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Cutaneous infection with *Leishmania major* mediates heterologous protection against visceral infection with *Leishmania infantum*

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Abstract

Visceral Leishmaniasis (VL) is a fatal disease of the internal organs caused by the eukaryotic parasite *Leishmania*. Control of VL would best be achieved through vaccination. However, this has proven to be difficult partly because the correlates of protective immunity are not fully understood. In contrast, protective immunity against non-fatal cutaneous Leishmaniasis (CL) is well defined and mediated by rapidly recruited, IFN- γ -producing, Ly6C⁺CD4⁺ T cells at the dermal challenge site. Protection against CL is best achieved by prior infection or live vaccination with *Leishmania major*, termed leishmanization. A long-standing question is whether prior CL or leishmanization can protect against VL. Employing an intra-dermal challenge model in mice, we report that cutaneous infection with *Leishmania major* provides heterologous protection against visceral infection with *Leishmania infantum*. Protection was associated with a robust CD4⁺ T cell response at the dermal challenge site and in the viscera. *In-vivo* labeling of circulating cells revealed that increased frequencies of IFN- γ ⁺CD4⁺ T cells at sites of infection is due to recruitment or retention of cells in the tissue, rather than increased numbers of cells trapped in the vasculature. Shortly after challenge IFN- γ producing cells were highly enriched for Ly6C⁺T-bet⁺ cells in the viscera. Surprisingly, this heterologous immunity was superior to homologous immunity mediated by prior infection with *Leishmania infantum*. Our observations demonstrate a common mechanism of protection against different clinical forms of leishmaniasis. The efficacy of leishmanization against VL may warrant the introduction of the practice in VL endemic areas or during outbreaks of disease.

INTRODUCTION

The leishmaniases consist of a broad range of cutaneous, mucocutaneous, and visceral diseases caused by different strains of the eukaryotic parasite *Leishmania*. *Leishmania*, which is transmitted to the human host by the bite of a sand fly vector, is an obligate

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intracellular pathogen that establishes chronic infection within phagocytes. Visceral forms of leishmaniasis are fatal if left untreated and while drugs are available they are expensive, highly toxic, and drug resistance is common (1, 2). Visceral leishmaniasis caused by *L. donovani* is endemic in northern India and East Africa, whereas *L. infantum*, also known as *L. chagasi*, is the causative agent of VL in South America and the Mediterranean basin, which has experienced several recent outbreaks (3-6). In some areas, *L. infantum* infection of dogs has reached epidemic proportions and dogs are believed to be a major reservoir of human disease (7).

No vaccine is currently available for any form of leishmaniasis in people. However, a deliberate, single needle inoculation of infectious *L. major* into the skin without the disease exacerbating factors co-egested during natural sand fly transmission, termed leishmanization, provides complete and long lasting homologous protection against sand fly transmitted cutaneous disease and has been used extensively as a live vaccine in humans (8-12). Despite its efficacy and the convenience of a single administration, leishmanization has largely been abandoned because of rare adverse reactions at the site of inoculation (13, 14); and the chronic nature of the infection raises concerns should leishmanized individuals become immune-compromised, although there are no reports of reactivation or dissemination of *L. major* in leishmanized individuals. A more justifiable use of leishmanization would be to vaccinate against strains that cause lethal visceral leishmaniasis (VL), for which the benefits of leishmanization may outweigh any risks. Cross-protection conferred by leishmanization against VL would suggest a common mechanism of resistance against *Leishmania* species that cause different clinical diseases, and suggest that different *Leishmania* species share a sufficient number of protective antigens to warrant their use in pan-*Leishmania* vaccines (15, 16). However, evidence that *L. major* infection cross-protects against VL in people is rare or difficult to interpret (17-23).

Experimentally, two prior studies have investigated this question and found that leishmanization either provided no protection (24) or enhanced visceral infection (25) following *L. infantum* challenge. However, these studies employed BALB/c mice that are susceptible to *L. major* infection due to a defect in the generation of Th1 immunity, a condition not typically observed in people infected with *L. major* (26). In contrast, leishmanized C57BL/6 mice more closely replicate the immune status of leishmanized humans (11). Therefore, we employed an intra-dermal challenge model of visceral infection caused by *L. infantum* in C57BL/6 mice leishmanized with *L. major* (27). We present evidence that leishmanization provides robust protection and similar correlates of protection against both cutaneous and visceral infection. Leishmanization may be a viable strategy for control of visceral disease.

MATERIALS AND METHODS

Parasites

L. major Friedlin strain was isolated from a patient who acquired his infection in the Jordan Valley (MHOM/IL/80/Friedlin). *L. infantum* (MHOM/ES/92/LLM-320; isoenzyme typed MON-1) was isolated from a patient with VL in Spain and was provided by Diane MacMahon-Pratt. *L. infantum*-RFP was generated as previously described (28). Parasites

were cultured *in vitro* at 26°C in complete medium 199 (CM199) supplemented with 20% heat-inactivated FCS (Gemini Bio-products), 100 U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 40mM Hepes, 0.1 mM adenine (in 50mM Hepes), 5mg/ml hemin (in 50% triethanolamine), and 1mg/ml 6-biotin. For *L. infantum* the CM199 was further supplemented with 2µg/ml 6-Biopterin (Sigma, St Louis). *L. infantum* and *L. major* infective-stage metacyclic promastigotes were isolated from stationary cultures (4-6 day old) by centrifugation through a Ficoll-step gradient as described (29). For leishmanization, *L. major* metacyclic promastigotes were isolated by negative selection of non-infective forms using peanut agglutinin (Vector Laboratories) (30).

Mice

Female C57BL/6 mice were obtained from Taconic. All mice were maintained in the National Institute of Allergy and Infectious Diseases animal care facility under specific pathogen-free conditions.

Leishmanization and challenge

Leishmanized mice were generated by injecting 10^4 *L. major* metacyclic promastigotes subcutaneously in the hind footpad in a volume of 40µl and used at 12 to 20 weeks post-primary infection when footpad lesions had completely resolved. Mice with a primary *L. infantum* infection were generated in the same manner. Naïve mice, leishmanized mice, or *L. infantum* infected mice were challenged with 2×10^6 *L. infantum* metacyclic promastigotes intra-dermally (i.d.) in the ear in a volume of 10µl. In some experiments mice were injected intravenously (i.v) in the tail vein with 2×10^6 *L. infantum* metacyclics promastigotes in a volume of 200µl.

Processing of different sites of infection and parasite quantification

Mice were perfused via intra-cardiac injection of 20 ml of cold PBS. Liver perfusion was performed by injection of 6 ml of cold PBS into the liver portal vein. The spleen, and ear draining LN (dLN) were removed, cut with tweezers, homogenized with a syringe plunger, and the cell suspension was filtered through a 70µm strainer. Liver were incubated for 45min with 2ml DMEM containing 250µg/ml of Liberase TL purified enzyme blend (Roche Diagnostic Corp.) and 10µg/ml of DNase. Liver cells were further purified using a Percoll gradient (31). In experiments employing direct intracellular staining (dICS), organs were processed in media pre-warmed to 37°C containing 20µg/ml of Brefeldine A (BFA) and incubated at 37°C and 5% CO₂ post-processing for a total time in BFA of 4 hours. Ear tissue was prepared as previously described (11). Briefly, ears were removed and placed in 70% ethanol for 2-5 minutes and then allowed to dry. Separated dorsal and ventral sheets of ears were then incubated at 37°C for 90 minutes in 1ml DMEM containing 160µg/ml of Liberase. Following Liberase treatment tissue was homogenized for 3½ minutes in a Medicon using a Medimachine (Becton Dickinson). The tissue homogenate was then flushed from the medicon with 10 ml RPMI media containing 0.05% DNase and filtered using a 50 µm-pore-size cell strainer. In experiments employing dICS, BFA at 20µg/ml was added to media pre-warmed to 37°C and the ear homogenate returned to 37°C and 5% CO₂ post-processing for a total time in BFA of 4 hours. In some experiments mice were not

perfused and red blood cells were removed from the spleen and liver using ACK lysing buffer for 5min at room temperature. Cells from each tissue were re-suspended in CM199 with 6-biopterin.

Parasite loads were determined by two fold serial dilutions in 96-well flat bottom microtiter plates, by overlaying 100µl of the diluted tissue suspension onto 50 µl NNN medium containing 20% defibrinated rabbit blood. The dilutions were made in duplicate. The plates were scored microscopically for growth and the number of parasites in each tissue was determined from the highest dilution at which parasites could be grown out after 7-10 days incubation at 26°C. In some experiments 25µg/ml of hygromycin B (Sigma) was added to the media to discriminate between parasites used for live vaccination versus parasites used for challenge.

Re-stimulation of tissue-derived cells for cytokine analysis by flow cytometry

Tissue derived cells were re-stimulated as described previously (11). Briefly, single-cell suspensions were incubated at 37°C in 5% CO₂ for 12-14 hours in flat-bottom 48-well plates with 0.5-1×10⁶ T cell-depleted (Miltenyi Biotech) naïve spleen cells (APCs), with or without 50 µg/ml freeze-thaw *Leishmania* antigen (*L.m.*-Ag and or *L.i.*-Ag) in a total volume of 1ml. During the final 4-6 hours of culture, 1µg/ml of Brefeldin A (Sigma) was added. Cells were then washed and labeled with Live/Dead fixable AQUA at a 1:500 dilution of the manufacturer suggested stock solution (Invitrogen) to exclude dead cells and anti-Fc III/II (CD16/32) receptor Ab (2.4G2), followed by PE-Cy7 anti-mouse CD4 (RM4-5), and in some experiments APC-eFluor 780 CD8a (53-6.7) for 20 minutes. In some experiments cells were stained with PerCp.Cy5.5 anti-CD4 (RM4-5), FITC anti-CD44 (IM7), APC-eFluor 780 anti-Ly6C (HK1.4) and PE-Cy7 anti-CD62L (MEL-14) for 20 minutes. Cells were then fixed with BD Cytfix/Cytoperm (BD Biosciences) according to the manufacturer instructions and stained with FITC anti-IFN-γ (XMG1.2), Per-CP eFluor710 anti-TNF-α (MP6-XT22), and in some experiments APC anti-TcR-β (145-2C11) and/or PE anti-human Granzyme B (GRB04, Invitrogen) and/or V500 anti-CD3 (145-2C11). In some experiments, samples were treated with the Foxp3 Fixation/permeabilization Buffer (eBioscience) per manufacturer's instructions, and subsequently stained for 1 hour at 4°C with PE anti-IFN-γ (XMG1.2) and eFluor 660 anti-Tbet (eBio4B10) antibodies. Isotype controls employed were rat IgG1 (R3-34) and rat IgG2b (A95-1 or eBR2a). All Abs were from eBiosciences or BD Biosciences. Data were collected using FACS DIVA software on a FACS CANTO flow cytometer (BD Biosciences), and analyzed using FlowJo software (TreeStar). Forward-scatter and side-scatter width was employed to exclude cell doublets from analysis.

Perfusion and in-vivo staining of circulating cells

In-vivo labeling of circulating cells was done as described previously (32). Briefly, 0.6µg of anti-TCR-β BV421 (clone H57-597 Biologend) was injected i.v in 200µl PBS via the tail vein. Three minutes after the injection, mice were sacrificed by isoflurane asphyxiation and perfused via intra-cardiac injection of 20 ml of cold PBS. Liver perfusion was performed by injection of 6 ml of cold PBS into the liver portal vein. Organs were then harvested as described above. Following re-stimulation and intracellular staining the frequency of TcR-β⁺ cells labeled by i.v. administration of TcR-β antibody was not reduced on T cells from the

liver (30.0+/-13 TcR- β^+ before re-stimulation versus 29.6+/-12 TcR- β^+ after re-stimulation and intracellular staining, n=14) and only slightly reduced on cells from the spleen (39.2+/-13 TcR- β^+ before re-stimulation versus 34.0+/-12 TcR- β^+ after re-stimulation and intracellular staining, n=12).

Statistics

Unless otherwise noted, data were compared using the Mann-Whitney test. Comparisons between multiple groups were done using ANOVA with Holm-Sidak's post test for comparisons between multiple groups. All p-values are two-sided. Statistical calculations were done in Graphpad PRISM 5.0c (www.graphpad.com). For figures depicting pooled data, error bars represent the standard error of the mean (SEM). For figures in which data points represent individual replicates error bars represent the standard deviation of the mean (SD). **** p<0.0001; *** 0.0001< p<0.001; ** 0.001<p<0.01; * 0.01<p<0.05.

Ethics Statement

All animal experiments were performed under the LPD-68E Animal Study Protocol approved by the NIAID Animal Care and Use Committee using guidelines established by the Animal Welfare Act and the PHS Policy on Humane Care and Use of Laboratory Animals.

RESULTS

The course of *L. infantum* infection in C57BL/6 mice following intra-dermal inoculation of the ear

In order to study cross protection against visceral infection, we challenged naïve or leishmanized C57BL/6 mice with 2×10^6 *L. infantum* parasites via the intra-dermal route in the ear. Although higher than the number of parasites deposited during natural sand fly transmission (33), we employed 2×10^6 parasites because this was the lowest dose at which *L. infantum* parasites disseminated from the skin to the spleen and liver in all naïve mice tested (Figure 1A). While no murine model replicates the development of progressive, fatal visceral leishmaniasis in people, the i.d. challenge model in C57BL/6 mice does allow for an immunological assessment of protective immunity in the skin, the physiological site of challenge (27). In addition, the i.d. challenge model results in the delivery of low doses of parasites into the visceral organs, something that is also likely to occur during natural infection, and far different from the massive doses that are rapidly delivered to the viscera during conventional i.v. challenge.

We initially determined using limiting dilution analysis (LDA) the course of *L. infantum* infection in naïve C57BL/6 mice following i.d. inoculation, since this has not been reported previously (Fig. 1B and C). Following i.d. inoculation, parasites were found in the ear, dLN, liver and spleen. In the ear and dLN parasites initially increased in number followed by a decline between 7 and 42 days, at which point parasite numbers stabilized for the remainder of the experiment. In the liver and spleen, parasites underwent significant expansion between 1 and 14-21 days p.i., peaking at 1737 in the spleen and 5011 in the liver. Parasite numbers then underwent a gradual reduction between 21 and 63 days, and by day 84,

parasites were no longer detected in the liver or spleen. This course of infection was in contrast to infection with *L. major*, which did not expand in the viscera but established stable, chronic infections in the skin and dLN following high dose inoculation of the ear (Fig. 1 D and E).

Leishmanization with *L. major* provides protection against visceral infection

Twelve to sixteen weeks post leishmanization with *L. major*, mice were challenged i.d. in one ear with *L. infantum*. Analysis of parasite loads in the ear, dLN, liver, and spleen of leishmanized animals revealed significant control of parasite numbers at all sites of challenge and at all time points tested versus control animals, with the exception of the dLN at 1 week post-challenge (Fig. 2A). At 3 weeks post challenge, 40% (8/20) of the spleens and 55% (11/20) of the livers from leishmanized animals had undetectable numbers of parasites and this increased to 88% (7/8) at both sites by 6 weeks post-challenge. To compare the efficacy of prior infection with *L. major* versus *L. infantum* to protect against challenge with *L. infantum*, mice chronically infected with either strain were challenged with drug resistant *L. infantum* parasites. At 1, 3, or 6 weeks post challenge parasites LDA was performed under drug pressure to eliminate the *Leishmania* employed for leishmanization. Leishmanization with *L. major* provided equivalent or enhanced protection versus leishmanization with *L. infantum* (Fig. 2B). This was most significant in the ear where the heterologous protection against *L. infantum* was similar to the level of protection against homologous challenge with *L. major* (Fig. 2C). At all time points tested, with the exception of the 6 weeks time point in the liver when control mice are beginning to clear parasites from this organ, protection was associated with significantly greater numbers of leishmanized versus control mice exhibiting sterilizing immunity in the spleen and liver as determined by the inability to culture organisms from these tissues ($p = 0.036$, Fisher's exact test). So far as we are aware, these observations demonstrate the first experimental confirmation that leishmanization with *L. major* provides protection against visceral infection.

Leishmanization is associated with a rapid and robust CD4⁺ T cell response in the skin and visceral organs following challenge

IFN- γ producing CD4⁺ T cells mediate the protective response conferred by leishmanization against homologous challenge with *L. major* (34, 35). In addition, the rapidity of the CD4⁺ response is the clearest correlate of protection against sand fly transmitted disease (11, 12). Therefore, we analyzed IFN- γ production by CD4⁺ T cells at early time points following heterologous challenge with *L. infantum* and found increased frequencies of CD4⁺ T cells with the capacity to make IFN- γ in the skin, dLN, spleen and liver of leishmanized versus control animals (Fig. 3A and 3B). As previously reported (11) a rapid CD4⁺ T cells response in the skin was observed within 3 days of i.d. challenge (Fig. 3B). We also observed post challenge responses in the viscera of leishmanized mice that were significantly greater than pre-challenge responses starting at day 3 (liver) or one-week (spleen) post challenge with *L. infantum*. This early response was similar to the response to homologous challenge with *L. major* (Fig. S1, A and B), suggesting that the early protective responses to *L. infantum* or *L. major* are the same despite the different clinical outcomes of infection with these two parasites. In addition, IFN- γ production was elicited equally well employing freeze-thawed

antigen from *L. infantum* or *L. major* (Ratio of response *L.m.*:*L.i.* 1:0.95, n=4, p=0.54, paired t-test), suggesting these two parasites also share immunogenic MHC class II restricted epitopes, as suggested by work with the sterol 24-c-methyltransferase protein (36).

Localization of IFN- γ ⁺CD4⁺ T cells associated with the liver and spleen employing *in vivo* i.v. staining

Leishmanized animals had low but detectable frequencies of CD4⁺ T cells with the capacity to make IFN- γ upon stimulation in the spleen and liver prior to challenge (Fig. 4A). However, similar cells were also found in the circulation making it difficult to conclude whether or not these cells were residing in the tissue prior to challenge or were simply trapped in the organ vasculature. In addition, trapped circulating cells may contribute to the increased frequencies of IFN- γ ⁺CD4⁺ T cells detected in the spleen and liver following challenge (Figure 3B). Although we routinely perfuse our mice, and perfusion does reduce the frequency of IFN- γ ⁺ cells in the liver and spleen (Fig. S2), recent observations have demonstrated that perfusion can be incomplete (32, 37). Therefore, in order to discern cytokine production from cells present in visceral tissues versus those trapped in the vasculature we *in-vivo* labeled circulating cells prior to euthanasia and perfusion (32). This technique labels cells on the luminal side of the liver and spleen vasculature as well as cells in the splenic red pulp. This analysis is important in the context of vaccine studies to determine if antigen-specific cells have actually infiltrated infected tissues to mediate effector function versus those that are circulating. Analysis of live CD4⁺ T cells from the blood following *in-vivo* labeling revealed greater than 99% were i.v.⁺ as expected (Fig. 4B). In the liver and spleen we found both i.v.⁻ and i.v.⁺ cells, despite whole body and liver specific perfusion (Fig. 4B). We then determined the frequency of cytokine producing cells that were in the tissue versus those trapped in the vasculature of the ear, spleen, and liver prior to and at 1 week following i.d. challenge, the peak of the CD4⁺IFN- γ T cell response. We also employed i.v. challenge with 2×10^6 *L. infantum*, which delivers large numbers of parasites directly into the visceral organs, particularly the liver (27), to act as a positive control for antigen driven recruitment and/or retention of IFN- γ ⁺CD4⁺ cells in the spleen and liver. Both prior to and following dermal challenge, the vast majority of IFN- γ ⁺ cells in the ear were i.v.⁻ (Fig. 4C, left panel), demonstrating these cells were residing in the tissue. In contrast, the majority of IFN- γ ⁺CD4⁺ cells in the spleen and liver were i.v.⁺, despite perfusion (i.v.⁻ versus i.v.⁺ p<0.001), demonstrating that these cells were trapped, circulating cells. This suggests that the majority of *Leishmania*-specific cytokine production attributed to cells associated with the visceral organs in Figure 3B is due to cells trapped in the vasculature, not cells that are actually mediating effector function in the organ. While i.v. tail vein challenge did not significantly change the data obtained from the spleen, there was a dramatic increase in the frequency of IFN- γ ⁺CD4⁺ T cells from the liver that were i.v.⁻, suggesting that antigen load can drive the recruitment and/or retention of IFN- γ producing cells residing in the liver parenchyma (Fig. 4C, right panel). Despite the fact that the majority of IFN- γ ⁺CD4⁺ T cells associated with the viscera were trapped i.v.⁺ circulating cells, dermal challenge significantly increased the frequency of IFN- γ ⁺CD4⁺ cells that were i.v.⁻ versus pre-challenge mice in both the spleen and liver (compare open circles in Fig. 4C). This was most dramatic in the liver following tail vein challenge (Fig. 4C, right panel). In order to correlate responses in the actual tissue with the reductions in parasite numbers

mediated by leishmanization observed in Figure 1, we also determined the frequency of cytokine producing cells within the total, i.v.⁻, or i.v.⁺ CD4⁺ T cell populations following challenge (Fig. 4, D-F). In both the spleen and liver there was a significant increase in the frequency of IFN- γ ⁺ cells within the i.v.⁻ population following i.d. challenge (Fig. 4E), demonstrating that leishmanization mediates a rapid response in the visceral organs similar to that observed at the dermal site of challenge. Analysis of i.v.⁺ cells revealed a trend towards increased frequencies of IFN- γ ⁺ cells following dermal challenge but this did not reach significance. These observations demonstrate that leishmanization with *L. major* mediates an early CD4⁺ T cells response in visceral sites of infection following i.d. challenge with *L. infantum*.

intravenous⁻, IFN- γ -producing cells in the viscera following challenge are enriched for Ly6C expression

We have previously reported that Leishmanization maintains high frequencies of short-lived CD44⁺CD62L⁻T-bet⁺Ly6C⁺ CD4⁺ T_{EFF} cells that are required for protection against re-infection with *L. major* in the ear dermis. The cells are not memory cells reactivated by secondary challenge, and while they produce cytokine *in-vivo* in an antigen-dependent manner, they can be rapidly recruited to dermal sites of challenge in an antigen independent manner (35). Therefore, we wished to determine if IFN- γ producing cells in the viscera at 3 days following dermal challenge of Leishmanized mice with *L. infantum* also expressed the Ly6C⁺ T_{EFF} phenotype (Figure 5). We employed direct intracellular staining (dICS) to identify IFN- γ producing cells (35) (Fig. 5A). Because dICS does not employ *ex-vivo* antigen or pharmacological re-stimulation, assessment of Ly6C and T-bet expression using this methodology likely reflects patterns of *in-vivo* expression. Non-circulating i.v.⁻CD44⁺CD62L⁻CD4⁺ T cells that expressed Ly6C were T-bet high, as described previously (35) (Fig. 5B), and were highly enriched for IFN- γ producing cells compared to Ly6C⁻ cells (Fig. 5C). In addition, the frequency of T-bet⁺Ly6C⁺ expressing cells was significantly higher among IFN- γ -producing i.v.⁻CD44⁺CD62L⁻CD4⁺ T cells versus IFN- γ non-producing i.v.⁻CD44⁺CD62L⁻CD4⁺ T cells (Fig. 5D). In contrast, IFN- γ production in naïve mice challenged with *L. infantum* was very low and no significant difference was observed between Ly6C⁺ and Ly6C⁻ cells. This data demonstrates that, similar to *L. major* challenge of the ear, IFN- γ production is highly associated with Ly6C expressing CD4 T cells in the viscera at acute time points following challenge with *L. infantum*.

Following challenge responding CD8 T cells are predominantly GzB⁺ but IFN- γ

While Th1 CD4⁺ T cells are associated with mediating protection against primary and secondary infection with *L. major* (35, 38), the role of CD8⁺ T cells in Leishmaniasis remains controversial and is dependent upon the dose, strain, and infection setting (primary or secondary) (1, 38-41). In visceral infection with *L. donovani*, CD8⁺ T cells have been shown to be both protective and dispensable (40, 42). We determined IFN- γ and Granzyme B (GzB) production by CD8⁺ T cells following challenge with *L. infantum* and, similar to observations in BALB/c mice (24), found a very low frequency of IFN- γ ⁺ cells regardless of the site of analysis (Fig. 6, A and B). In contrast, high frequencies of cells were found to be GzB⁺. Similar observations were made following challenge with *L. major* (Fig. S3). The postulated role of granzyme B in Leishmaniasis is evolving. Granzyme AxB^{-/-} double

knockout C57BL/6 mice are completely resistant to *L. major* infection (43) while CD8⁺GzB⁺ T cells have been associated with pathology, not parasite killing, during cutaneous *L. braziliensis* infection (41, 44, 45). Despite the high frequency of GzB⁺CD8⁺ cells we were unable to detect GzB by ELISA following antigen stimulation (data not shown). Employing *in-vivo* labeling of circulating cells we detected an increase in the frequency of GzB⁺ CD8⁺ T cells in the viscera following challenge, although this did not reach significance (Fig. S4).

DISCUSSION

Our results demonstrate for the first time, so far as we are aware, that leishmanization protects against heterologous visceral infection. Protection was associated with rapid activation and recruitment of IFN- γ producing Th1 cells at both the cutaneous site of challenge and visceral sites of infection, demonstrating that protection conferred by leishmanization is not site specific. Similar to what we have previously reported for *L. major* challenge in the skin, IFN- γ production was highly associated with CD44⁺CD62L⁻Tbet⁺Ly6C⁺ CD4⁺ T cells within the i.v.⁻ population in the viscera. At early time points following challenge these cells are recruited, infection-dependent, pre-existing T_{EFF} cells, not memory cells reactivated by challenge (35). Our observations suggest that the correlates of protective immunity against CL and VL may be sufficiently similar to warrant a pan-*Leishmania* vaccine provided the vaccine can replicate the correlates of immunity observed in leishmanized individuals (11, 12, 35). Our data also provides the best evidence to date that heterologous immunity can be identical, or even superior, to homologous immunity.

The controlled trials demonstrating the efficacy of leishmanization in humans is restricted to protection against CL caused by *L. major* or *L. tropica*. However, epidemiological studies suggest prior infection of humans with *L. major* may provide cross-protection against VL caused by *L. donovani*. Two longitudinal studies of VL in Sudan demonstrated that despite the presence of both leishmanin-skin test (LST) positive and negative individuals at the beginning of each study, all cases of VL were reported amongst the LST negative group (17, 18). Because LST positivity was highly associated with prior residence in an *L. major* endemic area, the authors hypothesized that prior infection with *L. major* provides protection against VL caused by *L. donovani*. Related observations in Sri Lanka reported that districts with high levels of CL caused by a naturally attenuated strain of *L. donovani* are almost devoid of VL (19). Experimentally, susceptible BALB/c mice infected with the attenuated *L. donovani* strain had significantly reduced parasite burden in the liver upon challenge with the *L. donovani* strain responsible for Sri Lankan VL (46). In a vervet monkey model of disease, Gicheru et. al. (47) reported the inverse result, that subclinical infection with *L. donovani* provided complete protection against cutaneous leishmaniasis following experimental challenge with *L. major*.

Experimental cross-protection mediated by infection (24, 25, 48-54) as well as T cell cross-reactivity with antigens from different *Leishmania species* (15, 16) has been studied extensively in animal models, primarily in an effort to define proteins that could be used in a pan-*Leishmania* vaccine. Genome sequencing has also revealed that 90% of the genome is

conserved between *L. major* and *L. infantum*, and 99% of the genes are syntenic (55, 56), suggesting a strong likelihood of shared antigens. Remarkably, only two experimental studies have investigated the cross-protection mediated by primary infection with *L. major* against subsequent visceral infection (24, 25). These studies employed BALB/c mice chronically infected with *L. major* and showed no protection against either i.v. or i.d. challenge with *L. infantum*. In contrast, we were able to demonstrate the protective effect of leishmanization against visceral infection employing C57BL/6 mice with a healed *L. major* infection, which more closely replicates the immune status of leishmanized humans. Our observations are limited to an assessment of protection against infection, not disease, because mice infected i.d. with visceralizing strains of *Leishmania* produce minimal pathology in the viscera. In addition, the i.d. model of infection does not establish long-term infection in naïve mice as determined by the detection of parasites in the liver and spleen by LDA at 12 weeks p.i. (Fig 1). Although this may be viewed as a limitation of the model, the use of the more conventional high dose i.v. challenge model would by-pass the potentially important initial step of parasite deposition in the skin, the natural site of infection. In addition, it should be noted that in humans the immunobiology of asymptomatic infection with visceralizing parasites is under-studied, and the scarcity of parasites in the viscera may in fact be a common finding in the field (57).

While the ideal challenge model would employ exposure to the bites of *L. infantum*-infected sand flies, we have yet to establish a reliable sand fly transmission model of *L. infantum* in our laboratory. Rather, we employed relatively early time points, i.e. 1 week post-needle challenge, to determine protective immunity in the viscera as early control of parasite numbers in the skin following needle challenge has proven to be an informative correlate of protection against cutaneous disease following challenge with *L. major* infected sand flies (11, 12).

Our observations suggest that the majority of cytokine production typically associated with adaptive T cell immunity in the spleen and liver is from circulating cells trapped in the vascular or the splenic red pulp at the time of euthanasia. Despite this observation, we were able to employ a combination of *in-vivo* labeling of circulating cells and pre-challenge leishmanized mice to demonstrate that leishmanization does mediate a rapid *Leishmania*-specific immune response in visceral tissue in response to dermal challenge with *L. infantum*. The use of the *in-vivo* labeling technique would appear to be critical in future studies to determine the relative contribution of circulating cells versus tissue resident cells to total cytokine production. It is important to note that even before challenge, we found low frequencies of i.v.⁻ cells with the capacity to make IFN- γ in the uninfected ear, non-draining LN, blood, liver and spleen of leishmanized mice, similar to what we have reported previously (35). Whether the i.v.⁻IFN- γ ⁺CD4⁺ T cells in the viscera are tissue resident memory T cells (T_{RM}) as has been reported for the lung (58) skin (59) and vaginal mucosa (60), or patrolling cells as has also been reported for the skin (61), is unknown. Where as pre-existing Ag-specific T cells are not required for the recruitment of highly protective *Leishmania*-specific T cells into a site of non-specific tissue damage (35), this does not preclude the possibility that they may enhance protection, regardless of their patrolling or resident nature (59). Extensive studies have identified a role for pre-existing CD8⁺ T_{RM}

cells in protective immunity (62). We found strong evidence for the existence of i.v.⁻, resident, *Leishmania*-specific GzB⁺CD8⁺ T cells in the viscera of leishmanized mice prior to challenge. The role of these cells in protection will be the topic of future research.

Our data reinforces the rationale for re-introducing the practice of leishmanization under conditions where the benefits, such as protection against fatal visceral disease, are deemed to outweigh the risks associated with administering live *L. major*. Numerous genetically modified or avirulent *Leishmania* strains have been studied in an effort to replicate the protective effect of leishmanization while reducing or controlling for the virulence of the parasite (52, 63-68). How effective these modified strains are compared to leishmanization with wild type *L. major* has not been addressed, with a single exception (68). Past observations (34, 35, 69, 70) suggest that attenuation resulting in sterile clearance rather than persistence will compromise long-term protection, especially against the more stringent conditions of infected sand fly challenge. The ideal situation would be to leishmanize with a parasite that minimizes pathology but also persists over the long term. An additional drawback of leishmanizing against VL is the wisdom of introducing *L. major* in areas where the parasite is not endemic, such as northeast India. However, *L. major* strains that establish persistent infection in the mammalian host but cannot produce transmissible infections in the sand fly midgut, such as various *lpg* knock out parasites, have already been developed that would address these concerns (67, 71-73).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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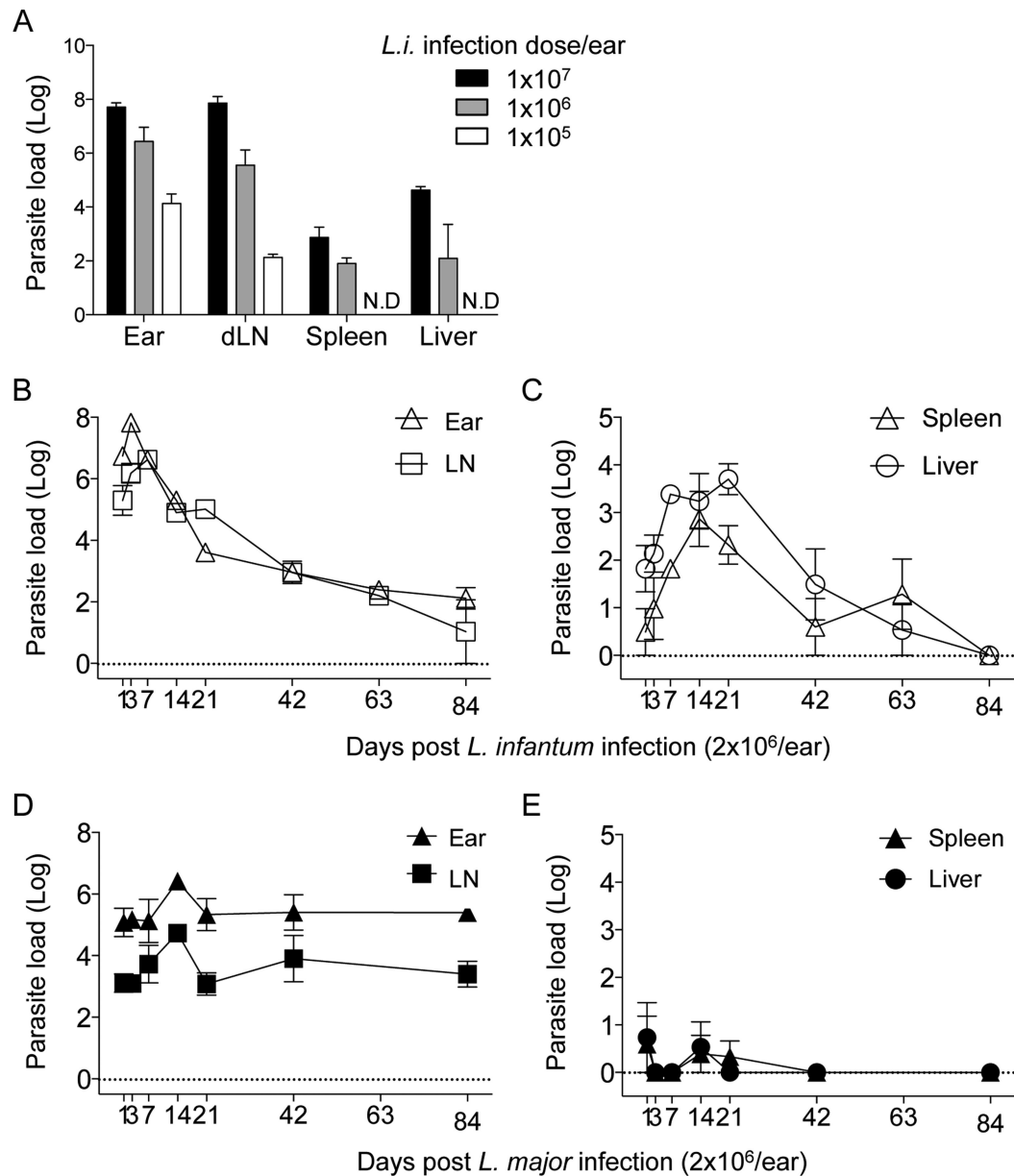
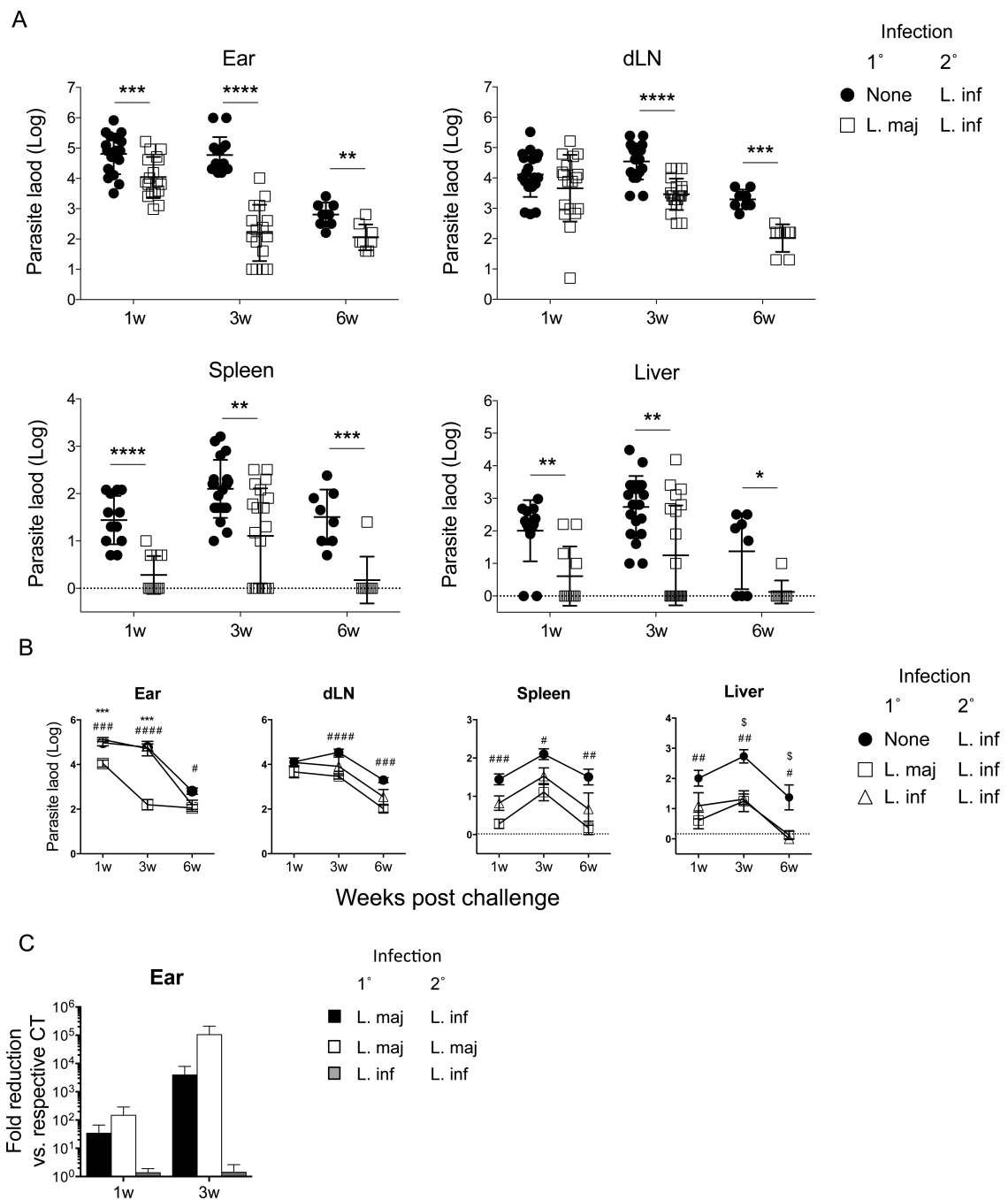


Figure 1. The course of primary infection with *L. infantum* or *L. major* in C57Bl/6 mice following intra-dermal inoculation of the ear

Naïve mice were infected with the indicated doses of *L. infantum* (A) or with 2x10⁶ *L. infantum* (B and C) or *L. major* (D and E) metacyclic promastigotes in the left ear pinnae. Parasite loads in individual ears and draining lymph nodes (A, B and D) or spleens and liver (A, C and E) was determined at 1 week (A) or the indicated time points (B-E) post-infection. n=4 (A) or n=3 (B-E) mice per time point. Representative results from 2 independent experiments. Mean and SEM are shown.



infantum; # $0.01 < p < 0.05$, ## $0.001 < p < 0.01$, ### $0.0001 < p < 0.001$, #### $p < 0.0001$ refer to differences between naive/*L. infantum* and *L. major*/*L. infantum*; \$ $0.01 < p < 0.05$ refers to differences between naive/*L. infantum* and *L. infantum*/*L. infantum*. (C) Mean (+/- SEM)fold parasite load reduction at the site of secondary challenge (ear) in mice with the indicated primary and secondary challenge infections versus the respective controls.

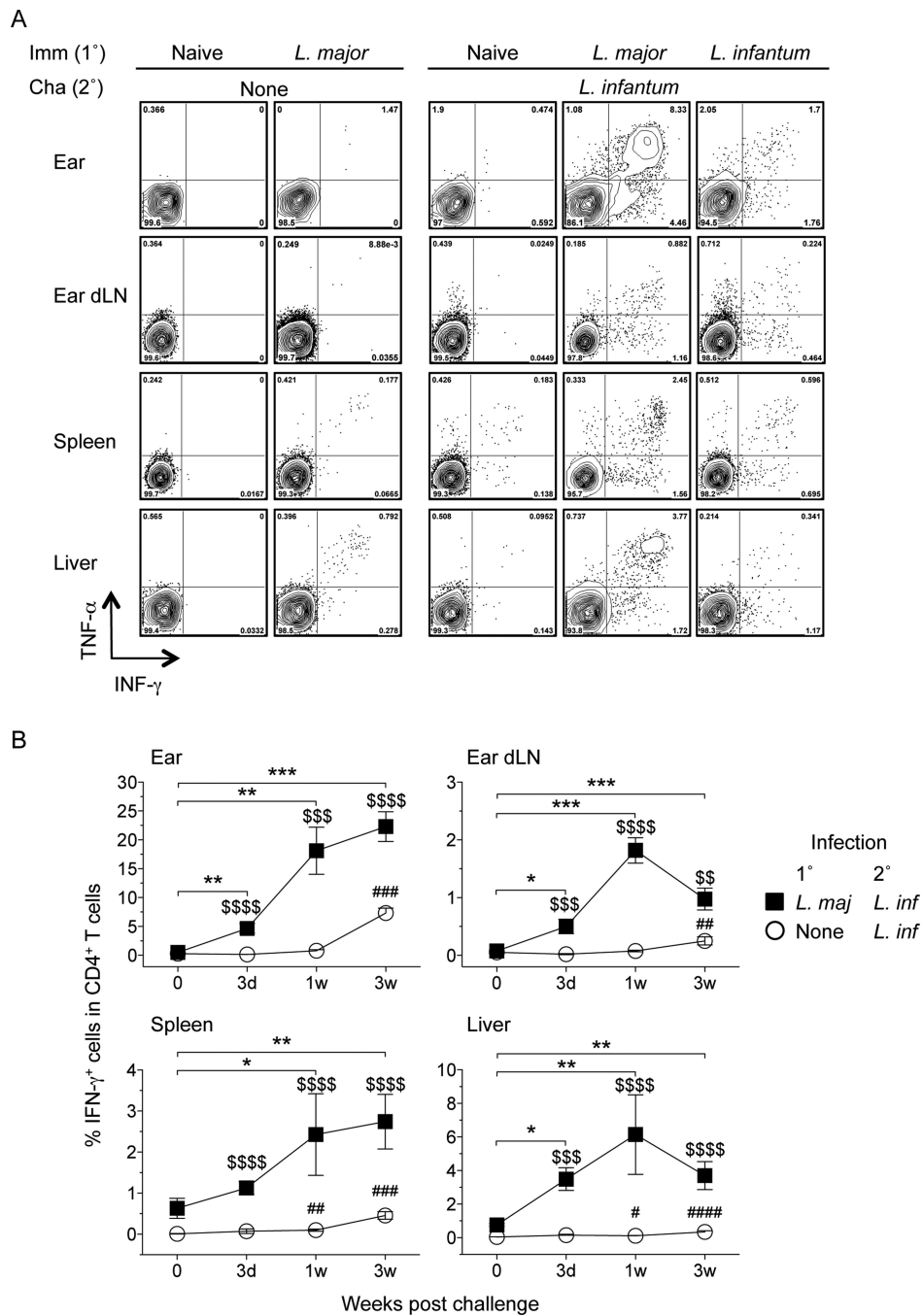


Figure 3. Kinetic of IFN- γ producing CD4⁺ T cells in the skin and viscera after challenge infection with *L. infantum*

At the indicated time post-challenge, cells from mice as shown in Figure 2A were restimulated *in vitro* with APCs plus a mixture of L.m.- and L.i.-antigen and analyzed by flow cytometry. A) Representative intracellular staining contour plots for cytokine-producing CD4⁺TCR β ⁺ T at 1 week post-challenge (B) Kinetic of IFN- γ production by CD4⁺TCR β ⁺ T cells in ears, dLNs, spleens, and livers. *0.01 < *p* < 0.05, **0.001 < *p* < 0.01, ***0.0001 < *p* < 0.001 refer to differences between leishmanized unchallenged mice and *L. major*/*L. infantum*.

infantum; # $0.01 < p < 0.05$, ## $0.001 < p < 0.01$, ### $0.0001 < p < 0.001$, #### $p < 0.0001$ refer to differences between naive/*L. infantum* and naive control mice; \$\$ $0.001 < p < 0.01$, \$\$\$ $0.0001 < p < 0.001$, \$\$\$\$ $p < 0.0001$ refer to differences between naive/*L. infantum* and *L. major*/*L. infantum*; n = 4–5 mice per group per time point. Data are the pool of five independent experiments including at least two time points (day 3, 1 wk, and/or 3 wk) per experiment.

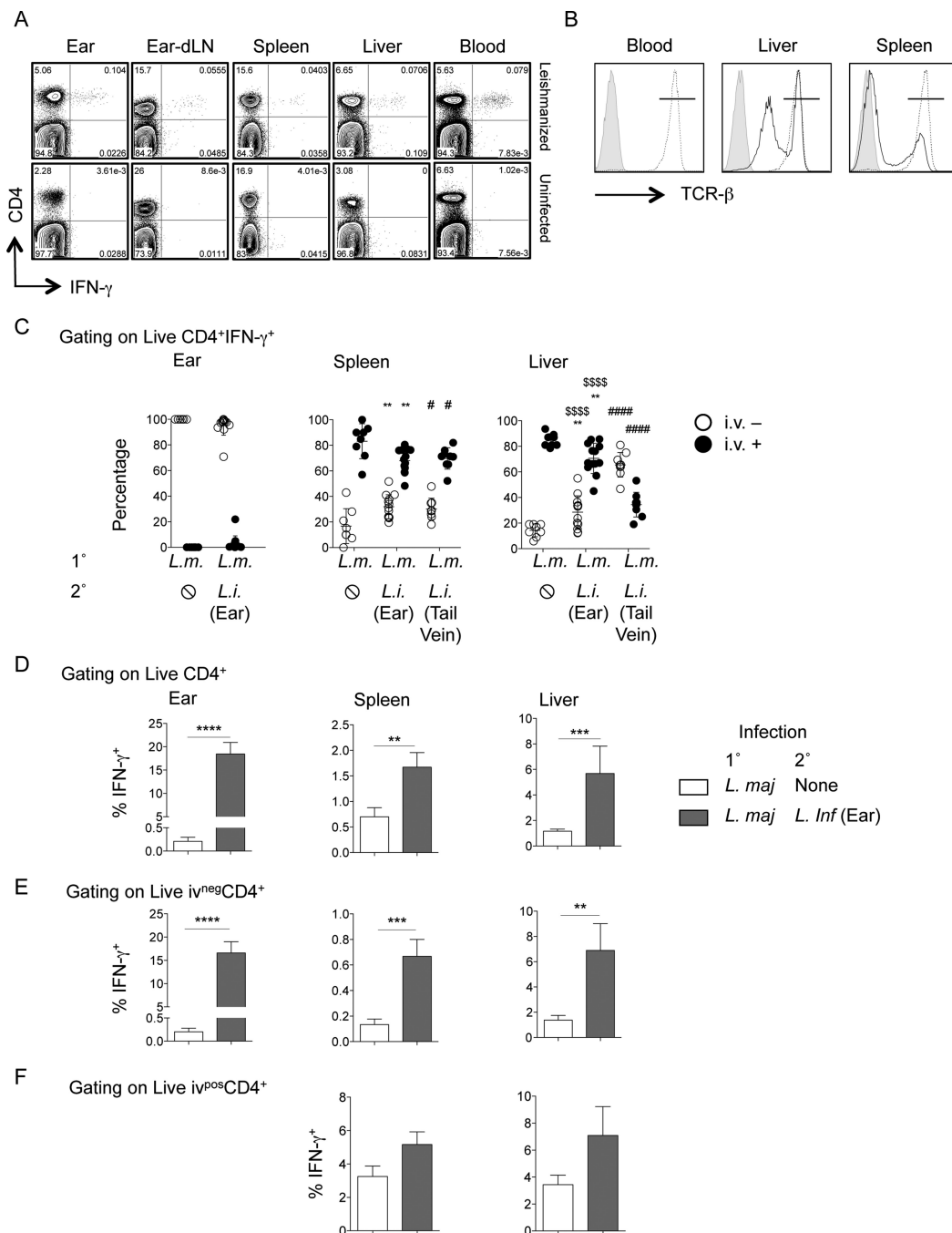


Figure 4. *In vivo* i.v. staining reveals localization of IFN-γ⁺CD4⁺ T cells in the spleen and the liver

(A) Prechallenge production of IFN-γ by CD4⁺TCRβ⁺ T cells from different organs of uninfected or leishmanized mice following *ex vivo* Ag restimulation as described in Fig. 3. (B) Representative histograms of i.v staining with anti-TCRβ-BV421 on CD4⁺ T cells in the blood, spleen and liver. (C) Proportion of i.v.⁻ or i.v.⁺ cells within the IFN-γ⁺ CD4⁺ T cell population (following *ex vivo* Ag restimulation) from the ear, spleen, or liver of leishmanized mice following no challenge (Lm-), i.d. challenge in the ear (Lm-Li (Ear)), or

i.v. challenge in the tail vein (Lm-Li (Tail Vein) with 2×10^6 *L. infantum* metacyclics. ** $0.001 < p < 0.01$, *** $0.0001 < p < 0.001$, **** $p < 0.0001$ refer to differences between Lm- and Lm-Li (Ear); # $0.01 < p < 0.05$, #### $p < 0.0001$ refer to differences between Lm- and Lm-Li (Tail Vein); \$\$\$\$ $p < 0.0001$ refers to differences between Lm-Li (Ear) and Lm-Li (Tail Vein). (D–F) Percentage of IFN- γ^+ cells in the total CD4 $^+$ T cell population (D), the i.v. $^-$ CD4 $^+$ T cell population (E), or the i.v. $^+$ CD4 $^+$ T cell population (F). One week after challenge data point is shown. Data are the pool of three independent experiments employing four to five mice per experiment (two experiments employed tail vein challenge). Li, *L. infantum*; Lm, *L. major*.

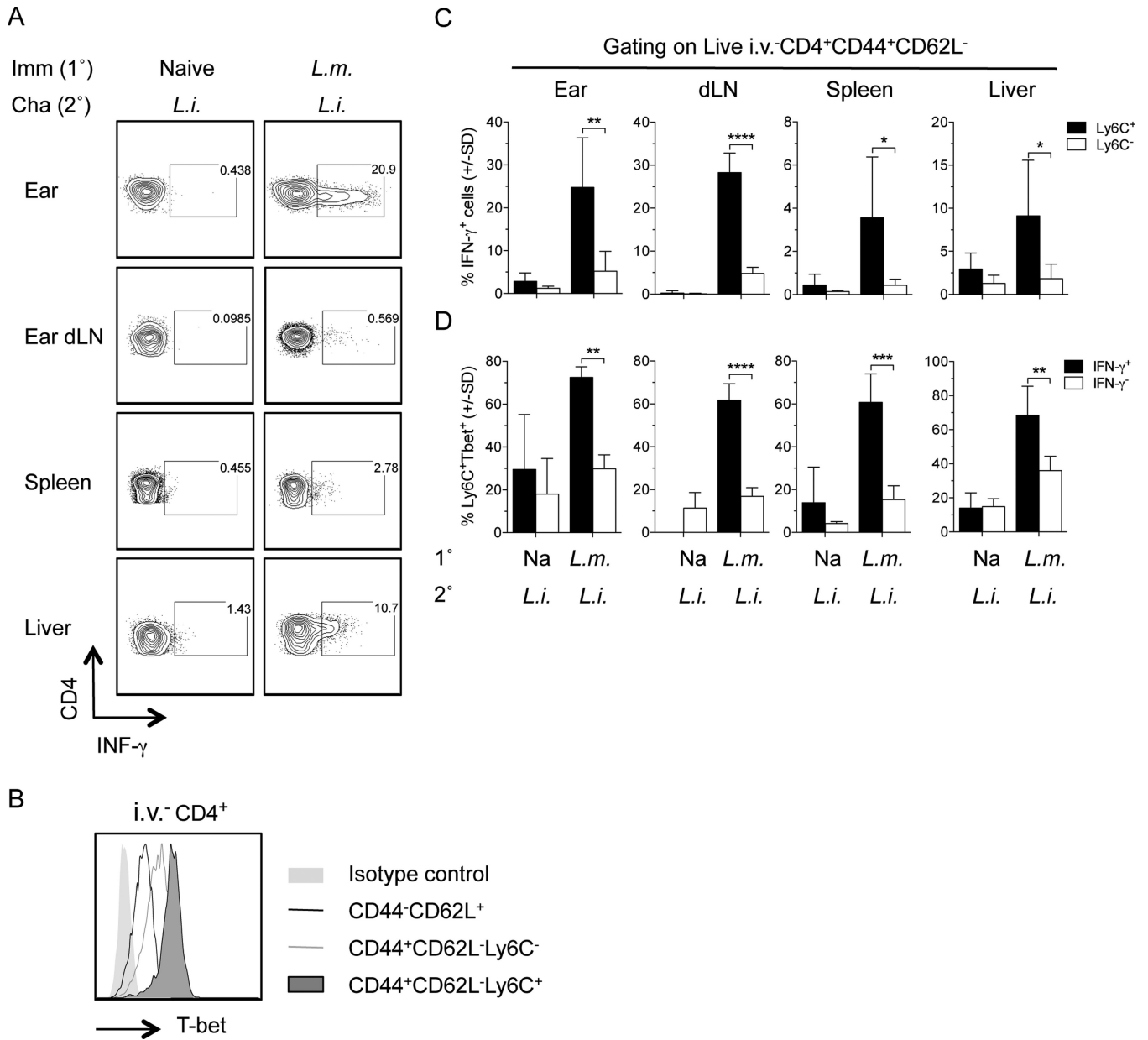


Figure 5. i.v.-CD4⁺CD44⁺CD62L⁻Ly6C⁺ CD4⁺ T cells in the viscera of leishmanized mice are enriched for IFN- γ producing cells following challenge

Naïve or Leishmanized animals were challenged with *L. infantum* as described in Figure 2.

On day 3 post-challenge CD4⁺TcR β ⁺ T cells were analyzed for CD44, CD62L, Ly6C, T-bet, and IFN- γ expression employing dICS. (A) Representative dot plots of IFN- γ production by i.v.-CD4⁺ T cells. B) Representative histogram of T-bet expression by the indicated cell populations in the spleen. C) Percentage of IFN- γ ⁺ cells within live

i.v.-CD4⁺CD44⁺CD62L⁻Ly6C⁺ or live i.v.-CD4⁺CD44⁺CD62L⁻Ly6C⁻ populations. D) Percentage of Ly6C⁺Tbet⁺ cells within the live i.v.-CD4⁺CD44⁺CD62L⁻IFN- γ ⁺ or live i.v.-CD4⁺CD44⁺CD62L⁻IFN- γ ⁻ population. In C and D, n=4 mice/group. Data are representative of 2 independent experiments. *0.01 < p < 0.05, **0.001 < p < 0.01, ***0.0001 < p < 0.001, ****p < 0.0001.

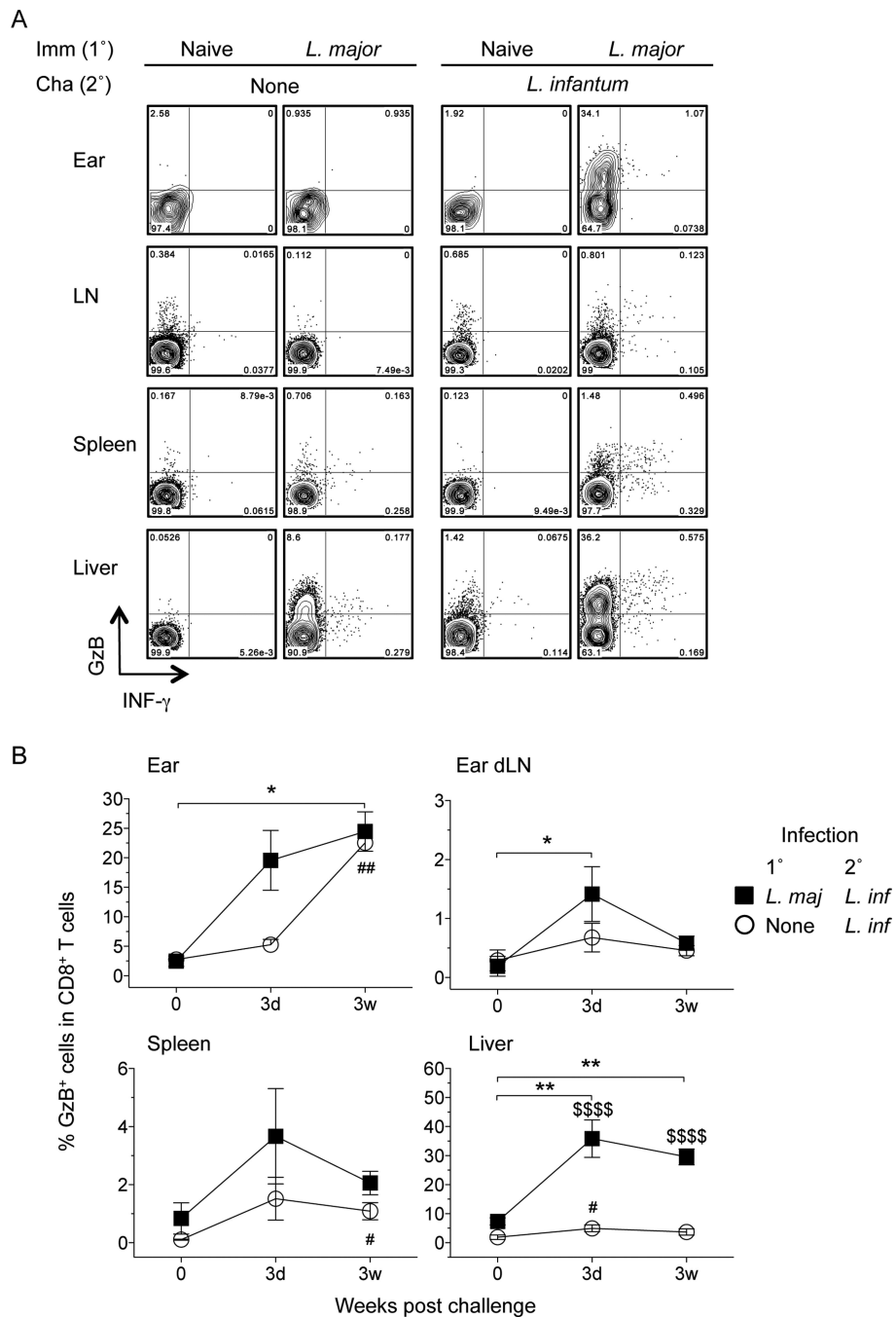


Figure 6. Kinetic of GzB producing CD8⁺ T cells in the skin and viscera after challenge with *L. infantum*

At indicated time post-challenge cells were re-stimulated *in vitro* with APCs plus a mix of L.m.- and L.i.-Ags and analyzed by flow cytometry. A) Representative intracellular staining contour plots for GzB- and IFN- γ -producing CD8⁺TCR β ⁺ T at 3 days post-challenge. (B) Kinetic of GzB production by CD8⁺TCR β ⁺ T cells in the ears, dLNs, spleens, and livers. *0.01 < p < 0.05, **0.001 < p < 0.01 refer to differences between leishmanized unchallenged mice and *L. major*/*L. infantum*; #0.01 < p < 0.05, ##0.001 < p < 0.01 refer to differences between naive/*L. infantum* and naive control mice; \$\$\$\$ p < 0.0001 refer to

differences between naive/*L. infantum* and *L. major/L. infantum*; n = 4–5 mice per group per time point. Data are the pool of three (ears) or four (other sites) independent experiments.

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