Inoculation of killed *Leishmania major* into immune mice rapidly disrupts immunity to a secondary challenge via IL-10-mediated process

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Recovery from natural or experimental *Leishmania major* infection, the causative agent of cutaneous leishmaniasis, results in development of durable immunity in mice and humans that is manifested as rapid control of parasite replication and resolution of cutaneous lesion after secondary challenge. This form of “infection-induced” immunity is thought to occur naturally in endemic areas and is generally considered the gold standard for any effective vaccine against cutaneous leishmaniasis. To determine factors that might heighten or abrogate infection-induced immunity, we investigated the impact of inoculating dead antigen in the form of killed *Leishmania* parasites into healed mice. We show that inoculation of killed parasites into mice that resolved their primary virulent *L. major* infection results in rapid and relatively sustained loss of infection-induced immunity. This loss of immunity was not due to the inability of killed parasites to induce inflammatory responses (such as delayed type hypersensitivity), but it was related to their failure to induce robust IFN-γ response. Furthermore, inoculation of killed *Leishmania* parasites into healed mice led to rapid expansion of IL-10-producing CD4+CD25+Foxp3+ T cells in lymph nodes draining the primary infection site. Treatment with anti-CD25 or anti-IL-10R mAb abolished killed parasite-induced loss of immunity. Our study suggests that vaccination with killed parasites could predispose naturally immune individuals to become susceptible to new infections and/or disease reactivation. This may account for the lack of efficacy of such vaccines in field trials in endemic regions. These findings have important implications for vaccine design and vaccination strategies against human cutaneous leishmaniasis.

Studies show that many individuals in cutaneous leishmaniasis endemic areas display strong leishmanin skin test (LST) and *Leishmania*-specific granzyme B reactivity (even in the absence of visible cutaneous lesions), strongly reflecting the presence of infection-induced immunity (12, 13). Our primary goal was to investigate the effect of inoculating killed *L. major* into such individuals. Therefore, we determined the impact of inoculation of killed, live, and live-attenuated parasites into mice that healed their primary, experimental virulent *L. major* infection. We show that the secondary immune response recalled by killed and live parasites in healed mice are qualitatively and quantitatively different. Remarkably, killed parasites caused a dramatic expansion of IL-10-producing CD4+CD25+Foxp3+ regulatory T cells in the draining lymph nodes (dLNs) of healed mice, resulting in unexpected rapid and sustained loss of infection-induced immunity.

**Results**

**Inoculation of Killed *L. major*** into Immune Mice That Resolved Their Primary *L. major* Infection Leads to Rapid and Sustained Loss of Infection-Induced Immunity. C57BL/6 mice that recovered from primary *L. major* infection developed infection-induced immunity, as evidenced by strong delayed type hypersensitivity (DTH) response and rapid parasite control after secondary *L. major* challenge (Fig. S1). Surprisingly, inoculation of killed (but not live) parasites into the footpad (primary infection site) or rump (distant site) of healed mice led to rapid loss of infection-induced immunity, as evidenced by the inability to control secondary *L. major* challenge (>104 more parasites than live or PBS controls; *P* < 0.01; Fig. 1 A and B). This loss of immunity was observed even when healed mice were challenged at 5 or 10 weeks after killed parasite inoculation (Fig. 1 A), suggesting that killed parasite-induced loss of immunity is relatively sustained and unrelated to nonspecific host innate immune response to killed parasite lysates. Interestingly, incorporation of bacillus Calmette–Guérin or cytokine phosphate guanosine oligodeoxynucleotide (CpG ODN), adjuvants that have been shown to enhance cellular immunity in vaccination studies (14–17), was insufficient to overcome killed parasite-induced loss of immunity in healed mice (Fig. 1C), suggesting that the immune-inhibitory effects of killed parasites are very strong and stable. Furthermore, inoculation of killed (but not live) parasites (without secondary virulent challenge) also led to disease reactivation and extensive proliferation of parasites at the primary infection site (Fig. 1D). Taken together, these results show that inoculation of killed parasites into healed mice leads to rapid, stable, and relatively

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sustained loss of infection-induced resistance, and this effect is independent of the site of killed parasite inoculation.

Inoculation of Live and Killed Parasites Induces Comparable Early Inflammatory Responses but Leads to Dramatic Differences in the Quality of Cytokine Recall Responses in Healed Mice. The striking and unexpected loss of resistance after inoculation of killed parasites into healed mice prompted us to investigate the mechanisms involved in this process. Healed mice inoculated with live or killed parasites (without secondary live challenge) developed comparable DTH response at the inoculation sites, and the number of cells in the dLNs was increased ∼3-fold (P < 0.05) over PBS controls (Fig. S2). Furthermore, antigen-specific proliferations of cells from the dLNs of healed mice inoculated with killed and live parasites were similar as assessed by vitro carboxyfluorescein diacetate succinimidyl ester (CFSE) dye dilution (Fig. 24).

Given that inoculation of live and killed parasites induced comparable DTH and proliferative responses in healed mice, we hypothesized they would also induce comparable IFN-γ recall response. Surprisingly, cells from mice inoculated with killed parasites produced 4- to 6-fold less IFN-γ (P < 0.05) and 3- to 4-fold more IL-10 (P < 0.01) upon in vitro restimulation with soluble Leishmania antigen (SLA) compared with those from mice inoculated with live parasites (Fig. 2B–D). This pattern of cytokine response was also seen by intracellular cytokine staining, where there was also 2- to 5-fold (P < 0.05) higher numbers of IFN-γ-producing CD4+ and CD8+ cells in healed mice injected with live parasites (Fig. S2). Furthermore, the mean fluorescence intensity (MFI) of IFN-γ-producing CD4+ T cells was also significantly different (live, 1564 ± 243 vs. killed, 736 ± 104; P < 0.05). Taken together, these results show that the nature of secondary anti-Leishmania immune responses recalled by live and killed parasites are qualitatively and quantitatively different.

Inoculation of Killed Parasites into Healed Mice Leads to Expansion of IL-10-Producing Regulatory T Cells at the Lymph Node Draining the Infection Site. Because Leishmania-specific, IL-10-producing CD4+CD25+Foxp3+ T cells (Tregs) have been shown to mediate parasite persistence and disease reactivation in healed mice under varying conditions (10, 18–20), we hypothesized that these cells play a key role in suppressing IFN-γ recall response and disrupting resistance in healed mice inoculated with killed parasites. The percentage and absolute number of CD4+CD25+ T cells in the dLNs of healed mice inoculated with killed parasites were 2- to 3-fold higher (P < 0.05) than those from mice injected with only PBS or live parasites (Fig. 3A and B), and these cells had a significantly higher MFI than those from mice given PBS or live parasites (336 ± 98, 472 ± 78, and 667 ± 104, P < 0.05, for PBS, live parasites, and killed parasites, respectively). Furthermore, the absolute numbers of CD4+CD25+Foxp3+ cells were 2- to 3-fold higher (P < 0.05) in mice inoculated with killed parasites than in those given PBS or live parasites (Fig. 3C). In addition, the percentages (Fig. S3 A–C) and absolute numbers (Fig. 3) of CD4+CD25+, CD4+CD25+IL-10+ and CD25+Foxp3+IL-10+ cells were 3- to 5-fold higher (P < 0.05) than in mice inoculated with killed parasites. Collectively, these results show that inoculation of killed parasites into healed mice leads to rapid expansion of CD25+Foxp3+IL-10+ T cells in the lymph nodes draining the primary infection sites.

Administration of Anti-CD25 or Anti-IL-10 mAb Before Inoculation of Killed Parasites Abolishes the Disruption of Infection-Induced Immunity in Healed Mice. To investigate the role of IL-10-producing CD4+CD25+Foxp3+ T cells in the loss of infection-induced resistance, we treated healed mice with depleting anti-CD25 mAb (clone PC61) or anti-IL-10R blocking mAb 1 day before inoculation with killed parasites and challenged treated mice with virulent.
**L. major** 7 days later. Whereas treatment with anti-IL-10R mAb had no effect on DTH, mice treated with anti-CD25 mAb had reduced DTH response (Fig. S4). Importantly, although mice inoculated with killed parasites and treated with control mAbs lost their immunity and had >1,000-fold higher parasite burden than their PBS controls ($P < 0.05$), mice treated with depleting anti-CD25 (>85% depletion of CD25+ cells) or anti-IL-10R mAb effectively controlled parasite proliferation (Fig. 4A). Treatment of naive mice with anti-CD25 or anti-IL-10R mAbs did not enhance their ability to control parasite burden (Fig. 4B), suggesting that the effects of these mAbs in healed mice were most likely related to depletion of the expanded CD25+IL-10+ population after inoculation of killed parasites.

Because healed mice inoculated with killed parasites also had significantly lower numbers of antigen-specific, IFN-γ-producing CD8+ T cells than those injected with live parasites (Fig. S2), we investigated whether this inability of killed parasites to recall *Leishmania*-specific CD8+ T cells contributed in part to the observed loss of infection-induced immunity. Depletion of CD8+ T cells in healed mice before injection with virulent or live-attenuated parasites did not affect the nature of cytokine response or resistance to virulent *L. major* challenge (Fig. S4). Taken together, these results show that increased expansion of IL-10-producing CD4+CD25+ T cells in healed mice after injection of killed parasites contributes to the loss of infection-induced resistance in these mice.

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**Fig. 2.** Inoculation of live and killed *L. major* elicits qualitatively different recall responses in healed mice. Healed mice were inoculated with live parasites, killed parasites, or PBS, and after 3 days, cells from the dLNs were labeled with CFSE dye, stimulated with SLA (50 μg/mL) for 5 days, and analyzed by flow cytometry (A). In some experiments, cells were cultured for only 3 days, and the culture supernatants fluids were assayed for IFN-γ (B), IL-4 (C), and IL-10 (D) by ELISA. Results are representative of three to four independent experiments (n = 4–5 mice per group) with similar results. Error bars show SEM. *, $P < 0.05$; **, $P < 0.01$.

**Fig. 3.** Expansion of IL-10-producing CD4+CD25+Foxp3+ T cells in the dLNs of healed mice inoculated with killed parasites. Direct ex vivo percentages (A) and absolute numbers (B) of CD4+CD25+ T cells in the dLNs of healed mice injected with PBS or inoculated with live or killed *L. major*. In some experiments, cells were directly stimulated ex vivo with PMA, ionomycin, and BFA for 5 h and stained intracellularly for Foxp3 and IL-10, and the absolute numbers of CD4+CD25+Foxp3+ (C) and CD25+IL-10+ (D), as well as the percentage of CD25+Foxp3+IL-10+ (E) and absolute numbers (F) cells were calculated. Results are representative of three (A–D) and two (E and F) independent experiments (n = 4–5 mice per group) with similar results. Error bars show SEM. *, $P < 0.05$; **, $P < 0.01$. 

**A**

- PBS
- Live
- Killed

**B**

- IFN-γ (pg/mL)
- IL-4 (pg/mL)

**C**

- IL-10 (pg/mL)

**D**

- CFSE

**E**

- CD4+CD25+ (10^5)
- Foxp3+ (10^6)

**F**

- CD25+IL-10+ (10^6)
- Foxp3+IL-10+ (10^6)
Inoculation of Live Avirulent L. major Does Not Induce Expansion of CD4+ CD25+ T Cells and Loss of Infection-Induced Resistance in Healed Mice. Next, we assessed the impact of two attenuated parasite strains, lpg2^- L. major (which does not induce pathology but resembles wild-type parasites because it persists indefinitely in infected mice) (21) and thymidine auxotroph, dhfr-ts^- L. major (which initially infects cells but is completely eliminated after 8 weeks) (22) on infection-induced resistance. Inoculation of virulent [Freidlin strain (FN)], attenuated (lpg2^- or dhfr-ts^-), or killed parasites into healed mice induced comparable DTH responses, consistent with previous observations (Fig. S5A). However, the percentages and absolute numbers of CD4^+ CD25^+ cells in the dLNs of mice inoculated with killed parasites were 2- to 3-fold higher (P < 0.05) than in those injected with virulent (FN) or attenuated (lpg2^- or dhfr-ts^-) L. major (Fig. 5A and B). In addition, upon in vitro stimulation with SLA, the percentages of proliferating CD4^+ Foxp3^+ and CD4^+ CD25^+ cells were higher in healed mice injected with killed parasites, and these cells underwent more divisions (more shift to the left, 32% vs. 12% for killed and dhfr-ts, respectively) than those from mice inoculated with live parasites (Fig. S5 B and C). Importantly, unlike killed parasites, injection of live-avirulent parasites (lpg2^- and dhfr-ts^-) did not result in loss of infection-induced resistance (Fig. 5C). Taken together, these results suggest that there is something inherent in killed parasites that causes expansion of CD4^+ CD25^+Foxp3^+ T cells and a concomitant loss of infection-induced resistance in healed mice.

Killed Trypanosoma congolense Does Not Elicit Expansion of CD4^+ CD25^+ T Cells or Induce Disruption of Immunity in Mice That Healed Primary L. major Infection. To determine whether expansion of CD4^+ CD25^+ Foxp3^+ T cells in healed mice inoculated with killed L. major is a general property of all killed protozoan parasites or is specific to Leishmania antigen, we inoculated healed mice with killed L. major and a related parasite, T. congolense, and after 3 days we measured DTH response and determined the numbers of CD4^+ CD25^+ cells in the dLNs. In contrast to killed L. major, which elicited a robust DTH response, killed T. congolense did not elicit significant DTH response in healed mice (Fig. 6A). Similarly, only mice given killed L. major had 2- to 3-fold elevation (P < 0.05) in the numbers of CD4^+ CD25^+ T cells in their dLNs (Fig. 6B) and were susceptible to virulent L. major challenge (Fig. 6C), suggesting that the induction of CD4^+ CD25^+ and loss of infection-induced resistance by killed parasites may be a Leishmania-specific-driven process. Furthermore, only Leishmania antigens (but not T. congolense antigens) were capable of inducing enhanced expression of Foxp3 and IL-10 on cells from the dLNs of healed mice injected with killed L. major, and these cells proliferated vigorously in response to SLA stimulation in vitro (Fig. S6). Together, these results suggest that the increased numbers of CD4^+ CD25^+ Foxp3^+ T cells in the dLNs of healed mice inoculated with killed L. major may be due to proliferative expansion of a subpopulation of T reg responding specifically to L. major antigens.
In this study, we evaluated the impact of live and killed parasites on immunity acquired after resolution of primary virulent L. major infection. We show that inoculation of killed parasites into immune mice leads to rapid and relatively sustained loss of infection-induced resistance, as evidenced by significantly higher parasite burden upon secondary challenge. Interestingly, the quality of secondary immunity recalled by killed parasites in healed mice was dramatically different from that recalled by live parasites. Live parasites recalled predominantly early and sustained IFN-γ response, leading to maintenance of infection-induced immunity. In contrast, killed parasites induced expansion of IL-10-producing CD4⁺ CD25⁺ Foxp3⁺ T cells. Injection of anti-CD25 or anti-IL-10R mAb before inoculation of killed parasites abolished the disruption of infection-induced immunity, implicating regulatory T cells and IL-10 in this process.

To our knowledge, rapid loss of anti-Leishmania immunity in the face of parasite persistence has not been previously described. Loss of infection-induced immunity has been linked previously to manipulations that result in complete parasite clearance (10, 11). The sustained loss of immunity (>5–10 weeks) after inoculation of killed parasites suggests this could not have been related to a nonspecific effect of host innate immune response to killed parasites. Indeed, we found that killed and live parasites induced comparable early inflammatory responses in healed (Fig. 2A and Fig. S1) and naive mice (23). Furthermore, inoculation of killed parasite did not cause clearance of persistent parasites in healed mice, but resulted in expansion of IL-10-producing CD4⁺ CD25⁺ Foxp3⁺ cells, leading to reactivation of primary infection and loss of resistance. Thus, the data presented here indicate that although persistent parasites are necessary, they are by themselves not sufficient to maintain infection-induced immunity in healed animals, particularly in response to killed parasite inoculation. They further suggest that after recovery from primary infection, a critical but delicate balance is established between regulatory T cells and persistent parasites for maintenance of infection-induced immunity, and perturbing each of these components could lead to sterile cure or loss of immunity and reactivation disease.

The rapid expansion (within 3–7 days) of CD4⁺ CD25⁺ cells and coexpression of FoxP3 only in healed mice inoculated with killed but not live virulent or attenuated (lpq2⁻ or dhfr-ts⁻) parasites suggest these cells are not derived from activated effector cells responding to their cognate antigen. In addition, the sustained loss of immunity after inoculation of killed parasites indicates the expanded T regs persist over a long period. Furthermore, only Leishmania antigens (but not T. congolense antigens) were capable of inducing expansion in vivo and in vitro of FoxP3⁺ and IL-10⁺ cells in healed mice inoculated with killed L. major (Fig. S6). This is consistent with previous reports showing that T regs from healed mice could be expanded with leishmanial antigen in vitro (19), are the major producers of IL-10 in L. major-infected mice, and play a critical role in disease reactivation and recrudescence (10). In contrast, Anderson et al. (24) found CD4⁺ Tbet⁻ IFN-γ⁻ cells were the major producers of IL-10 in chronic L. major infection. These discrepancies may be related to differences in parasite strains; Anderson et al. (24) used a strain of L. major that causes chronic, nonhealing disease, whereas we and others used Friedlin, a strain that induces the classic healing disease in C57BL/6 mice. In line with this, previous studies by the same laboratory that used FN implicated CD4⁺ CD25⁺ FoxP3⁺ cells as the major IL-10 producers in infected mice (10). In addition, our finding that live parasites do not expand T regs differs from that reported by Mendez et al. (18). Although this report did not examine the effect of killed parasites (making direct comparisons impossible), we think the differences may arise from various technical sources, such as the site studied (primary site vs. dLNs in our study) and/or viability of the parasite inocula. A recent study suggests viability can be a factor in parasite virulence (25). The parasites used in our study were more than 95% viable, whereas this information was not provided by Mendez et al. (18).

An interesting but surprising finding was the inability of CpG ODN and bacillus Calmette–Guerin to abrogate killed parasite-induced loss of immunity. In particular, CpG had been shown to act directly on CD4⁺ T effector cells, thereby making them resistant to suppressive effects of T regs (26). It is plausible that T regs in our model may be acting directly on infected macrophages to cause their alternative activation, and thereby inhibit their leishmanicidal ability (27). In line with this, we found there was no difference in DTH and antigen-specific T-cell proliferation in vitro and in vivo in mice inoculated with killed and live parasites, despite the significant differences in percentages and absolute numbers of regulatory T cells. Alternatively, it is possible that in addition to T regs, other mechanisms contribute to killed parasite-induced loss of immunity.

Several vaccination trials have been conducted in several parts of the world using heat-killed whole Leishmania with or without bacillus Calmette–Guerin as immunogen, with disappointing results (15, 28). The failure of heat-killed Leishmania vaccine to induce significant protection or durable immunity has been attributed to its inability to induce sustained anti-Leishmania memory cells (3, 29). Many people in endemic regions who would be classified as healthy likely have asymptomatic infections through natural infection with L. major naturally by infected sand fly bite. A majority of these individuals will present little or no evidence of clinical disease and will subsequently develop strong infection-induced resistance (akin to our healed mice) (13, 30). As in mice, primary infection with L. major in human results in persistence of parasites and accumulation of natural regulatory T cells at the site of infection (31). Therefore, vaccinating these individuals with heat-killed L. major could lead to expansion of these Leishmania-specific T regs, which could abolish the induction of protective immunity or result in loss of natural infection-induced resistance, thereby rendering these individuals susceptible to new infections or reactivation of latent infections. Although mice inoculated with killed parasites showed some protection over naive mice when challenged after 10 weeks, the complete loss of immunity (at 2 and 5 weeks) and significantly impaired protection at 10 weeks indicate there is a window when exposure to killed parasites leads to complete or partial breakdown of immunity, which could predispose to susceptibility to new infections.

In summary, we have shown that inoculation of killed L. major in healed mice leads to a rapid expansion of IL-10-producing CD4⁺ CD25⁺ cells resulting in rapid and sustained loss of infection-induced resistance. A proposed model to explain this loss of resistance is presented in Fig. S7. Although the effects of killed parasites have not been tested in humans, it is conceivable that vaccination with heat-killed Leishmania could produce similar effects, particularly in endemic areas where many people with subclinical infections (LST⁺) akin to infection-induced resistance in mice exist.

Materials and Methods

Mice. Female C57BL/6 mice (6–8 weeks old) were obtained from the University of Manitoba Central Animal Care Services breeding facility. All mice were maintained in a specific pathogen-free environment, and all experiments were approved by institutional ethics committee in accordance with the Canadian Council for Animal Care guidelines.

Parasites and Infections (Primary and Challenge Infections). L. major parasites [MHOM/IL/80/Friedlin (FN)] were grown in Grace insect medium (Invitrogen, Life Technologies) supplemented with 20% heat-inactivated FBS, 2 mM-l-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 25 mM Hepes (complete parasite medium). The strains and method of derivation of the attenuated (avirulent) LPG2-deficient L. major (lpq2⁻) and conditional auxotrophic L. major (dhfr-ts⁻) have been described previously (21, 22). The dhfr-ts parasites were grown in complete parasite medium supplemented with 10 μM thymidine and 10 μM mycophenolic acid.
and some cells were then used for intracellular cytokine staining as described 72 h, the supernatant fluids were collected and assayed for cytokines by ELISA, glutamine, 100 units/mL penicillin, 100 g/mL). After 72 h, the supernatant fluids were collected and assayed for cytokines by ELISA, and some cells were then used for intracellular cytokine staining as described previously (32). Some cells were labelled with CFSE dye as described previously (32), cultured in 96-well, round-bottom culture plates (2 × 10^5 per well in 200-μL aliquots) with SLA or freeze-thawed L. major or T. congolense (10^5 per milliliter) for 5 days, and routinely stained for CD4, CD25, IL-10, and Foxp3 expressions. Stained cells were acquired on a FACS discriminatory machine and analyzed by using CellQuest Pro (BD Biosciences).

**Cytokine ELISAs.** The concentrations of IL-4, IL-10, and IFN-γ in the culture supernatant fluids were determined by sandwich ELISAs using Ab pairs purchased from BD Biosciences according to the manufacturer’s suggested protocols.

**DTH Measurement and Determination of Parasite Burden.** DTH in the primary or challenge infection site was determined in immune mice inoculated with live or killed L. major parasites at 72 h after inoculation (challenge) by measuring footpad swelling by using digital calipers (Fisher Scientific). Parasite burden was determined by limiting-dilution assay, as described previously (33).

**Depletion of CD25+ Cells and Blockade of IL-10 Signaling in Vivo.** Age-matched naive and healed mice were injected (i.p.) with 100 μg of anti-CD25 mAb (PC61) or anti-IL-10 mAb at day −1 before inoculation with killed parasites and then once weekly for 3 weeks (for anti-IL-10R). Previous dose titration studies indicate this concentration of PC61 mAb was sufficient to deplete between 85% and 95% of CD4+ CD25^high cells for more than 7 days.

**Statistical Analysis.** Two-tailed Student’s t test was used to compare mean and SEM between two groups. In some other experiments, a one-way ANOVA (non-parametric) was used to compare mean and SEM of more than two groups. Tukey’s test was further used when the ANOVA test revealed significant differences. Differences were considered significant at P < 0.05.

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