

Chronic *Leishmania donovani* Infection Promotes Bystander CD8⁺-T-Cell Expansion and Heterologous Immunity

Rosalind Polley,[†] Stephanie L. Sanos,[‡] Sara Prickett,[§] Ashraful Haque,
and Paul M. Kaye*

Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine,
London WC1E 7HT, United Kingdom

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It has been proposed that long-lived memory T cells generated by vaccination or infection reside within a memory compartment that has a finite size. Consequently, in a variety of acute infection models interclonal competition has been shown to lead to attrition of preexisting memory CD8⁺ T cells. Contrary to expectations, therefore, we found that chronic *Leishmania donovani* infection of *Listeria*-immune mice results in heightened protection against subsequent *Listeria* challenge. This protection was associated with bystander expansion of *Listeria*-specific CD8⁺ T cells and a bias in these cells toward a central memory T-cell phenotype with an enhanced capacity for gamma interferon production. We propose that splenomegaly, which is characteristic of visceral leishmaniasis and other tropical infections, may help promote heterologous immunity by resetting the size of the memory compartment during chronic infection.

Memory CD8⁺-T-cell responses play an essential role in mediating natural and vaccine-induced immunity to a range of important human pathogens, and the dynamics of the CD8⁺-T-cell response to a number of prototypic microbial infections has been extensively studied. Activation of naïve CD8⁺ T cells results in rapid and extensive clonal expansion, followed by a clonal contraction phase. Subsequently, memory CD8⁺ T cells persist at a higher frequency than their naïve precursors (at approximately 5% of the maximal clone size) and occupy space in a finite memory compartment (1, 7). The mechanisms regulating memory cell persistence in this memory compartment are varied. For example, whereas cross-reactivity can promote the survival of preexisting memory CD8⁺ T cells (18–20, 22), clonal competition in the absence of cross-reactivity can promote attrition of preexisting memory CD8⁺ T cells (4, 19). In addition, inflammatory cytokines are also thought to play an important role in regulating memory CD8⁺-T-cell persistence (21). In the latter case, recent mathematical modeling suggests that clonal contraction should occur rapidly to maintain homeostasis (1).

In contrast to these studies with acute infection models, memory cell behavior during chronic infection is less well understood. A recent study demonstrated that in contrast to mice with acute lymphocytic choriomeningitis virus infection, mice with a chronic infection do not develop memory CD8⁺ T cells with the capacity for long-term antigen-independent persis-

ence. Memory CD8⁺ cells in these mice exhibit reduced expression of interleukin-7 (IL-7) and IL-15 receptors and fail to undergo homeostatic proliferation (24). However, the impact of chronic infection on the persistence of preexisting host-protective memory CD8⁺ T cells has not been described previously. Here, we demonstrate that *Leishmania donovani* infection of *Listeria*-immune mice results in significantly enhanced protection against lethal challenge and long-term bystander expansion of *Listeria*-specific memory CD8⁺ T cells. We propose that infection-associated splenomegaly may reset the size of the memory compartment to avoid interclonal competition and allow the maintenance of preexisting immunity.

MATERIALS AND METHODS

Mice and pathogens. BALB/c mice were obtained from Charles River U.K. (Margate, United Kingdom) and were maintained under barrier conditions at the Biological Services Unit, London School of Hygiene and Tropical Medicine. Female mice that were 8 to 12 weeks old were used in all experiments. *L. donovani* amastigotes (LV9) were obtained from the spleens of infected Syrian hamsters and were isolated as described elsewhere (6). Mice were infected intravenously via the lateral tail vein with 2×10^7 amastigotes. *Listeria monocytogenes* was grown overnight in static culture at 37°C in tryptone soya agar (TSA) broth (Oxoid, United Kingdom). Bacteria were then washed twice in phosphate-buffered saline before resuspension in 30% glycerol-phosphate-buffered saline for storage at –70°C. For vaccination and challenge, bacterial stocks were freshly thawed and serially diluted in sterile saline. The injected dose was confirmed by plating on TSA plates. All animal procedures were approved by the London School of Hygiene and Tropical Medicine Animal Procedures Ethical Committee and were subject to United Kingdom Home Office Regulations.

Vaccination and challenge. Mice were inoculated with a vaccinating nonlethal dose containing $\sim 5 \times 10^4$ CFU *Listeria*, after which bacilli were cleared within 2 weeks (9; data not shown). At week 6 after vaccination, groups of mice ($n = 12$) were infected with *L. donovani* or retained as controls. Approximately 10 weeks later, groups of mice were killed to assess the fate of *Listeria*-specific CD8⁺ T cells or were challenged with 6×10^5 to 8×10^5 CFU *Listeria*, and the levels of protection were determined on day 3 after challenge. Naïve mice infected with this dose did not survive beyond day 3 (data not shown). Livers and spleens were removed and homogenized in fixed volumes of sterile saline and RPMI, respectively, and serial dilutions were plated in triplicate on TSA plates. After incubation for 24 h at 37°C, the number of colonies was determined, and total number of organ CFU was calculated.

* Corresponding author. Present address: Immunology and Infection Unit, Department of Biology, University of York, PO Box 373, York YO10 5YW, United Kingdom. Phone: 44 1904 328840. Fax: 44 1904 328844. E-mail: pmk2@york.ac.uk.

[†] Present address: Lymphocyte Biology Section, Laboratory of Immunology, NIAID/National Institutes of Health, Bethesda, MD 20892.

[‡] Present address: Skirball Institute of Biomolecular Medicine, NYU School of Medicine, 540 First Avenue, New York, NY 10016.

[§] Present address: Infection and Immunity, Walter and Eliza Hall Institute for Medical Research, 1G Royal Parade, Parkville, Victoria 3050 Australia.

In vitro restimulation of *Listeria*-immune CD8⁺ T cells. Dendritic cells (DC) were obtained by culturing BALB/c or C57BL/6 bone marrow cells in granulocyte-macrophage colony-stimulating factor for 7 days by using conventional protocols. Dendritic cells were plated in six-well plates and were infected at a multiplicity of infection of 10:1 with either *Listeria* or amastigotes of *L. donovani* OVA, a transgenic line of *L. donovani* expressing chicken ovalbumin (OVA) (15a). When appropriate, control cultures received 5 μg/ml LLO₉₁₋₉₉ or OVA₂₅₇₋₂₆₄. *Listeria* infection was terminated using 50 μg/ml gentamicin. After 4 h, DC were irradiated (3,000 R) and then plated overnight. CD8⁺ T cells were isolated from spleens of *Listeria*-immune mice by using magnetic sorting and were added to DC at a 10:1 ratio. In control experiments, OVA-specific OT-1 T cells were used. Proliferative responses were assessed at day 3 using thymidine incorporation, and gamma interferon (IFN-γ) production by bulk CD8⁺ T cells and by tetramer-positive CD8⁺ T cells was determined on day 3 following incubation for 5 h in the presence of brefeldin A as described below.

Flow cytometry. CD8⁺ cells were positively selected from spleen cell suspensions by magnetic sorting using anti-CD8α (Ly-2) MicroBeads (Miltenyi Biotec). Four-color flow cytometry was performed with spleen cell populations using fluorescein isothiocyanate-conjugated CD8 (Caltag, Burlingame, CA), phycoerythrin-conjugated anti-CD62L (MEL-14), biotinylated anti-CD44 (Pgp-1; detected with streptavidin-PerCP), allophycocyanin (APC)-conjugated anti-IFN-γ (XMG1.2), or APC-conjugated rat immunoglobulin G1 as an isotype control (all obtained from BD Pharmingen, Oxon, United Kingdom). H2K^d/LLO₉₁₋₉₉ tetramers were obtained from Proimmune (Oxford, United Kingdom) and used to detect LLO₉₁₋₉₉-specific CD8⁺ T cells. For intracellular detection of IFN-γ, spleen cells (1 × 10⁷ cells/ml) were restimulated in the presence or absence of 5 μg/ml of LLO₉₁₋₉₉ (Proimmune) for 1 h, followed by 4 h in the presence of 10 μg/ml brefeldin A (Sigma). Data were collected for >50,000 events using a FACSCalibur and were analyzed using the Cell Quest II software (BD). All mice (four to six mice per group per experiment) were analyzed individually.

Statistical analysis. Statistical analysis was performed using Student's *t* test, and a *P* value of <0.05 was considered significant. Each experiment was repeated independently at least twice, with similar results.

RESULTS AND DISCUSSION

To determine how *L. donovani* infection affected established CD8⁺-T-cell-dependent protective immunity to a heterologous pathogen, we first vaccinated mice with a sublethal dose of *L. monocytogenes*, prior to *L. donovani* infection. When chronic *L. donovani* infection was well established, mice were challenged with an otherwise lethal dose of *Listeria*. Control vaccinated mice were significantly, although moderately, protected in terms of tissue CFU (Fig. 1A). Similar levels of protection were also observed in control unvaccinated mice infected with *L. donovani* (Fig. 1A and B), most likely as a result of enhanced local macrophage effector function (14) or bystander cytokine-dependent activation of irrelevant memory CD8⁺ T cells (2, 12). In contrast, *Listeria*-immune mice infected with *L. donovani* showed remarkably high levels of protection. In the spleen, the number of CFU was reduced to undetectable levels (Fig. 1A), whereas in the liver, the protection was ~1.5 log greater than that seen either in *L. donovani*-infected, *Listeria*-naïve mice or in control vaccinated mice (Fig. 1B). These data demonstrate that *L. donovani* infection both enhanced innate immunity to *Listeria* challenge and also significantly augmented vaccine-induced protection.

During chronic *L. donovani* infection, mice exhibited marked splenomegaly (Fig. 2A) that was accompanied by an increase in the number of total spleen memory CD44^{hi} CD8⁺ T cells (2.6 × 10⁵ ± 0.3 × 10⁵ and 21 × 10⁵ ± 8 × 10⁵ cells in naïve and chronically infected mice, respectively; *P* < 0.005). To determine whether this expansion of memory CD8⁺ T cells had an effect on preestablished CD8⁺ *Listeria*-specific memory CD8⁺ T cells, we determined the numbers of H2K^d/LLO₉₁₋₉₉ tetramer-positive cells (3). *Listeria*-immune mice had signifi-

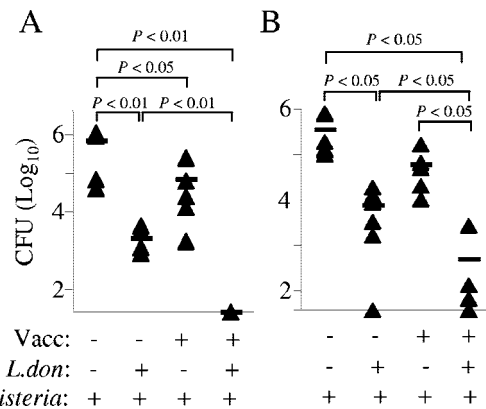


FIG. 1. *L. donovani* infection enhances protection against *Listeria*. *Listeria*-naïve or *Listeria*-immune BALB/c mice were infected with *L. donovani* and at 68 days after infection were challenged with 6 × 10⁵ CFU *Listeria*. The bacterial burdens in the spleens (A) and livers (B) were determined by colony counting as described in Materials and Methods. Each symbol represents an individual mouse (five or six mice per group), and statistical significance between groups was determined using Student's *t* test. Vacc, vaccination; *L.don*, *L. donovani*.

cantly greater numbers of tetramer-positive cells than *Listeria*-naïve mice (Fig. 2B). More importantly, almost fivefold more tetramer-positive cells could be recovered from *L. donovani*-infected, *Listeria*-immune mice than from mice that were not infected with *L. donovani* (Fig. 2B). Thus, *L. donovani* infection, rather than causing attrition of preexisting CD8⁺ memory T cells, appeared to favor bystander expansion. To rule out the possibility that tetramer-positive cells in *L. donovani*-infected mice merely had a greater potential for proliferation than the cells in noninfected mice, we also determined the number of

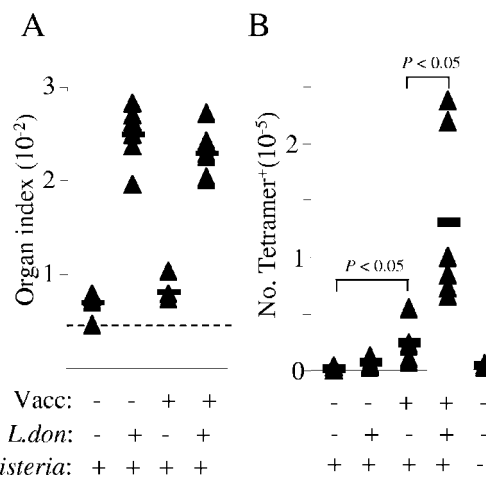


FIG. 2. *L. donovani* infection causes splenomegaly and expansion of LLO-specific CD8⁺ T cells. Spleen cells were isolated from groups of mice that were treated 3 days after *Listeria* challenge, enriched for CD8⁺ T cells by magnetic sorting, and then analyzed for binding of the H2K^d/LLO₉₁₋₉₉ tetramer. The degree of splenomegaly (A) was calculated relative to body weight. The dashed line indicates the mean value for control uninfected and nonvaccinated mice. The tetramer data are expressed as the absolute number of tetramer-positive CD8⁺ T cells in the spleen (B). Each symbol represents an individual mouse (five or six mice per group), and statistical significance between groups was determined using Student's *t* test. Vacc, vaccination; *L.don*, *L. donovani*.

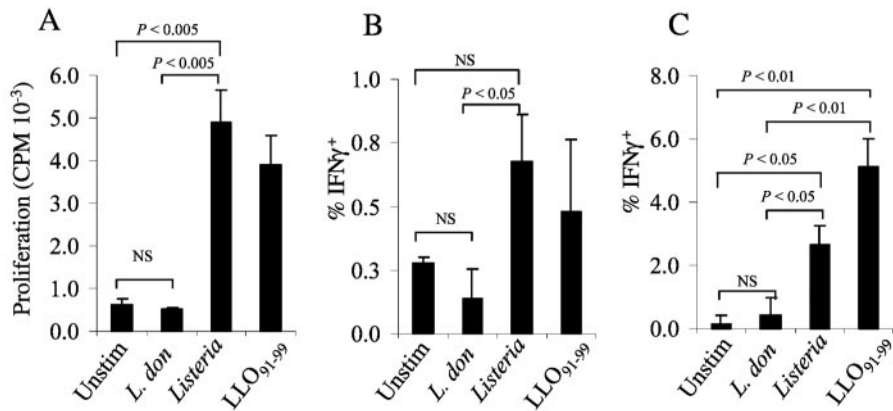


FIG. 3. *Listeria*-specific CD8⁺ T cells do not recognize *L. donovani*-infected dendritic cells. CD8⁺ T cells were isolated from *Listeria*-immune BALB/c mice and stimulated with dendritic cells infected with either *Listeria* or *L. donovani* OVA (multiplicity of infection, 10:1). Proliferation (A) and IFN-γ secretion (B) of CD8⁺ T cells were determined on day 3. (C) CD8⁺ T cells gated on tetramer staining prior to analysis. Unstim, unstimulated; *L. don*, *L. donovani*.

tetramer-positive cells in mice before challenge with *Listeria*. The spleens of control *Listeria*-immune mice contained $10.3 \times 10^3 \pm 2.24 \times 10^3$ tetramer-positive cells, whereas the spleens of *L. donovani*-infected, *Listeria*-immune mice contained $44 \times 10^3 \pm 10.16 \times 10^3$ tetramer-positive cells ($P < 0.05$; $n = 4$). Together, these experiments demonstrated for the first time that *L. donovani* infection expands a population of memory CD8⁺ T cells specific for a heterologous pathogen.

Although the number of tetramer-positive cells was not increased by *L. donovani* infection of naïve mice, it was still possible that LLO₉₁₋₉₉-specific memory CD8⁺ T cells might show some cross-reactivity with *L. donovani* that was more readily expressed due to the lower threshold for activation of memory cells than for activation of naïve T cells. To address this possibility, we assessed the capacity of CD8⁺ T cells from *Listeria*-immune mice to recognize either *Listeria*- or *L. donovani*-infected dendritic cells in vitro. Whereas bulk populations

of CD8⁺ T cells proliferated (Fig. 3A) and secreted IFN-γ (Fig. 3B) in response to *Listeria*-infected dendritic cells and to LLO₉₁₋₉₉-pulsed dendritic cells, no such response was evident with dendritic cells infected with a transgenic line of *L. donovani* expressing OVA. Furthermore, tetramer-positive cells also failed to respond to these *L. donovani*-infected dendritic cells (Fig. 3C). The lack of response in these cultures was not due to a failure in class I presentation, as DC infected with these transgenic *L. donovani* cells were capable of stimulating responses in OVA-specific class I-restricted OT-1 cells (data not shown). Based on these findings together with the in vivo data described above, we concluded that there is not significant recognition of *L. donovani* either by bulk *Listeria*-specific CD8⁺ T cells or by the LLO₉₁₋₉₉-specific tetramer-positive cells that expand during chronic *L. donovani* infection of *Listeria*-immune mice.

Recently, memory T cells have been subdivided on the basis

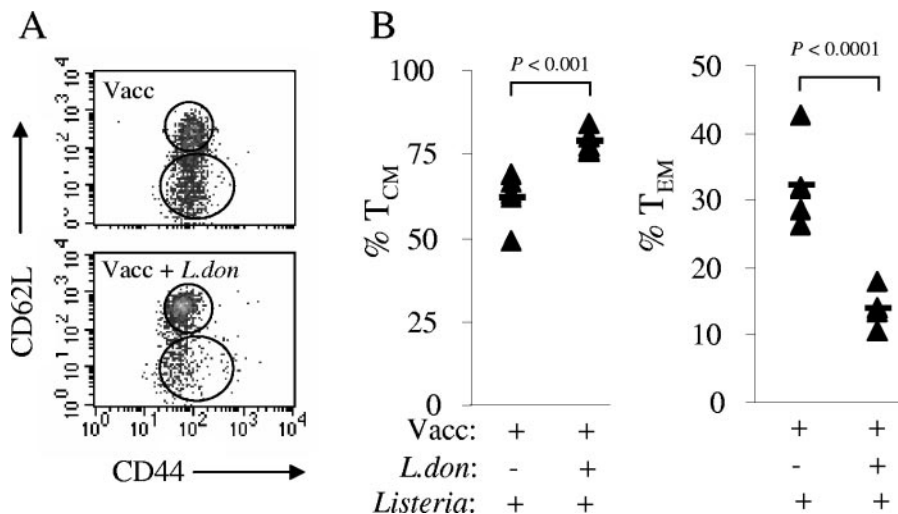


FIG. 4. *L. donovani* infection alters the balance of LLO-specific T_{EM} and T_{CM} cells. CD8⁺ T cells from groups of mice were isolated before and after *Listeria* challenge, stained with the H2K^b/LLO₉₁₋₉₉ tetramer, and analyzed for expression of CD44 and CD62L. (A) Representative dot plots. (B) Data for each group of mice. Each symbol represents an individual mouse (five or six mice per group), and statistical significance between groups was determined using Student's *t* test. Vacc, vaccination; *L. don*, *L. donovani*.

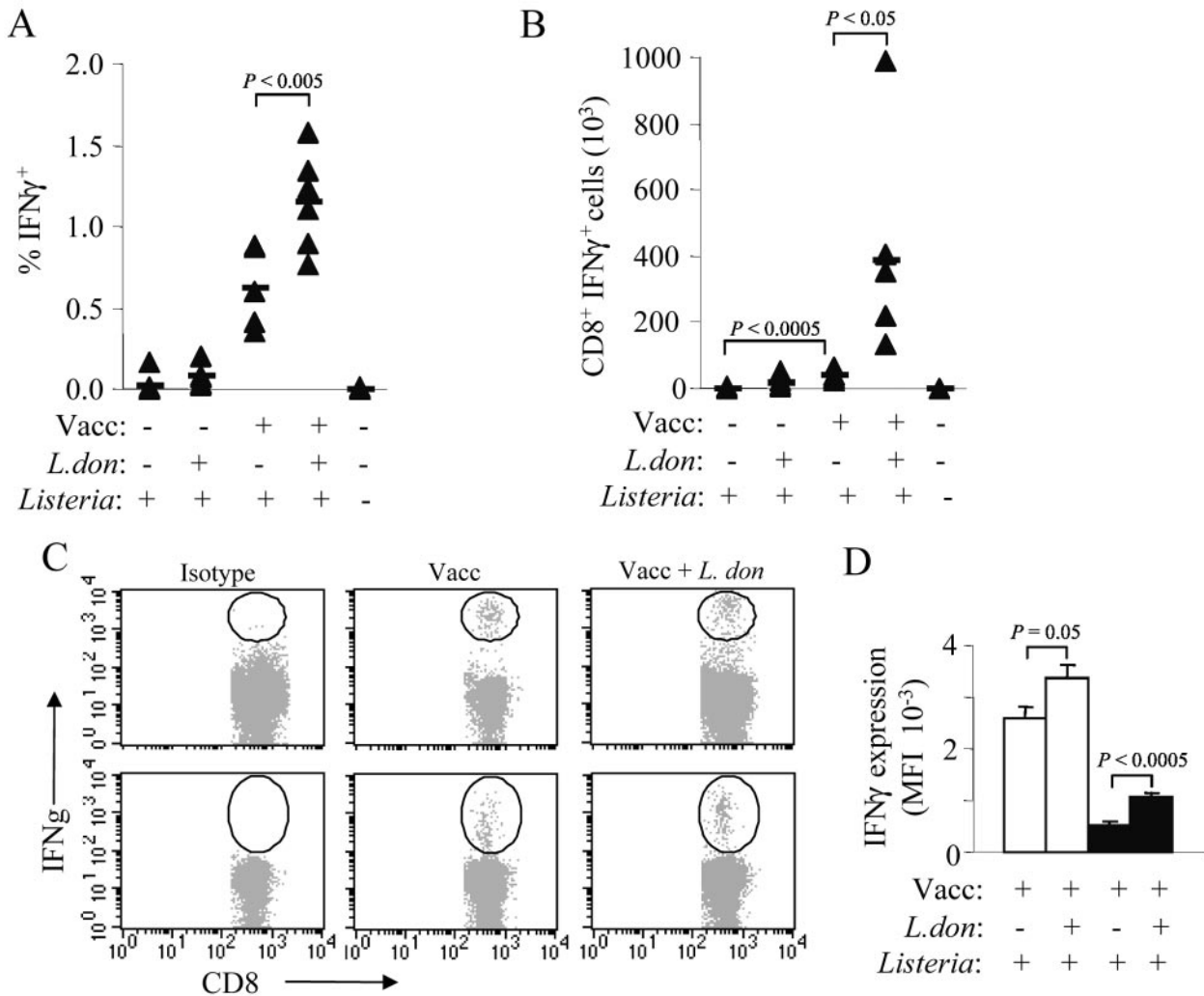


FIG. 5. IFN- γ production by LLO-specific CD8⁺ T cells. Bulk splenocytes were purified from individual mice in groups and restimulated in vitro with LLO₉₁₋₉₉ in the presence of brefeldin A. The frequency (A) and absolute number (B) of IFN- γ -positive CD8⁺ T cells are shown. (C) Representative dot plots showing the IFN- γ levels produced by CD8⁺ T cells taken from mice before challenge (top panels) and after challenge (bottom panels) with *Listeria*. Data for all mice analyzed in this way before challenge (open bars) and after challenge (solid bars) are shown in panel D. The bars indicate IFN- γ production expressed as the mean fluorescence intensity (MFI), and the error bars indicate standard deviations (four to six mice per group). Data were analyzed using Student's *t* test. Vacc, vaccination; *L. don*, *L. donovani*.

of CD44 and CD62L expression into two subsets. CD44^{hi} CD62L^{lo} T effector memory (T_{EM}) cells represent cells that traffic to inflammatory sites and act as rapid effectors after reexposure to antigen (15, 23). In contrast, CD44^{hi} CD62L^{hi} T central memory (T_{CM}) cells have the capacity to migrate into peripheral lymphoid tissues (16). The responsiveness of these two subsets of memory cells to cytokine-driven bystander proliferation has been partially characterized in vitro (8, 25). However, the impact of chronic infection on the relative frequencies of heterologous antigen-specific T_{EM} and T_{CM} cell populations in vivo has not been documented previously. Examination of tetramer-positive cells in *Listeria*-immune mice indicated that approximately 60% were T_{CM} cells, as defined by CD44 and CD62L staining. However, in *Listeria*-immune mice infected with *L. donovani*, the frequency of T_{CM} cells was almost 80%, and reciprocal changes were observed in the T_{EM}-

cell population (Fig. 4A and B). In absolute terms, the number of tetramer-positive T_{CM} cells was approximately sevenfold greater in *L. donovani*-infected, *Listeria*-immune mice than in control *Listeria*-immune mice ($102 \times 10^3 \pm 27 \times 10^3$ and $14.9 \times 10^3 \pm 6.4 \times 10^3$ cells, respectively; $P < 0.05$; $n = 5$ or 6). Thus, *L. donovani* infection significantly skewed the LLO₉₁₋₉₉-specific memory CD8⁺-T-cell pool toward a T_{CM} phenotype. A number of possibilities could explain this finding: the greater potential for homeostatic proliferation of T_{CM} cells than of T_{EM} cells (25) may be maintained in response to cytokines present in the inflammatory environment caused by *L. donovani* infection (6); the rate of conversion from T_{EM} cells to T_{CM} cells (25) might be enhanced in the environment of the *L. donovani*-infected spleen, by factors independent of the turnover rate; or tetramer-positive T_{EM} cells may have been preferentially recruited from the spleen to other sites of *L. dono-*

vani infection (e.g., the liver). Further studies are required to differentiate between these possibilities.

To assess the functional capacity of tetramer-positive cells expanded as a consequence of *L. donovani* infection, we restimulated spleen cells with 5 µg/ml LLO₉₁₋₉₉ and then determined the frequency of IFN-γ-producing cells by flow cytometry. In nonimmune mice, very few LLO₉₁₋₉₉-specific CD8⁺ T cells were detectable after *Listeria* challenge, whether the mice had been infected with *L. donovani* or not (Fig. 5A and B). However, in *Listeria*-immune mice, LLO₉₁₋₉₉-specific CD8⁺ T cells were readily stimulated in vitro to produce IFN-γ. For spleen cells from *L. donovani*-infected, *Listeria*-immune mice, but not for spleen cells from *L. donovani*-infected, *Listeria*-naïve mice, there was a marked increase in the frequency of LLO₉₁₋₉₉-specific CD8⁺ T cells able to make IFN-γ (Fig. 5A). The impact of *L. donovani* infection on the total capacity of the mice to make IFN-γ in response to LLO₉₁₋₉₉ was even more evident after we adjusted for the total number of CD8⁺ T cells in the spleen (Fig. 5B). The levels of IFN-γ produced by CD8⁺ T cells in response to restimulation with LLO₉₁₋₉₉ were also different in these groups of mice. CD8⁺ T cells from control *Listeria*-immune mice produced significantly more IFN-γ when they were directly restimulated in vitro than CD8⁺ T cells produced when they were restimulated 3 days after challenge infection (Fig. 5C and D). These data may reflect down-regulation of the T-cell receptor and associated molecules on the recently antigen-reactivated memory cells (10). More strikingly, in mice infected with *L. donovani*, LLO₉₁₋₉₉-specific CD8⁺ T cells produced higher levels of IFN-γ than the cells in control *Listeria*-immune mice produced (Fig. 5C and D). Hence, not only does *L. donovani* infection increase the numbers of LLO₉₁₋₉₉-specific CD8⁺ T cells in the spleens of *Listeria*-immune mice, but these cells have a heightened capacity to produce IFN-γ when they are reexposed to antigen.

Concluding remarks. Our results identified a number of features associated with in vivo bystander CD8⁺-T-cell expansion that have not been reported previously. First, we found that infection with *L. donovani* augments vaccine-induced immunity to *Listeria*, expanding and enhancing the effector function of a host-protective LLO₉₁₋₉₉-specific memory CD8⁺-T-cell population. The in vivo regulation of bystander expansion is likely to be both complex and highly dynamic and controlled by cytokines and costimulatory receptor-ligand interactions (4, 5, 11, 13, 26), and many of these parameters remain to be fully evaluated in our model. However, it is known that there is increased accumulation of IL-2 mRNA in mice with chronic *L. donovani* infections (6), and more recent preliminary studies indicate that IL-7 mRNA accumulation was not altered, while IL-15 mRNA accumulation was reduced in the spleens of infected mice compared to the spleens of naïve mice (Polley, unpublished data). Second, our data indicate that as a consequence of *L. donovani* infection, LLO₉₁₋₉₉-specific memory CD8⁺ T cells acquire an enhanced functional capacity, as measured at the single-cell level by intracellular IFN-γ production. The basis for this enhanced responsiveness remains to be determined, but the responsiveness is consistent with previous findings which showed that there was bystander enhancement of IFN-γ production (12). Finally, the increase in heterologous memory CD8⁺ T cells that accompanies *L. donovani* infection was contrary to our expectations based on the concept of a

finite size for the memory pool (7) and evidence from viral infection models indicating that non-cross-reactive infections can cause attrition of preexisting memory (17, 18). In this respect, it is tempting to speculate that the splenomegaly associated with *L. donovani* infection, and indeed with other chronic parasitic infections, such as malaria and schistosomiasis, may override some of the constraints normally imposed on the memory compartment under steady-state conditions, resetting a new level of homeostasis in the enlarged spleen. Given the global burden of these infections, the extent to which chronic or recurrent splenomegaly and/or lymphadenopathy may affect the memory CD8⁺-T-cell compartment clearly warrants further experimental and clinical study.

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