasite isolate and serially passaged in vitro in hemoflagellate minimal essential medium (HOMEM) for 6 years. *L. major* amastigotes (strain WHOM/IR/-/173) were derived by homogenization of infected mouse footpads and grown as promastigotes in Schneider's insect medium with 20% FCS, 20 mM L-glutamine, and 50 μg/ml gentamicin at 26°C. All promastigotes were used in stationary phase of growth. Metacyclic *L. major* were isolated by selection with peanut agglutinin as described (29, 30).

Construction of viscerotropic DHFRTS *gene-targeting vectors*

Cosmid S1.2 bearing the *L. donovani DHFRTS* gene was obtained by screening an *L. donovani* cosmid library (31) with an *L. major DHFRTS* probe. A *Sac*I 7-Kb fragment containing the *L. donovani DHFRTS* gene and at least 2 Kb of 5′ and 3′ flanking DNA was inserted into the *Sac*I site of a modified pBluescript vector (lacking the polylinker sites between *Hin*dIII and *Sac*II sites), yielding pDDSac7 (strain B3505). To replace *DHFR-TS* with a *NEO* marker, an *Eco*RI/*Sty*I fragment containing the 5′ untranslated region and most of the coding region of *DHFR-TS* was replaced by the *Eco*RI/*Ppu*MI fragment from pX63NEO (32) containing the *L. major DHFR-TS* splice acceptor and *NEO*. This construct is pDD-Sac7NEO (B3497). The *HYG* replacement construct was made by replacing the NEO coding region in pDD-Sac7NEO with the HYG coding region contained in the *Bam*HI/*Spe*I fragment from pX63HYG (32) (pDD-Sac7HYG).

Generation of gene knockout L. chagasi *and* L. donovani *lacking both alleles of* DHFR-TS *(*dhfr-ts$^-$*)*

We used the above *L. donovani*-based vectors for the replacement of the *L. chagasi DHFR-TS* genes. A *dhfr-ts*$^-$ derivative of the virulent *L. chagasi* strain MHOM/BR/00/1669 (33) was constructed by two successive rounds of targeted gene replacement with plasmids pDD-Sac7NEO and pDD-Sac7HYG as described (32). Before electroporation, the targeting DNAs were cut with *Sac*I, and the ends were made blunt by treatment with T4 DNA polymerase in the presence of 100 μM dNTPs. The resulting clonal *L. chagasi* line of *dhfr-ts*$^-$ (*Lcdhfr-ts*$^-$) line, N/H 15, was recloned on agar plates, and one of these (N/H 15.5) was used throughout these studies. An "add-back" control *Lcdhfr-ts*$^{-/+}$ DHFR-TS line (N/H 15.5$^+$) was made, in which an episomal *L. donovani DHFR-TS* gene was reintroduced into N/H 15.5 by transfection with plasmid pXGTKNEO-ldDHFR-TS (B2722) and selecting for thymidine prototrophy in semidefined M199. Thus the genotype of the *Lcdhfr-ts*$^-$ knockout is DHFR-TS:ΔNEO/DHFR-TS:ΔHYG and the genotype of the "add-back" control *Lcdhfr-ts*$^{-/+}$ DHFR-TS is DHFR-TS:ΔNEO/DHFR-TS:ΔHYG [pXGTKNEO-DHFRTS]. In this work, we will refer to them as L*cdhfr-ts*$^-$ and L*cdhfr-ts*$^{-/+}$DHFR-TS, respectively.

The *L. donovani dhfr-ts*$^-$ knockout strain was derived from a long-term laboratory passaged 1S2D line by a slightly different procedure. A *DHFR-TS/NEO* heterozygote was obtained by transfection with plasmid pDD-Sac7NEO as described above. This line was subjected to anti-DHFR-TS selection by growth in medium containing methotrexate and thymidine. As in *L. major*, survivors were obtained that had undergone loss of heterozygosity and were now homozygous for the *NEO* replacement allele (34). This line was recloned, yielding the *L. donovani dhfr-ts*$^-$ strain (DHFR-TS:ΔNEO/DHFR-TS:ΔNEO) or *L. donovani* line of *dhfr-ts*$^-$ organisms (*Ldhfr-ts*$^-$).

The absence of *DHFR-TS* sequences in *Lcdhfr-ts*$^-$ and *Lddhfr-ts*$^-$ lines was confirmed by Southern blot hybridization and phenotypic testing for thymidine auxotrophy (data not shown) (35). The line of *dhfr-ts*$^-$ *L. major* previously reported to protect against s.c. *L. major* infection was used (25).

dhfr-ts$^-$ parasites were grown in HOMEM supplemented with 10 μg of thymidine/ml. During selection, they were maintained in 9 μg G418/ml and 16 μg hygromycin B/ml. After characterization, they were maintained without drug, although their resistance to both antibiotics was periodically verified. *Lcdhfr-ts*$^{-/+}$*DHFR-TS* were maintained in G418 throughout. To assess the contribution of in vitro cultivation for the attenuation of the knockout strains, a culture of the same *L. chagasi* organisms used to generate the original knockouts, passaged an equivalent number of times on agar or in liquid, was used as the control in immunization experiments.

Immunization and challenge

Stationary phase *L. chagasi* or metacyclic *L. major* promastigotes were washed and resuspended at 10^8/ml, 10^5/ml, or 10^3/ml in HBSS containing 5% heat-inactivated virus-free BALB/c mouse serum (VFMS). One hundred microliters of parasite suspension or 5% VFMS/HBSS (control mice) were injected s.c. into the dorsum of the neck of BALB/c mice. Four or 8 wk after immunization, mice received a challenge infection with 10^7 stationary phase *L. chagasi* promastigotes via the tail vein. At the peak of infection (4 wk after i.v. challenge), mice were sacrificed and impression smears were made from the livers and spleens. Slides were stained with Diff-Quik (American Scientific Products, McGraw Hill, IL). The estimated number of parasites per organ was determined microscopically as (number of amastigotes per 500 mononuclear cells) × (organ weight in mg) × (2×10^5) (36). Student's *t* test was used to compare the mean parasite burdens in different groups.

To study the effects of immunization in a model of cutaneous leishmaniasis, mice were challenged in the footpad with 10^6 metacyclic *L. major* promastigotes selected with peanut lectin (agglutinin) as described (30). Footpad thickness was documented with calipers. Five weeks after infection, the infected foot was removed and homogenized.

Listeria monocytogenes *challenge*

The virulent 10403S strain of *L. monocytogenes* was provided by Dr. John Harty (University of Iowa, Iowa City, IA). *L. monocytogenes* were grown in tryptic soy broth (Difco, Detroit, MI) with streptomycin (50 μg/ml) until they reached an A_{600} reading of 0.1, equivalent to 1.5×10^8 CFU/ml. Bacteria were diluted in pyrogen-free saline to a concentration of 5×10^4 CFU/ml. Dilutions of this *L. monocytogenes* suspension were plated on tryptic soy agar (Difco) with 50 μg streptomycin/ml, and CFUs were counted 24 h later to verify the estimated inoculum size. Four weeks after immunization with virulent *L. chagasi* promastigotes or buffer, BALB/c mice were challenged with 200 μl containing ~1 LD_{50} of *L. monocytogenes*. Forty-eight hours later, livers and spleens were weighed, and a section of each was homogenized and serially diluted. Twenty microliters from three consecutive 10-fold dilutions of each organ homogenate was plated as above and overnight colony counts were used to calculate total organ CFUs.

Splenocyte cultures and cytokine assays

Four weeks after immunization or challenge infection, spleens were removed and passed through stainless steel mesh (No. 100; Bellco Biotechnology, Vineland, NJ) to produce single-cell suspensions. Erythrocytes were lysed hypotonically in 1% NH_4Cl and 0.01 M HEPES (pH 6.8) for 2 min, and cells were washed in HBSS with 0.3% BSA. APCs were presumably macrophages, dendritic cells, and B cells within these populations. Total splenocytes were suspended at 2×10^6/ml in 200 μl of RPMI 1640 with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 μM 2-ME, 2 mM sodium pyruvate, 20 mM HEPES, and 50 μg gentamicin/ml in 96-well flat-bottom microtiter plates (reagents from University of Iowa Cancer

Center, Iowa City, IA). FCS was omitted from cultures used to quantify TGF-β. Triplicate wells contained no Ag, 5 μg Con A/ml, or 3×10^6 *L. chagasi* promastigotes/ml. Supernatants were collected 72 h later. Our prior determinations showed that live promastigotes provide a more potent antigenic stimulus than a promastigote lysate (11).

Cytokine concentrations in supernatants were measured with 2-sandwich ELISAs as previously reported (11). IFN-γ was captured on plates coated with mAb HB170 (American Type Culture Collection (ATCC), Manassas, VA) and detected by sequential incubations in polyclonal rabbit anti-IFN-γ, peroxidase-conjugated goat anti-rabbit IgG (Accurate, Westbury, NY), and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Zymed, San Francisco, CA). Values were compared with a standard curve prepared from rIFN-γ (Amgen, Thousand Oaks, CA). The IL-10 ELISA used SXC-2 Ab as capture and biotinylated SXC-1 as detection Abs (ATCC), using rIL-10 to prepare a standard curve (provided by Satish Menon, DNAX, Palo Alto, CA). IL-4 was captured with 11B11 (HB191; DNAX) and detected with biotinylated BVD6, using rIL-4 (PeproTech, Rocky Hill, NJ) for the standard curve. TGF-β was captured with MAB240 and detected with biotinylated polyclonal anti-TGF-β1 serum BAF240 (R&D Systems, Minneapolis, MN). The standard curve was prepared using rTGF-β1 (R&D Systems) suspended in culture medium. Sensitivities of the ELISAs were 30 pg/ml for IFN-γ, 20 pg/ml for IL-4, 500 pg/ml for IL-10, and 30 pg/ml for TGF-β. Statistical analyses used Student's *t* test.

NK cell depletion

Ab against asialo-GM1 (Wako Chemicals, Richmond, VA) was diluted 1:6 in PBS, and 200 μl were inoculated i.p. into BALB/c mice every 4 days for a total of three injections. Control mice received nonimmune rabbit serum in PBS. Six days after the first injection, Ab-treated or control mice were immunized s.c. with 10^7 stationary phase *L. chagasi* promastigotes or buffer. One month after the s.c. inoculation, mice were challenged with 10^7 stationary *L. chagasi* promastigotes i.v. The parasite burden was calculated 4 wk after challenge infection as described above.

^{51}Cr release cytolytic assay for NK activity

We used a standard assay of lysis of ^{51}Cr-labeled Yac-1 target cells (37). Briefly, Yac-1 target cells were labeled with ^{51}Cr and incubated with varied dilutions of splenocytes (effector cells) in DMEM. After 12 h at 37°C, released ^{51}Cr in supernatants was quantified on a gamma counter. Specific lysis was calculated by subtracting spontaneous ^{51}Cr released into wells containing target but lacking effector cells.

CD4$^+$/CD8$^+$ T cell depletion

Splenocytes were suspended in RPMI 1640 with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (RPMI-5) and incubated in buffer, mAb RL-172 (anti-CD4), or 3-168 (anti-CD8) at 4°C. Cell lines were provided by Dr. John Harty (University of Iowa). After 30 min, cells were washed and exposed to rabbit complement (Pel-Freeze Biologicals, Brown Deer, WI) and diluted 1:8 for 1 h at 37°C. The incubation with Ab and complement was repeated, and viable cells were recovered by density separation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). Viable splenocytes were cultivated as described above except that protease peptone-elicited syngeneic peritoneal macrophages (3×10^4/well) were included as APCs (38). The efficacy of depletion was documented on each experiment by FACS as previously described (11).

Results

Subcutaneous immunization with virulent, but not with attenuated L. chagasi *protects mice against* L. chagasi *infection*

Differing numbers of virulent stationary phase *L. chagasi* promastigotes or diluent alone were administered s.c. to BALB/c mice, which are genetically susceptible to *L. chagasi* infection, in the dorsal cervicothoracic junction. Subcutaneous immunization did not result in disseminated disease, as judged by microscopy and culture of liver and spleen after 4 or 8 wk, similar to a previous report of *L. donovani* footpad infection (39). Protection did not result from low-dose (10^2 promastigotes) immunization (Fig. 1*A*). However, 4 wk after s.c. immunization with 10^7 live stationary phase *L. chagasi* promastigotes, mice were significantly protected against challenge infection with *L. chagasi* promastigotes administered i.v. Immunization with intermediate numbers of organisms (10^4) caused intermediate amounts of protection. Eight repeat experiments yielded similar results. Additionally, there was significant protection (88% reduction in the parasite burden) when mice were challenged with promastigotes 8 wk after s.c. immunization with 10^7 live parasites (data not shown). Inoculation of the immunizing dose of *L. chagasi* promastigotes into a different location (s.c. in the gluteus) also resulted in significant protective immunity against reinfection with *L. chagasi* (data not shown). Consistent with our previous findings, the heaviest burden of infection occurred 4 wk after i.v. challenge with the parasite (Fig. 1*A*) (11). Therefore, subsequent assessments of parasite load were performed 4 wk after the challenge infection. Parasite burdens in the spleens were too low to interpret at these time points, consistent

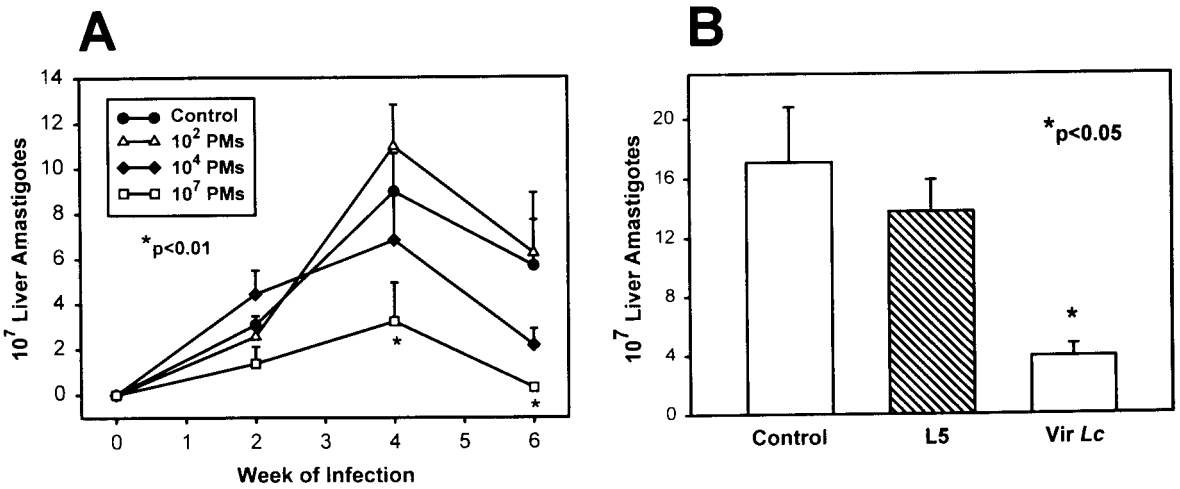

FIGURE 1. Effect of s.c. immunization with live *L. chagasi*. *A*, BALB/c mice were immunized by s.c. inoculation of 0 (control), 1×10^2, 1×10^4, or 1×10^7 live stationary phase *L. chagasi* promastigotes in buffer (VFMS/HBSS). After 4 wk, they were challenged with 1×10^7 virulent *L. chagasi* promastigotes i.v. Two, 4, or 6 wk later, parasites were enumerated on impression smears of the livers. Bars represent the mean ± SE liver amastigotes in a group of five mice from a representative study. *B*, Mice were immunized s.c. with buffer, 10^7 virulent stationary phase *L. chagasi* promastigotes (Vir *Lc*), or 10^7 attenuated *L. chagasi* L5 promastigotes. After 4 wk, they were challenged i.v. with 10^7 virulent *L. chagasi* promastigotes, and the liver parasite burden was enumerated on the 4th week of infection. Data are the mean ± SE for five mice in a representative of three experiments. Statistical analyses were performed with Student's *t* test.

with previous findings that local growth of *L. chagasi* and local immune responses differ between the livers and spleens (11, 40).

Virulent organisms used for the above immunizations are passaged through hamsters to maintain their infectivity and used within 3 wk of isolation. An attenuated strain of *L. chagasi*, L5, has been passaged strictly in vitro for 6 yr. L5 has a markedly reduced capacity to cause systemic infection when administered i.v. to mice (33). Similar to other attenuated *L. chagasi*, L5 exhibits decreased expression of the surface proteins GP63 and GP46 and altered developmental regulation of GP63 mRNA and protein (41, 42). BALB/c mice were immunized s.c. with high doses (10^7) of L5 or virulent promastigotes and challenged i.v. with virulent *L. chagasi* promastigotes. Four weeks later, the L5 strain did not effect a significant degree of protection against challenge infection, compared with virulent *L. chagasi* promastigotes (Fig. 1*B*). This suggests that virulence of the immunizing organisms is necessary to induce protective immunity.

The use of heterologous organisms for immunization or challenge

Although there have historically been impressions that protective immunity against one *Leishmania* sp. may not protect humans against infection with another *Leishmania* sp., this notion has recently been challenged (43, 44). We examined whether there is protection against a visceralizing species of *Leishmania* after s.c. immunization with a species that causes cutaneous leishmaniasis in humans (*L. major*). Subcutaneous injection of 10^7 *L. major* into the footpad or base of the tail of susceptible BALB/c mice causes local ulceration and ultimately dissemination (45, 46), whereas inoculation into the dorsal anterior trunk is unlikely to cause disease (47). Therefore, we immunized mice with high and low numbers of *L. major* promastigotes inoculated s.c. into the dorsum of the neck, to avoid ulceration at the site. There was no significant protection against *L. chagasi* infection induced by immunization with either dose of *L. major* (Fig. 2*A*). Similarly, immunization of BALB/c mice with 0, 10^2, or 10^7 *L. chagasi* s.c. did not elicit protective immunity against a challenge infection with 10^6 *L. major* promastigotes in the footpads using the above immunization route (data not shown). However, s.c. immunization with *L. major* induced protection against challenge with *L. major* in the footpad. Similar to published data, immunization with a low dose of *L. major* induced protection, whereas high-dose (10^7) immunization was not protective (Fig. 2*B*). Indeed, when challenge infection occurred 16 wk rather than 4 wk after immunization with *L. major*, it was apparent that high-dose immunization significantly exacerbated *L. major* disease (Fig. 2*B*) (26). Thus, both s.c. immunization with *L. major* and *L. chagasi* could protect against challenge with the same organism but there was not cross-protection between species in this model. Furthermore, high-dose immunization with *L. chagasi* and low-dose immunization with *L. major* were required to induce protective immunity.

We tested whether the protective immunity induced by s.c. immunization was specific for *Leishmania* or due to nonspecific stimulation of cellular responses. Four weeks after s.c. immunization with virulent *L. chagasi* promastigotes or buffer, BALB/c mice were challenged with 1 LD_{50} of virulent *L. monocytogenes*, a bacterium that is also controlled by cell-mediated immune responses (48). Fig. 2*C* shows that s.c. immunization did not cause a significant reduction in the numbers of *L. monocytogenes* recovered from livers and spleens. Instead, more bacteria were recovered from the immunized than the unimmunized mice in this and two replicate experiments. Thus, s.c. immunization with *L. chagasi* does not protect against all intracellular pathogens.

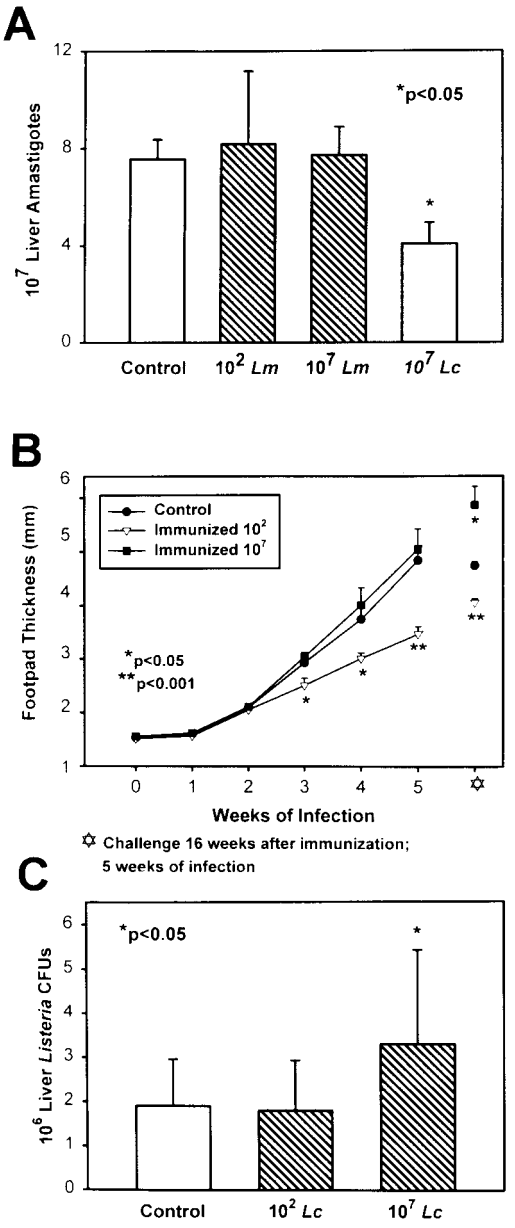

FIGURE 2. Immunization or challenge with heterologous organisms. *A*, Subcutaneous immunization with *L. major*. To determine whether there was cross-protective immunity between *Leishmania* sp., mice were immunized with 0, 10^2, or 10^7 stationary phase *L. major* or 10^7 *L. chagasi* promastigotes s.c. Four weeks later they were challenged with 10^7 *L. chagasi* promastigotes i.v. Shown are the mean ± SE liver parasite burdens in groups of four to five mice calculated at the peak of infection, 4 wk after *L. chagasi* challenge. Data are representative of two experiments, each with five mice per time point. *B*, Subcutaneous immunization with *L. major* protects against *L. major* challenge. Mice were immunized s.c. with low- or high-dose *L. major* as in *A* and challenged 4 wk later by inoculation of 10^6 metacyclic *L. major* promastigotes in the footpad. Footpad thickness was measured with a calipers, and the panel shows the mean ± SE for four mice at the indicated times after infection. A separate group of mice was challenged 16 wk after immunization with *L. major*. The mean ± SE footpad thickness at 5 wk after challenge infection is shown to the right of the panel ($n = 5$). *C*, Lack of protection against a heterologous genus. Mice were immunized s.c. with 0, 10^2, or 10^7 *L. chagasi* promastigotes. After 4 wk, mice were challenged i.v. with 1 LD_{50} of virulent *L. monocytogenes*. Forty-eight hours later, their livers were homogenized and CFUs were enumerated. Bars represent the mean ± SE CFUs in groups of three mice. Data are representative of three experiments. Statistical analyses used Student's *t* test.

NK cells are not required for the development of protective immunity

The cytokine milieu as measured in lymph nodes draining the site of a *L. major* infection early in disease correlates with subsequent outcome of infection. High levels of IFN-γ produced ex vivo 3 days after *L. major* inoculation correlate with subsequent expansion of Th1 cells and a resistant phenotype, and the inclusion of IL-12 with an immunizing Ag can bias toward a protective Th1 response (13, 49). NK cells are a potent source of IL-12-induced IFN-γ early in *L. major* disease (8). We reasoned that protective immunity induced by s.c. *L. chagasi* promastigotes likely also requires IL-12- induced IFN-γ at the inoculation site. Therefore, we studied whether NK cells are necessary to develop a protective immune response after s.c. immunization with *L. chagasi* promastigotes. NK cells were depleted from BALB/c mice by treatment with mAb against asialo-GM1, which recognizes a surface glycosphingolipid on NK cells, several days before and after s.c. immunization (50, 51). Control mice were given the comparable amount of nonimmune rabbit serum. Anti asialo-GM1 treatment thoroughly depleted NK cells, shown by the inability of splenocytes to lyse the NK target Yac-1 (Fig. 3*A*). Asialo-GM1 is also present on other cell types, such as macrophages, so treatment is not entirely NK-specific. Nonetheless, NK depletion did not arrest the development of protective immune responses induced by high-dose s.c. immunization (Fig. 3*B*).

Cytokines elicited by s.c. immunization

One month after s.c. immunization, splenocytes of mice that received high- but not low-dose immunization with *L. chagasi* promastigotes produced significantly more parasite Ag-induced IFN-γ than control mouse splenocytes (Fig. 4*A*). Curiously, low-dose immunization with 10^2 organisms actually lowered the spontaneous IFN-γ release by splenocytes cultivated with live *L. chagasi* promastigote Ag. The mitogen Con A caused splenocytes from all groups of mice to produce similar readily detectable amounts of IFN-γ (data not shown). To discern which subset of splenic T cells secreted IFN-γ, $CD4^+$, or $CD8^+$, cells were depleted from splenocytes of immunized mice with Ab plus complement. Splenocytes depleted of $CD4^+$ T cells secreted negligible amounts of IFN-γ in response to promastigote Ag, whereas splenocytes depleted of $CD8^+$ T cells produced ample Ag-specific IFN-γ. Thus, $CD4^+$ cells were the main source of IFN-γ induced by s.c. immunization in our model (Fig. 4*B*).

Levels of cytokines potentially inhibiting the protective immune response were also examined. Splenocytes of mice immunized with high-dose *L. chagasi* paradoxically produced significantly greater amounts of Ag-induced IL-10 and IL-4 than did controls (Fig. 5*A*), whereas low-dose immunization had no effect on either. Apparently, these potentially inhibitory cytokines did not prevent development of a type 1 response, reminiscent of our prior findings after i.v. immunization (40). Of interest, after s.c. immunization at a different body site (gluteus), there was not a significant change in either IL-10 or IL-4 (Fig. 5*B*).

In contrast to the above, splenocytes derived from mice immunized with low-dose *L. chagasi* produced a significantly greater amount of TGF-β than splenocytes from control (buffer-immunized) mice. Splenocytes from mice immunized with a high dose of promastigotes did not release significantly more TGF-β into culture medium than did cells from control mice (Fig. 5*C*). TGF-β inhibits IFN-γ responses by suppressing macrophage IL-12, inhibiting macrophage activation, and suppressing the IFN-γ or curative response (40, 52–55). In combination with Fig. 4*A*, the above results suggest that a type 1 response to *L. chagasi* Ag is actively inhibited by TGF-β in low-dose immunized mice, whereas a type 1 response develops unopposed in mice immunized with a high dose of *L. chagasi* promastigotes. The net result is development of a parasite-specific type 1 immune response after high-dose immunization.

We previously reported that infection of mice with *L. chagasi* causes an increase in TGF-β, whereas TGF-β is suppressed in mice that were previously immunized i.v. with promastigotes before challenge infection (40). We investigated whether a similar suppression occurred after s.c. immunization. Mice were immunized s.c. with a high dose of *L. chagasi* promastigotes either at the

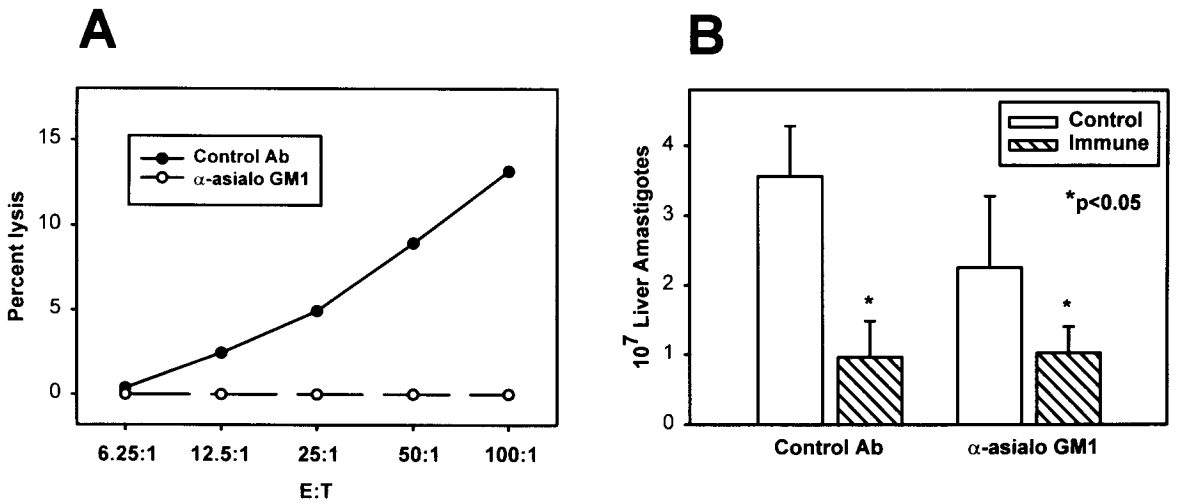

FIGURE 3. NK cells are not required to develop protective immunity. *A*, BALB/c mice received either mAb against asialo GM1 to deplete NK cells or nonimmune rabbit serum i.p. every 4 days for three doses. One day after the third dose, NK cytotoxic activity was examined in splenocytes of NK-depleted or control mice using a standard ^{51}Cr release assay to assess lysis of Yac-1 target cells. Shown is the percent lysis for the indicated E:T ratios. *B*, NK-depleted or control mice were challenged with *L. chagasi*. On day 6 (after the second Ab dose) of the above depletion protocol, mice received either 0 or 10^7 stationary phase *L. chagasi* promastigotes s.c. Four weeks later, mice were challenged i.v. with 10^7 stationary phase *L. chagasi* promastigotes. Four weeks after challenge, the liver parasite burden was enumerated by microscopic evaluation of impression smears of liver. Bars represent the mean ± SE amastigotes in each group of five mice from one representative study of two experiments. Statistics were performed with Student's *t* test.

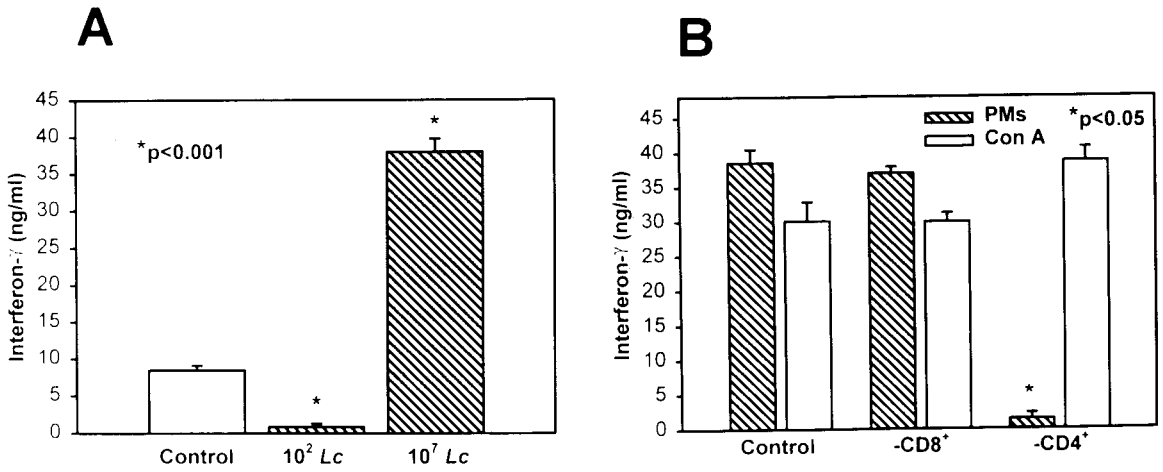

FIGURE 4. IFN-γ induced by immunization. *A*, Splenocytes derived from mice 4 wk after s.c. immunization with either buffer (control) or the indicated dose of *L. chagasi* (*Lc*) were isolated 4 wk after immunization. Shown is the IFN-γ in the medium after 3 days of cultivation with promastigote Ag, quantified by ELISA. Bars represent the mean ± SE from three separate experiments, each containing six replicate wells with pooled splenocytes from five mice. *B*, Splenocytes were isolated from mice immunized with 10^7 *L. chagasi* promastigotes s.c. and treated with Ab and complement to deplete $CD4^+$ or $CD8^+$ cells. Control conditions were treated with nonspecific Ab plus complement. Splenocytes were cultured with no Ag, promastigote Ag, or Con A (5 μg/ml) for 3 days. Bars represent the mean ± SE for six replicate wells from Ag-stimulated conditions in a representative experiment of two, each containing five mice.

base of the neck or in the gluteus and challenged with *L. chagasi* 4 wk later. At the peak of infection (4 wk after challenge), splenocytes from mice immunized in the neck produced a significantly lower amount of TGF-β than did control mice (81.4 ± 13.1 vs 359.4 ± 26.2 pg/ml, respectively; $p < 0.005$). Similarly, mice immunized in the gluteus produced significantly less TGF-β than did control mice (data not shown; $p < 0.05$). In separate experiments, we compared the levels of TGF-β produced by splenocytes from mice that had been immunized s.c. with buffer, low-dose (10^2) promastigotes, or high-dose (10^7) promastigotes before challenge infection. TGF-β levels were 167.1 ± 21.2, 153.3 ± 18.1, and 50.0 ± 2.2 pg/ml, respectively ($p < 0.001$, comparing control with 10^7 immunized mice). We conclude that s.c. immunization with a high dose of promastigotes suppresses the TGF-β response that was usually elicited in response to *L. chagasi* infection.

Immunization with dhfr-ts⁻ *organisms*

We queried whether immunization with "genetically attenuated" recombinant organisms, rendered avirulent by knockout of a critical metabolic enzyme pathway, could induce protective immunity. Because a loss of virulence induced by in vitro cultivation can result in inability to induce protective immunity (Fig. 1*B*), the outcome of this experiment was not obvious. Promastigotes lacking both alleles of the *DHFR-TS* locus were generated from a virulent isolate of our strain of *L. chagasi* (*Lcdhfr-ts*⁻). Other *dhfr-ts*⁻ organisms were generated from virulent *L. major* (*Lmdhfr-ts*⁻) and *L. donovani* (*Lddhfr-ts*⁻). "Add-back" *L. chagasi* were generated from the above *dhfr-ts*⁻ knockout *L. chagasi* in which the *DHFR-TS* locus was restored on an episomal plasmid (*Lcdhfr-ts*$^{-/+}$*DHFR-TS*). Groups of five BALB/c mice were immunized s.c. at the base of the neck with the above recombinant promastigotes. Control mice were immunized with a virulent recent isolate of *L. chagasi* or the line of *L. chagasi* used to generated *dhfr-ts*⁻ promastigotes passaged an equivalent amount of time in vitro. Note that by the time they were ready for assay, control parasites constituted a long-term passaged *L. chagasi* line that was distinct from L5. Parasite loads were compared with mice immunized with buffer alone (PBS). Four weeks after s.c. immunization, all mice were infected with 10^7 virulent *L. chagasi*.

Fig. 6 shows the magnitude of parasite load in immunized or control mice at the peak (4th wk) of infection. As before, mice immunized s.c. with virulent organisms were significantly protected against the infection. Similar to our findings with the attenuated L5 strain, the control (wild-type) promastigotes passaged for the same length of time as recombinant organisms did not induce significant protection against disease. Immunization with *Lcdhfr-ts*⁻ knockout parasites also did not afford protection. The other recombinant *Leishmania* tested (*Lcdhfr-ts*$^{-/+}$*DHFR-TS*, *Lddhfr-ts*⁻, and *Lmdhfr-ts*⁻; Ref. 25) were unable to induce protective immunity against *L. chagasi* challenge infection. Subcutaneous immunization of BALB/c mice with *Lcdhfr-ts*$^{-/+}$*DHFR-TS* caused splenocytes to release IFN-γ in response to parasite Ag before challenge infection (0.64 ng/ml after 3 days of in vitro cultivation with parasite Ag, compared with 0 ng/ml released by control splenocytes and 1.79 ng/ml by splenocytes from mice immunized with 10^7 *L. chagasi*).

Inoculation of *Lcdhfr-ts*⁻ or *Lcdhfr-ts*$^{-/+}$*DHFR-TS* promastigotes i.v. into BALB/c mice did not result in an infection that could be measured microscopically. However, 1 mo after infection viable organisms were recovered from homogenates of spleens and livers of mice inoculated each of the recombinant strains. Recovered organisms retained their antibiotic resistance characteristics (data not shown). This is consistent with the reported finding that *dhfr-ts*⁻ *L. major* survive up to 2 mo after introduction into a host (25). Thus, the failure of immunization with recombinant *dhfr-ts*⁻ strains could not be attributed to a lack of viability of the recombinant organisms, although we cannot be sure that all strains survived at the s.c. site of inoculation. We also cannot be certain whether introduction of organisms in alternate sites or immunization schedules might stimulate a protective immune response in these mice or in alternate hosts such as humans. However, the above data do indicate that the efficacy of s.c. immunization with several different species of *dhfr-ts*⁻ *Leishmania* is not as great as

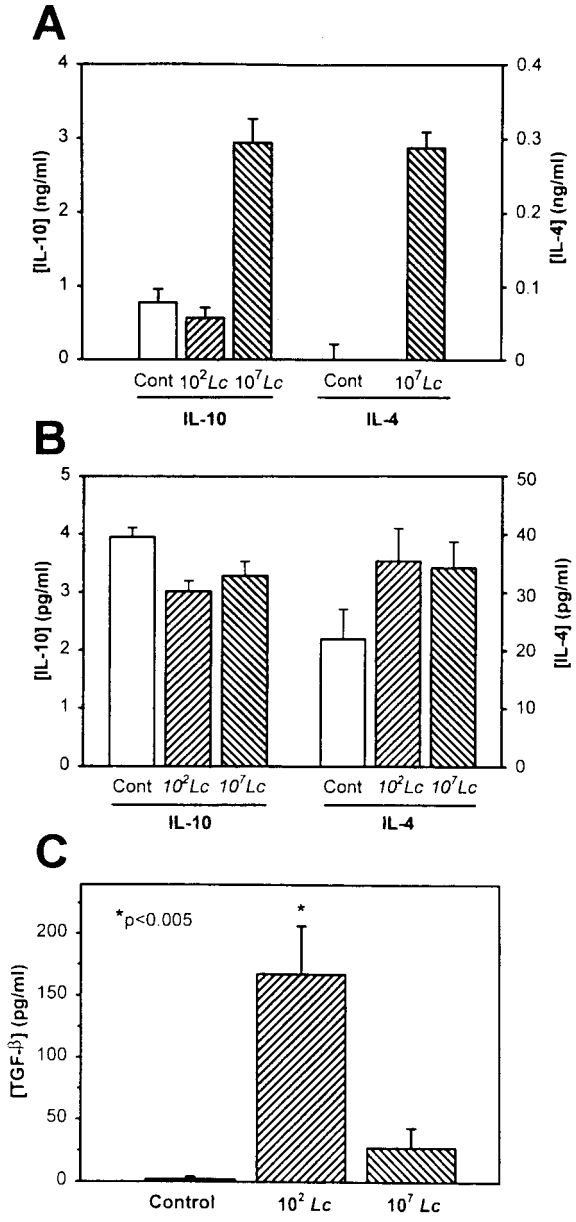

FIGURE 5. IL-10, IL-4, and TGF-β after s.c. immunization. *A*, BALB/c mice were immunized s.c. at the base of the neck with buffer (control), 10^2 virulent *L. chagasi* promastigotes (10^2 *Lc*), or 10^7 virulent *L. chagasi* (10^7 *Lc*). Four weeks later (before challenge infection), splenocytes were cultivated in vitro. Shown are the mean ± SE amounts of IL-10 or IL-4 in supernatants after 3 days of culture with promastigote Ag, measured by ELISA. *B*, Mice were immunized s.c. over the gluteus. Splenocytes were harvested 4 wk later and cultivated in vitro for 3 days with parasite Ag. Supernatants from these cultures were assayed for IL-10 and IL-4 by ELISA. *C*, Four weeks after immunization with 10^2 or 10^7 *L. chagasi* promastigotes at the base of the neck, splenocytes were harvested and cultivated in vitro for 3 days. TGF-β in the medium was quantified using an ELISA.

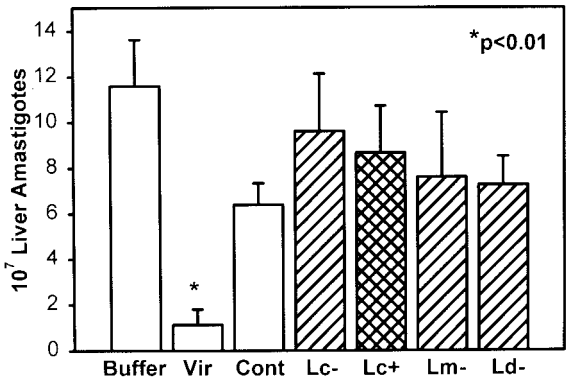

FIGURE 6. Immunization with *dhfr-ts*$^-$ knockout promastigotes. BALB/c mice were immunized s.c. at the base of the neck with buffer alone (buffer), virulent *L. chagasi* promastigotes (Vir), or a strain of *L. chagasi* used to raise knockout organisms, passaged in vitro an equivalent number of times as knockouts (control). Other mice received *L. chagasi* lacking both alleles of the *DHFR-TS* locus (*Lc*$^{[minus]}$), or *dhfr-ts*$^-$ lines of *L. major* (*Lm*$^-$) or *L. donovani* (*Ld*$^-$). *Lc*$^+$ denotes *dhfr-ts*$^{-/+}$ *DHFR-TS L. chagasi* parasites in which the *DHFR-TS* genes were reintroduced on an extrachromosomal plasmid. All immunizations were performed with 1×10^7 promastigotes introduced s.c. over the dorsum of the neck. Four weeks later, mice were challenged with 1×10^7 virulent *L. chagasi* i.v. Shown are the mean ± SE parasite load in groups of five mice, 4 wk after challenge infection, in a representative experiment.

the reproducible and efficient immunization observed after s.c. inoculation of virulent *L. chagasi*.

Discussion

Asymptomatic human infection with *L. chagasi* often results in long-term protective immunity against reinfection (56). A goal of antileishmanial vaccine development is to replicate this naturally acquired protective immunity through immunization with parasite Ags. Live attenuated vaccines have historically provided efficient protection against some infectious diseases (57). During this study, we generated a model of asymptomatic *L. chagasi* infection in susceptible BALB/c mice that provided immunity against parasite rechallenge. We used this model to investigate whether protective immune responses were elicited by immunization with live parasites that are attenuated either by virtue of knocking out a conditionally essential gene locus or by long-term cultivation in vitro.

Many previous studies have documented murine immune responses to *L. major*, a cause of self-healing cutaneous ulcers in humans. The usual outcome of *L. major* infection of susceptible BALB/c mice is progressive local infection and disseminated disease in the setting of Ag-specific Th2 cell expansion. In contrast, infection of genetically resistant mice (e.g., C3H, C57BL/6) results in expansion of Th1-type CD4$^+$ cells and self-healing lesions. Several manipulations can lead to protection of BALB/c mice against *L. major* infection. Depleting CD4$^+$ cells by irradiation or anti-CD4 Abs, altering the early cytokine milieu by adding exogenous IL-12/IFN-γ or neutralizing IL-4 or inducing tolerance to a dominant Th2-stimulating *Leishmania* Ag (LACK), all lead to a Th1 response that effects resistance to infection (49, 58–61). Immunization with Ags plus an appropriate adjuvant or with recombinant organisms (vaccinia, bacillus Calmette-Guérin, *Salmonella* sp.) expressing *Leishmania* Ags has provided partial immunity against parasite challenge (15, 17–19, 26, 27). Bretscher et al. (26) showed that protective cellular immune responses also ensue after BALB/c mice are immunized with low, but not with high, numbers of *L. major* promastigotes s.c. in the footpad. The protective response to low-dose Ag results from stimulation of delayed-type hypersensitivity-producing Th1-type CD4$^+$ T cells, whereas high-dose Ag stimulates potentially exacerbating Th2-type and Ab responses (27, 62).

Our work focused on *L. chagasi*, a cause of fatal disseminated visceral leishmaniasis in humans. We found that a high-dose s.c.

inoculum of *L. chagasi* promastigotes was required for development of protection against reinfection, whereas low-dose immunization either had no effect or slightly exacerbated disease. In contrast, immunization with a low dose but not with a high dose of *L. major* induced protective immunity against autologous challenge. Consistent with previous reports of *L. donovani* infection, inoculating high doses of *L. chagasi* promastigotes s.c. into BALB/c tissues did not result in metastatic infection involving the liver or spleen (39). Subcutaneous immunization with a high dose of promastigotes instead caused an undetectable infection resulting in protective immunity and Ag-specific, IFN-γ-producing $CD4^+$ cells in the spleen. In contrast, low-dose *L. chagasi* promastigote immunization resulted in production of TGF-β and a suppressed IFN-γ response. TGF-β is thought to promote parasite growth, and its presence after immunization may suppress the development of protective immunity (40, 55).

The site of cutaneous inoculation of *Leishmania* sp. yields different outcomes, possibly because of local variations in APCs or in lymphatic microvasculature (47, 63, 64). We found that protective immunity developed in mice immunized at both caudal and cranial sites, but there was a paradoxical increase in both IL-4 and IL-10 after cranial but not after caudal s.c. immunization. Although we did not find a physiologic consequence of these increases, this finding underscores the differences in immune responses at distinct bodily sites. The site of immunization differed between this study and that of Bretscher et al. (26), precluding a direct comparison between the studies. Nonetheless, we produced a result parallel to that of Bretscher et al. in that s.c. immunization with low-dose *L. major* protected mice against a challenge infection with *L. major* in the footpad, whereas high-dose immunization exacerbated subsequent disease. First, we can conclude that the local immune responses after high- or low-dose immunization must reflect unique biological characteristics of each pathogen. Second, s.c. immunization with the appropriate dose of either virulent *L. chagasi* or *L. major* quite efficiently elicits protective immunity against homologous but not against heterologous challenge in this model system.

The use of "genetically attenuated" gene knockout parasites for immunization is preferable to using parasites attenuated by long-term cultivation, because the former should be incapable of regaining virulence in vivo. Additionally, genetically attenuated parasites should be superior to recombinant proteins or killed parasite vaccines because they can persist in tissues and provide prolonged antigenic stimulation that may stimulate long-lasting immunity. Verifying the utility of this approach, immunization of BALB/c mice with recombinant *L. major* lacking both alleles of the *DHFR-TS* locus provided protection against challenge infection with virulent *L. major* (25). The small degree of sequence divergence between the viscerotropic *Leishmania* sp. (65) allowed the successful use of the *L. donovani*-based vectors for the replacement of *L. chagasi* genes, generating an analogous gene knockout *L. chagasi* line lacking both alleles of the *DHFR-TS* locus called *Lcdhfr-ts*$^-$.

Theoretically, *Lcdhfr-ts*$^-$ should protect against challenge infection with *L. chagasi*, similar to the *L. major* result. However, among other biologic differences between the species, *L. chagasi* becomes attenuated more rapidly than *L. major* in culture. Indeed *L. chagasi* virulence diminishes over even a few weeks of culture. We found that attenuated (L5) *L. chagasi* were unable to elicit protective immune responses when inoculated s.c. at high dose into BALB/c mice. This could have reflected either a loss of critical antigenic epitopes needed to elicit protective immune responses during in vitro attenuation or the fact that attenuated organisms did not establish a subclinical infection required to elicit protective responses. Consistent with this finding, the *Lcdhfr-ts*$^-$ line was also unable to elicit protective immune responses against *L. chagasi* challenge in BALB/c mice. The process of raising recombinant *L. chagasi* requires weeks to months of cultivation on solid and liquid media. Therefore, it is possible that the inability of *Lcdhfr-ts*$^-$ to elicit protective immunity in mice is due to their cultivation-associated loss of critical antigenic epitopes and other virulence characteristics.

L. major does not have the same propensity as *L. chagasi* for rapidly losing virulence during in vitro cultivation. The possibility of using *dhfr-ts*$^-$ mutants of *L. major* for cross-immunization against species that rapidly lose their virulence (e.g., *L. donovani*, *L. chagasi*) is quite attractive. However, neither virulent *L. major* nor *dhfr-ts*$^-$ knockout lines of either *L. major* or *L. donovani* caused heterologous protection against *L. chagasi* infection when delivered at a dose of 10^7 promastigotes s.c. at the base of the neck. Thus, these species and mutants are not as efficient as wild-type *L. chagasi* in eliciting protective immunity using our model system. Different routes of *L. major* delivery were found to protect mice against challenge infection with *L. major* infection in the past (25, 26). Therefore, to thoroughly examine the possibility of cross-protection between species we would need to exhaustively test alternate routes and doses of immunization. Using our s.c. immunization model, we conclude that, similar to human disease, protective immunity against the visceralizing species of *Leishmania* can be established by subclinical infection of mice. This protection requires virulence and/or antigenic characteristics that are lost during cultivation of *L. chagasi* in vitro. The issues of rapid parasite attenuation and cross-species protection must be carefully considered in designing a strategy for immunization against the visceralizing species of *Leishmania*.

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