Skin-resident memory CD4+ T cells enhance protection against *Leishmania major* infection

Nelson D. Glennie,[1] Venkata A. Yeramilli,[1] Daniel P. Beiting,[1] Susan W. Volk,[1] Casey T. Weaver,[2] and Phillip Scott[1]

[1]Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104
[2]Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35233

Leishmaniasis causes a significant disease burden worldwide. Although *Leishmania*-infected patients become refractory to reinfection after disease resolution, effective immune protection has not yet been achieved by human vaccines. Although circulating *Leishmania*-specific T cells are known to play a critical role in immunity, the role of memory T cells present in peripheral tissues has not been explored. Here, we identify a population of skin-resident *Leishmania*-specific memory CD4+ T cells. These cells produce IFN-γ and remain resident in the skin when transplanted by skin graft onto naive mice. They function to recruit circulating T cells to the skin in a CXCR3-dependent manner, resulting in better control of the parasites. Our findings are the first to demonstrate that CD4+ T_{RM} cells form in response to a parasitic infection, and indicate that optimal protective immunity to *Leishmania*, and thus the success of a vaccine, may depend on generating both circulating and skin-resident memory T cells.

CORRESPONDENCE
Phillip Scott:
pscott@vet.upenn.edu

Abbreviations used: GSEA, gene set enrichment analysis; T_{RM}, tissue-resident memory T cell.

The development of effective vaccines for several intracellular microbial pathogens, such as *Mycobacteria*, *Toxoplasma*, *Plasmodium*, and *Leishmania,* remains an elusive goal. Despite substantial efforts to define the mechanisms required for resistance, to develop new adjuvants, and to identify protective antigens, the long-lived cellular immunity generated in response to infection has not been recapitulated by vaccination. To address this problem in leishmaniasis, we have focused on defining the memory T cells that mediate infection-induced immunity.

C57BL/6 mice show robust immunity to reinfection after resolution of a primary *Leishmania major* infection (referred to here as immune mice), providing a useful model to interrogate the factors that might contribute to a successful vaccine. Previous studies have shown that immune mice contain circulating CD4+ T cells with effector, effector memory, and central memory phenotypes (Scott et al., 2004; Colpitts et al., 2009; Colpitts and Scott, 2010). Each of these T cell subsets likely plays a role in resistance to reinfection, with the effector subsets rapidly migrating into tissues to provide protection and central memory T cells proliferating in the draining lymph node to provide a pool of new effector cells. However, whereas adoptive transfer of either effector or central memory T cells to naive mice enhances immunity to reinfection (Zaph et al., 2004), neither subset alone or in combination provides the same level of protection as that seen in intact immune animals.

In addition to circulating memory T cells, an additional memory T cell subset resides in the tissues as resident memory T cells (T_{RM}; Kim et al., 1999; Hogan et al., 2001a; Masopust et al., 2001; Clark et al., 2006; Gebhardt et al., 2009; Wakim et al., 2010; Hofmann and Pircher, 2011; Jiang et al., 2012; Mackay et al., 2012; Schenkel et al., 2013). Several studies have described T_{RM} cells that mediate immunity to acute viral infections, such as vaccinia, herpes simplex, influenza, and lymphocytic choriomeningitis virus (Gebhardt et al., 2009; Teijaro et al., 2011; Jiang et al., 2012; Schenkel et al., 2013). These T_{RM} cells can be found in the gut, brain, lung, and skin (Kim et al., 1999; Clark et al., 2006; Wakim et al., 2010; Teijaro et al., 2011), and their location allows them to respond immediately to control a challenge infection without the delay associated with the mobilization of circulating T cells. Additionally, T_{RM} cells can promote rapid

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recruitment of effector cells from the circulation (Schenkel et al., 2013) and induce antigen-independent innate immunity (Ariotti et al., 2014; Schenkel et al., 2014), thereby accelerating and amplifying resistance to infections.

$CD8^+$ T_{RM} cells are fairly well characterized, but less is known about $CD4^+$ T_{RM} cells. Nevertheless, recent studies using Kaede-Tg mice to facilitate tracking of T cells in the skin indicate that a population of $CD4^+$ T cells appear to be skin-resident under homeostatic conditions (Bromley et al., 2013). In addition, $CD4^+$ T_{RM} cells in the lung and vaginal mucosa have been reported to enhance resistance to influenza and herpes simplex virus, respectively (Hogan et al., 2001b; Teijaro et al., 2011; Iijima and Iwasaki, 2014; Laidlaw et al., 2014), and a population of human tissue-resident $CD4^+$ T cells remain in the skin after circulating T cells have been depleted (Clark et al., 2012; Watanabe et al., 2015). However, the potential role of $CD4^+$ T_{RM} cells in establishing resistance to chronic parasitic infections, such as *L. major*, is virtually unknown.

Here, we identify for the first time a tissue-resident population of $CD4^+$ T cells that seed the skin after *L. major* infection. These cells are observed in skin sites far from the primary infection site, and persist long term in immune mice. They produce IFN-γ in response to stimulation with *L. major* and, during a secondary challenge, act as sentinels to rapidly recruit circulating memory cells, resulting in enhanced protection against reinfection. Thus, our results suggest that these previously unidentified memory $CD4^+$ T cells are instrumental in protection against *L. major* parasites and should now be considered during vaccine development.

RESULTS

L. major–specific $CD4^+$ T cells are present in skin far from the primary infection site

C57BL/6 mice infected with *L. major* in the ear develop a lesion that resolves by 12 wk. These mice are resistant to reinfection, and are referred to as immune mice. To determine if *L. major*–specific T cells were present in skin sites distant from the primary infection site, we isolated cells from the uninfected ear (contralateral ear) and the flank. We also isolated cells from the primary infection site (primary ear) of immune mice, and from the ears and flank of naive mice. The cells were incubated with or without *L. major*–infected dendritic cells overnight, and IFN-γ production was assessed as a measure of T cell activation. We used *IFN-g/Thy1.1* knock-in mice in these experiments (Harrington et al., 2008) so that we could use surface staining for Thy1.1 to sensitively detect transcription of the IFN-γ gene. As expected, *L. major*–responsive cells were absent in ear and flank skin from naive mice, whereas ∼25% of $CD4^+$ T cells from the primary ear of immune mice produced IFN-γ in the presence of *L. major*–infected DCs, likely because of a low level of persistent infection (Fig. 1, A and B). However, when we stimulated cells from two uninfected sites of immune mice, the contralateral ear and the flank skin, we also detected a population of $CD4^+$ T cells that responded to *L. major*–infected DCs (Fig. 1, A and B). These cells did not respond to uninfected DCs, demonstrating their specificity. In separate experiments, we used intracellular staining to confirm the production of IFN-γ protein (Fig. 1 C). A similar population of IFN-γ responsive $CD4^+$ T cells in immune mice was also recently observed (Peters et al., 2014). Importantly, no parasites could be detected in either the contralateral ear or the flank skin after lesion resolution by culture, qRT-PCR for ribosomal RNA, or PCR for kinetoplast DNA (unpublished data).

To determine if the *L. major*–responsive cells we observed in the contralateral ear and flank of immune mice might be in the vasculature of the skin, we used a technique developed to distinguish resident and circulating T cells in tissues (Anderson et al., 2012). Immune mice were injected with FITC anti-CD45.2 antibody intravenously, and 3 min later the blood and skin were collected and assessed for responsiveness to *L. major*–infected dendritic cells. The IFN-γ^+ cells from the blood were $FITC^+$, whereas cells from the skin were not, indicating that the *L. major*–responsive cells were indeed contained within the skin, and thus sequestered from circulation (Fig. 1 D). Consistent with the potential for these cells to be resident in the skin, we found that >80% of the *L. major*–responsive cells in the contralateral ear and flank were $CD69^+$ and all of the cells were CCR7 negative (unpublished data). Finally, when we compared the frequency of *Leishmania*-responsive cells in the skin to the spleen, we found a significant enrichment in the skin, suggesting that there may be preferential retention of these cells in the tissue (Fig. 1 E). Together, our results identify a previously unrecognized population of T cells distributed broadly throughout the skin, positioned to act as a frontline defense against *Leishmania* infection.

L. major–specific $CD4^+$ T cells are resident in immune skin

To determine how long after infection *L. major*–specific $CD4^+$ T cells could be detected in skin far from the primary infection site, we incubated infected DCs with skin cells from immune mice at various time points after infection. *L. major*–specific $CD4^+$ T cells persisted in skin sites distant from the primary infection site at every time point we observed, up to one year after resolution of disease (Fig. 2 A). Although these data confirmed that *L. major*–responsive $CD4^+$ T cells are a persistent population in the skin, they did not distinguish whether these T cells were resident in skin or were instead being replenished from circulation.

To address this issue, we grafted uninfected flank skin from immune CD45.2 mice originally infected in the ear onto naive CD45.1 recipients, and determined how long donor *L. major*-specific T cells remained in the grafts by quantifying the number of *Leishmania* memory cells present within donor skin at the time of grafting (t = 0), and in harvested grafts 1, 2, and 4 wk later. At all time points, we were able to identify *L. major*–specific donor cells resident within the graft skin (Fig. 2 B). The difference between the number of *L. major*–specific cells at the time of grafting and later time points was not significant but did trend toward a decrease, suggesting that some cells might recirculate as well. Regardless, *L. major*–responsive

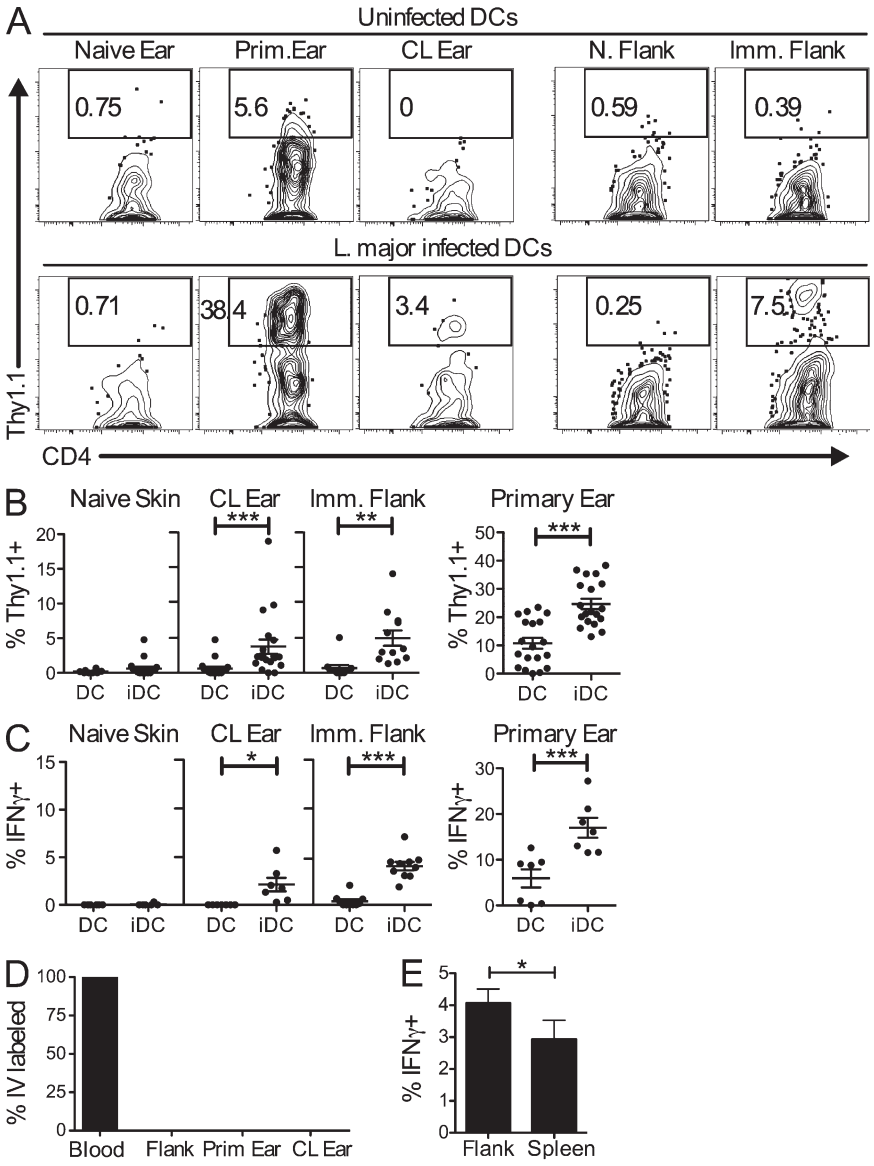

Figure 1. *L. major*–specific CD4⁺ T cells are present in skin distant from the primary infection site. Skin was collected from either naive C57BL/6 mice (wild-type or IFN-γ–reporter mice) or mice that had been previously infected with *L. major* in the left ear and resolved their lesions (referred to as immune mice). Skin isolated from infected mice included the left ear (primary ear), contralateral ear (CL), and immune (Imm.) flank; skin from naive mice (N.) included the ear and the flank. (A–C) Cells from the skin were incubated with either uninfected BM-derived DCs or BMDCs that were infected with *L. major* (iDC) and, 12 h later, IFN-γ production in CD4⁺ T cells (gated on live, CD45, CD90.2, CD4 cells) was assessed by expression of Thy1.1 (A and B) or by IFN-γ intracellular staining (C). (A) Representative flow plots. (B and C) Each dot represents an individual mouse, and data are combined from at least three experiments. (D) FITC anti-CD45.2 antibody was injected i.v. into immune mice (15 wk after infection) and 3 min later blood, flank, primary ear (Prim.), and contralateral (CL) ear were collected and incubated with infected BMDCs as above, and FITC staining was assessed on CD4⁺ IFN-γ⁺ cells. Data shown are from one representative experiment of 2 ($n = 5$ mice). (E) Cells isolated from the flank or spleen of immune mice were incubated with *L. major* infected BMDCs as above, and each bar represents the mean (±SEM) percent of IFN-γ–producing CD4⁺ T cells detected by flow cytometry (gated on live, CD45, CD4 cells). Data shown are combined from 3 experiments ($n = 10$ mice per group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

CD4⁺ T cells were retained for at least 4 wk in immune skin in the absence of circulating memory cells, and thus form a tissue-resident population.

L. major–immune mice rapidly up-regulate IFN and chemokine signaling pathways after challenge

To determine if *L. major*–specific T_{RM} cells could contribute to the immune response early after a challenge infection, we injected previously uninfected flank skin from naive and immune mice with *L. major*, harvested the inoculated skin 12 h later, and compared gene transcript levels. We found 145 differentially regulated genes between naive and immune skin (fold change ≥ 2; false discovery rate ≤ 0.05; Fig. 3 A). Strikingly, competitive gene set enrichment analysis revealed IFN signaling to be the most highly up-regulated canonical signaling pathway in immune mice (Fig. 3 B). Chemokine and chemokine receptor signaling were also among the most significantly enhanced gene sets (Fig. 3 C).

To confirm that the transcriptional changes we observed were caused by cells resident in immune skin and independent of any cells that might have been recruited, we isolated flank skin cells and incubated them with *L. major* in vitro. We assessed the transcriptional response of a select group of genes found to be up-regulated by global transcriptional profiling using qRT-PCR and observed a significant increase in expression of several IFN-γ responsive genes (Fig. 3 D). Notably, CXCL9 and CXCL10 were highly expressed in restimulated flank skin both in vitro and in vivo. These two chemokines interact with CXCR3 on activated T cells to drive T cell recruitment (Nakanishi et al., 2009). This data, along with recent evidence in the literature (Schenkel et al., 2013; Ariotti et al., 2014), led us to hypothesize that *L. major*-specific T_{RM} cells

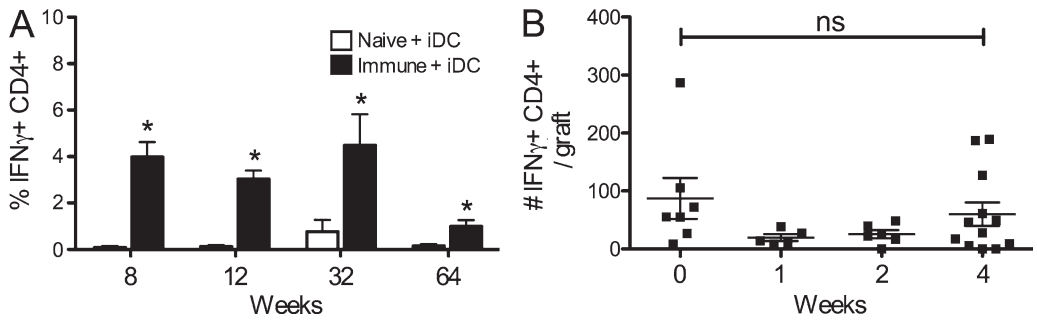

Figure 2. *L. major*–specific CD4 T cells are resident in immune skin. (A) Flank skin was collected from naive or immune mice at various time points after *L. major* infection, and IFN-γ–producing CD4+ T cells detected as in Fig. 1. Data shown represent the mean (±SEM) percent IFN-γ–producing CD4+ T cells detected by flow cytometry (n = 3–4 mice per time point). (B) Flank skin from immune mice (13–14 wk after infection) was grafted onto naive mice, and at various times the grafts were harvested and IFN-γ–producing CD4+ T cells were detected as in Fig. 1. Quantification of *L. major*–specific cells in skin grafts before grafting (time 0) and 1, 2, or 4 wk later is shown. Data are combined from two separate experiments and each dot represents one mouse. *, $P < 0.05$.

might function by activating a program to rapidly recruit circulating effector T cells to the site of infection after challenge.

L. major–specific T_{RM} cells enhance T cell recruitment after challenge

To compare the recruitment of effector T cells to an *L. major* challenge site with and without *L. major*–specific T_{RM} cells, we transferred CFSE-labeled splenocytes from immune mice into naive or immune recipients, infected the flank skin, and measured the number of cells recruited from the blood to the challenge site 20 h later. Although equivalent pools of circulating cells were available in the blood of naive and immune recipients, there was significantly enhanced recruitment of both $CD4^+$ and $CD8^+$ T cells to the skin of *Leishmania*-immune animals (Fig. 4, A and B). Increased recruitment was not a response to tissue injury alone, as PBS injection was insufficient to increase recruitment in immune animals compared with naive mice (Fig. 4 C).

Although the activation of *L. major* T_{RM} appeared to be antigen-specific, we hypothesized that after antigen recognition these T_{RM} cells could recruit any circulating memory T cell (Schenkel et al., 2013). Indeed, when we transferred LCMV immune T cells to naive and *L. major* immune mice, we also observed increased recruitment of T cells to immune skin after challenge (Fig. 4 D). Thus, these results show that antigen-specific stimulation of immune skin can promote increased recruitment of circulating memory T cells nonspecifically.

To determine if T cell recruitment was dependent on the $CD4^+$ T cells in the immune skin, we compared the recruitment of transferred memory cells to immune and $CD4^+$ T cell–depleted immune mice. $CD4^+$ T cells were depleted from both the recipient and donor cell populations, so we focused on the recruitment of $CD8^+$ T cells for these studies. After anti-CD4 mAb treatment, no $CD4^+$ T cells could be identified in the skin of immune mice (Fig. 4 E), and the enhanced recruitment of circulating memory cells observed in *L. major*–immune skin was absent in anti-CD4 mAb–treated mice, suggesting that the enhanced recruitment was $CD4^+$ T cell dependent (Fig. 4 F).

We next assessed the role of chemokines in promoting the T cell recruitment to challenge sites by treating immune mice with anti-CXCR3 mAb. The chemokine receptor CXCR3 interacts with CXCL9, CXCL10, and CXCL11, and anti-CXCR3 mAb blocks CXCL10 and CXCL11 signaling in vitro (Uppaluri et al., 2008). We transferred labeled *L. major*–immune cells to intact and anti-CXCR3 mAb-treated immune mice, challenged in the flank skin, and compared the recruitment of T cells to the challenge site 20 h later. The increase in recruitment to immune flank skin was completely blocked in anti-CXCR3 mAb-treated mice, indicating that this chemokine axis indeed promotes the enhanced T cell recruitment seen in immune mice (Fig. 4 G).

Finally, to determine if skin-resident cells were sufficient to drive the increased recruitment of activated T cells to immune skin, we grafted naive and immune flank skin in parallel onto opposite sides of naive recipients that lack any *L. major* memory cells. We then transferred circulating *L. major* immune cells, challenged both the naive and immune graft with *L. major*, and compared recruitment of the labeled cells 20 h later. We observed enhanced recruitment of circulating cells to the immune graft compared with the naive graft, indicating that cells from the skin alone could indeed mediate this effect (Fig. 4, H and I). Together, these data indicate that *L. major*–responsive $CD4^+$ T cells in the skin promote the early recruitment of circulating effector cells, and thus have the potential to improve the outcome of a secondary infection.

L. major–specific T_{RM} cells provide increased protection against secondary challenge

Finally, we sought to determine if T_{RM} cells could contribute to the control of *L. major* infection in immune mice. To specifically assess the effects of T_{RM} cells in the absence of circulating memory T cells, we grafted both naive and immune skin onto a naive recipient and challenged both skin grafts.

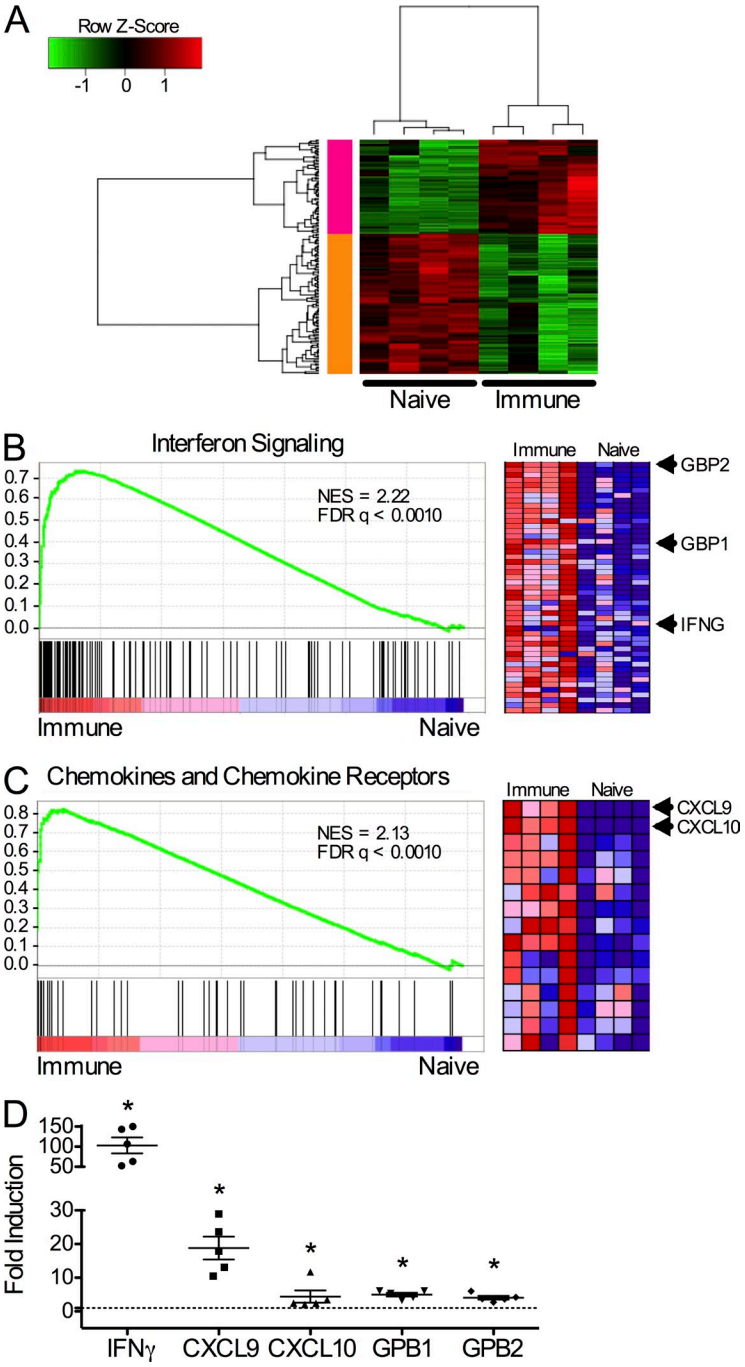

Figure 3. *L. major*–immune mice rapidly up-regulate IFN and chemokine signaling after challenge. Naive or immune mice (20 wk after infection) were infected in the flank with *L. major*, and the flank skin was harvested 12 h later for transcriptional profiling by microarray. (A) Heat map of 145 differentially expressed genes between 4 naive and 4 immune mice challenged in the flank (fold change ≥ 2, false discovery rate ≤ 0.05). (B and C) Gene set enrichment analysis (GSEA) plot for gene sets IFN signaling (B) and chemokines and chemokine receptors (C). Heat maps show the top up-regulated genes from the IFN-signaling gene set or chemokines and chemokine receptors gene set between naive and immune mice (red, high; blue, low). Normalized enrichment score (NES) with false discovery rate q-value as calculated by GSEA. (D) Cells were isolated from the flank skin of immune mice (20 wk after infection) were incubated with or without *L. major* parasites for 12 h and gene expression assessed by qRT-PCR. Data shown are the fold induction of IFN-regulated genes after exposure to *L. major*. Each dot represents an individual animal, and is from one representative experiment of 2. *, $P < 0.05$.

After 2 wk, we compared the parasite burden in each graft and found no significant difference between naive and immune grafts (Fig. 5 A). Thus, T_{RM} cells did not appear to provide a significant level of protection by themselves. To assess if T_{RM} cell–driven recruitment of circulating cells was important, we grafted naive mice with both naive and immune skin as before, but in addition we adoptively transferred *L. major*–specific memory cells isolated from immune mice. As expected, the presence of circulating *L. major*–specific cells significantly reduced the parasite burden, even ithe naive graft, 2 wk after infection (Fig. 5 A). However, the parasite burden in the immune graft was further significantly decreased compared with the naive graft, indicating that immune skin was indeed better protected.

To assess the level of protection achieved by transferring both circulating and skin-resident immune cells to naive mice, we compared skin-grafted mice to intact immune mice 2 wk after *L. major* challenge in the flank (Fig. 5 B). As expected, intact immune mice were significantly protected compared with naive mice, and were also better protected than naive graft skin that had access to circulating cells alone. However, there was no difference between the level of protection

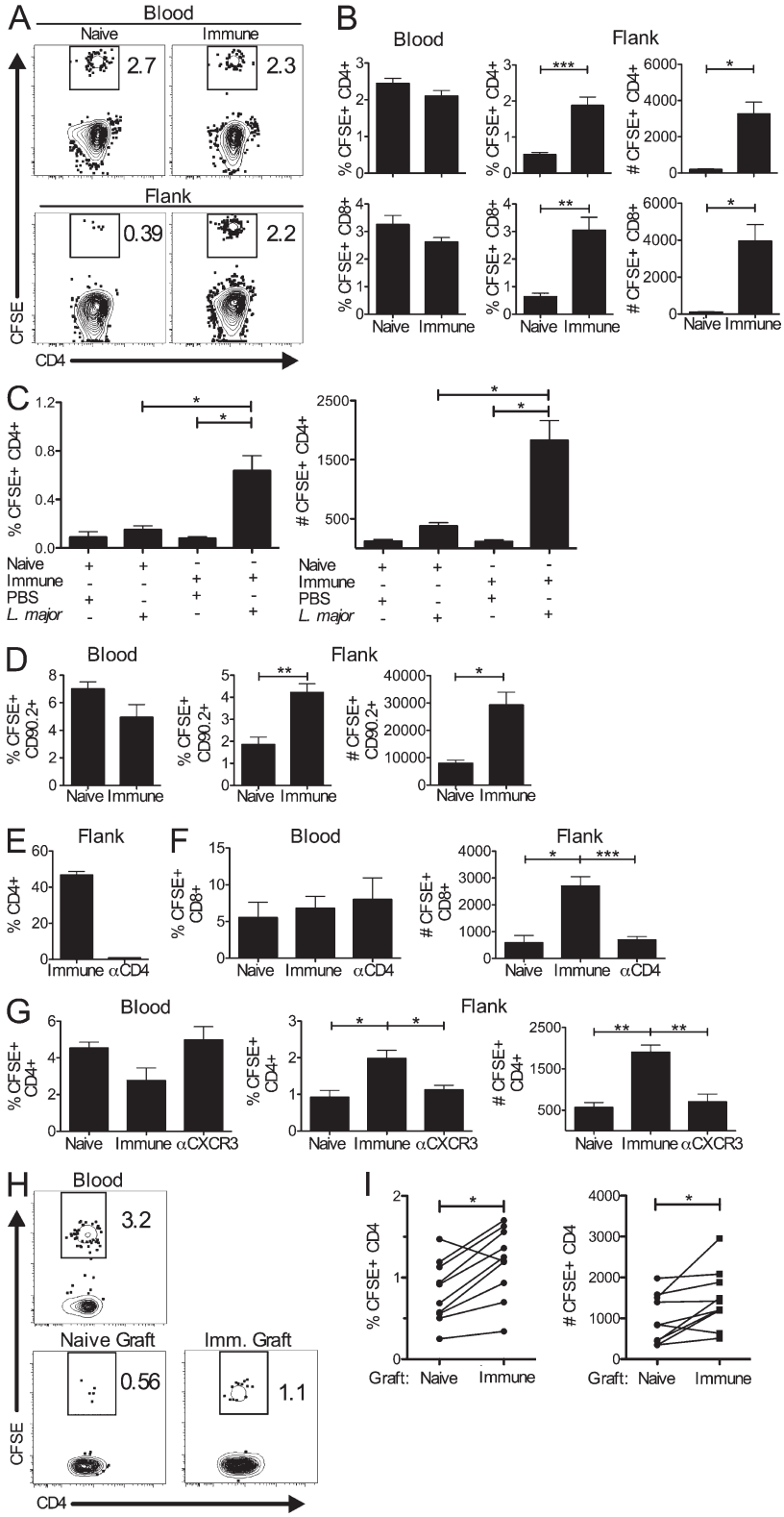

Figure 4. *L. major*–specific T_{RM} cells enhance T cell recruitment after challenge with *L. major*. Splenocytes from *L. major* (A–C) or LCMV (D) immune mice were CFSE labeled to track them after transfer. They were injected i.v. into either naive or *L. major*–immune mice (20 wk after infection) that were then challenged with *L. major* or PBS in the flank. Recruitment of cells to the challenge site was assessed 20 h later. (A) Representative flow plots of CFSE$^+$ CD4$^+$ T cells comparing the blood and the flank in naive or immune mice challenged with *L. major*. (B) Frequency and number of transferred *L. major* immune CD4$^+$ and CD8$^+$ T cells in the blood and flank skin of naive and immune mice 20 h after challenge with *L. major*. (C) Frequency and number of transferred *L. major*–immune CD4$^+$ T cells 20 h after challenge with *L. major* or injection of PBS in naive or immune mice. (D) Frequency and number of transferred LCMV immune lymphocytes in the blood and flank skin of naive and immune mice 20 h after challenge with *L. major*. (E) *L. major*–immune mice were treated with anti-CD4 mAb, and the frequency of CD4$^+$ T cells in the skin of untreated or anti-CD4–treated immune mice is shown. (F and G) Naive *L. major*–immune or *L. major*–immune mice treated with anti-CD4 mAb (F) or anti-CXCR3 mAb (G) received CFSE-labeled splenocytes from immune mice, and were then infected in the flank with *L. major*. After 20 h, the frequency and number of the transferred cells in the blood and flank was determined. (H and I) Flank skin from naive or immune mice was grafted onto the flank of naive mice. After 7 d, the recipient mice were injected i.v. with CFSE-labeled immune cells, each graft was infected with *L. major*, and, 20 h later, cells were isolated from the grafts. (H) Representative flow plots of CFSE$^+$ CD4$^+$ T cells comparing the blood and naive or immune skin grafts challenged with *L. major*. (I) Frequency and number of transferred *L. major*–immune CD4$^+$ T cells in naive or immune skin grafts (cells were gated on live, CD45, CD90.2, CD4 cells). All data are from one representative experiment of two or more with three or more mice per experiment, and each bar represents the mean (±SEM) of individual mice (A–G) or combined data from two experiments where each dot represents a mouse (H and I). Immune mice used in all these experiments were 14 to 20 wk after infection.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

observed in intact immune mice and skin-grafted mice challenged in immune flank skin, suggesting that the presence of circulating and skin-resident T cells was sufficient for optimal protection.

Finally, to confirm that this protective phenotype was long lived, we grafted naive and immune skin onto naive mice and rested them for 30 d. We then transferred in *L. major*–immune cells, challenged each graft, and compared parasite burdens at

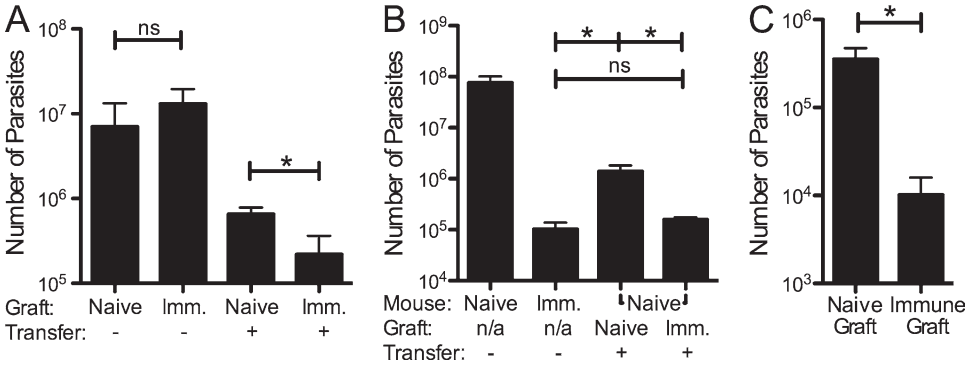

Figure 5. ***L. major*–specific T_{RM} cells provide increased protection against secondary challenge.** Flank skin from naive or immune mice was grafted onto the flank of naive mice. After 7 or 30 d, grafts from both the naive and immune mice were challenged with *L. major* and, 2 wk later, the grafts were isolated and parasite burdens were measured. Some mice received 20 × 10^6 splenocytes (i.v.) from *L. major*–immune mice at the time of challenge. (A) Naive or immune skin grafts in the presence or absence of adoptively transferred *L. major*–immune cells were challenged with *L. major* and the parasite burden was assessed at 2 wk. Data shown are the mean (±SEM) parasite burden. (B) Intact naive or immune mice, or naive and immune skin grafted onto naive mice were challenged with *L. major*. The mice with skin grafts also received *L. major*–immune cells as above, and the parasite burden was assessed at 2 wk. The data shown are the mean (±SEM) of the parasite burden. (C) Naive or immune skin was grafted onto naive mice, and 30 d later mice received *L. major*–immune cells as above and both grafts were challenged with *L. major*. The data shown are the mean (±SEM) of the parasite burden 2 wk after *L. major* challenge. Data from A and B are representative of 2 experiments (n = 5 mice per group), and C is combined data from 2 experiments (n = 6 mice). Immune mice used in all these experiments were 14 to 20 wk after infection. *, $P < 0.05$.

2 wk. Even 1 mo after grafting, the parasite burdens were significantly lower in immune skin, suggesting that *L. major*–specific T_{RM} cells were still mediating protective effects (Fig. 5 C). Together, these data support a model in which *L. major* T_{RM} cells act as sentinels in the skin to rapidly recruit circulating memory cells and restrict parasite replication. Consequently, immunizations that do not induce *L. major* T_{RM} cells may result in delayed recruitment of effector cells and impaired control of infection.

DISCUSSION

We discovered that tissue-resident memory $CD4^+$ T cells are present in the skin after resolution of *Leishmania* infection. Although resident memory T cells generated in response to acute viral infections have been extensively studied (Kim et al., 1999; Hogan et al., 2001a; Gebhardt et al., 2009; Wakim et al., 2010; Teijaro et al., 2011; Jiang et al., 2012; Schenkel et al., 2013; Iijima and Iwasaki, 2014), we identify for the first time a population of skin-resident $CD4^+$ T cells that form in response to a chronic parasitic infection. These T_{RM} cells persist in the absence of circulating *L. major*–specific T cells, are retained long after resolution of disease, produce IFN-γ, and enhance the recruitment of circulating memory cells to the site of *Leishmania* challenge. This rapid mobilization of pathogen-specific T cells significantly reduced the parasite burden, indicating that $CD4^+$ T_{RM} cells contribute to protective immunity to *Leishmania*.

Although several subsets of *L. major*–specific T cells are present in mice that have resolved a primary infection, adoptive transfer of these cells into naive mice never recapitulates the level of protection seen in intact immune mice. Such results could stem from an inability to adoptively transfer adequate numbers of *L. major*–specific T cells; however, our current results documenting the ability of skin-resident *L. major* memory cells to rapidly recruit circulating effector cells to the site of infection support an alternative hypothesis: that the absence of T_{RM} cells in recipient mice is responsible for the lower level of observed protection. Nevertheless, our results also show that these T_{RM} cells are insufficient to mediate protection alone, and require a circulating pool of *L. major*–specific $CD4^+$ T cells. These circulating cells have been well-characterized, and include effector ($CD62L^{lo}$, $IL\text{-}7R^{lo}$), effector memory ($CD62L^{lo}$, $IL\text{-}7R^{hi}$), or central memory ($CD62L^{hi}$, $IL\text{-}7R^{hi}$) $CD4^+$ T cells (Colpitts et al., 2009; Colpitts and Scott, 2010). Most recently, the circulating *Leishmania*-specific T cells have been further characterized, and the most protective cells were shown to be $Ly6C^+$ $CD4^+$ effector T cells (Peters et al., 2014). Collectively, our data indicate that an optimal vaccine would need to generate both circulating protective $CD4^+$ T cells and T_{RM} cells.

An important attribute of the *L. major*–specific $CD4^+$ T_{RM} cells is their persistence in the absence of chronic parasites. Specifically, *L. major*–specific skin-resident memory T cells transferred by skin graft to uninfected mice persist for several weeks. This is in contrast to effector cells, which depend on low numbers of persisting parasites to be maintained (Belkaid et al., 2002), and is most likely why optimal protection requires chronic infection (Uzonna et al., 2001; Belkaid et al., 2002; Mendez et al., 2004; Sacks, 2014). Nevertheless, some protection can be maintained in the absence of persisting parasites, which we previously attributed to the parasite-independent maintenance of central memory T cells (Zaph et al., 2004). Our discovery that $CD4^+$ T_{RM} cells are maintained in skin in the absence of persistent parasites now identifies another parasite-independent memory T cell population that is an ideal target for a T cell vaccine.

The $CD4^+$ T_{RM} cells that we identified in *L. major*–infected mice appear to function by recruiting circulating effector cells. Transcriptional profiling of immune skin challenged with *L. major* showed increased expression of many IFN-γ/STAT1–dependent genes, including the IFN-γ–inducible chemokines CXCL9 and CXCL10. By blocking the receptor for these chemokines, CXCR3, we demonstrate that chemokine receptor signaling is important for the enhanced recruitment to challenge sites in immune mice. Although a similar role for $CD8^+$ Trm cells has been previously described (Schenkel et al., 2013; Ariotti et al., 2014), other studies suggest that $CD8^+$ T_{RM} cells can control viruses by themselves, even when the recruitment of circulating cells is suppressed by treatment of mice with FTY-720 (Hofmann and Pircher, 2011; Jiang et al., 2012). This was not the case in *L. major* infection, which might be caused by inherent differences between $CD4^+$ and $CD8^+$ T_{RM} cells, or more likely might be related to the nature of the pathogen. Unlike acute viral infections that are quickly controlled even in naive hosts, leishmaniasis is a chronic disease that takes months to heal, suggesting that rapid control of secondary challenges may require a particularly strong immune response. Thus, although *L. major*–specific T_{RM} cells are an important component in the protection seen in immune mice, they also require the presence of circulating immune cells to promote protection.

Multiple strategies have been applied to *Leishmania* vaccine development, including the use of different live or killed parasites, various adjuvants, and a multitude of DNA constructs and protein subunits (Coler and Reed, 2005; Kedzierski et al., 2006; Dunning, 2009). Although some of these have provided a degree of protection (Palatnik-de-Sousa, 2012), there still is no human vaccine against *L. major* (Noazin et al., 2008), and none of the experimental vaccines induce the level of protection seen in immune mice. Our results provide a new model for how leishmanial infections generate resistance to reinfection, one in which both circulating and tissue-resident cells work together to provide robust protective immunity. Taking this into account, new approaches to the development of a vaccine will need to consider not only the generation of circulating memory T cells, but also how best to generate a population of T_{RM} cells, which may be the missing link in the quest for a successful *L. major* vaccine.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from the National Cancer Institute (National Institutes of Health). *Ifng/Thy1.1* knock-in mice were provided by C. Weaver (University of Alabama, Birmingham, AL). All mice were maintained in a specific pathogen–free environment at the University of Pennsylvania Animal Care Facility. This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the protocol was approved by the Institutional Animal Care and Use Committee.

***L. major* and LCMV infections.** *L. major* (WHO/MHOM/IL/80/Friedlin) parasites were grown in Schneider's insect medium (Invitrogen) supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Metacyclic enriched promastigotes were used for infection (Späth and Beverley, 2001). For all the primary infections to generate immune mice, mice were infected with 2×10^6 *L. major* in the left ear (referred to as the primary ear). The contralateral ear (right ear) was not infected. Mice that resolved a primary infection in the left ear (immune mice) were challenged with 2×10^6 *L. major* in the flank or in the skin grafted onto the flank. Immune mice were between 14 and 20 wk after infection, except where otherwise noted. C57BL/6 mice were injected i.p. with 2×10^5 pfu LCMV Armstrong, and cells for adoptive transfer collected 21 d after infection. The parasite burden in lesion tissues was assessed using a limiting dilution assay as previously described (Zaph et al., 2004). Detection of parasites by PCR was done in two ways. DNA was isolated from skin using the REDExtract-N-Amp kit (Sigma-Aldrich) and PCR was performed using primers for the kinetoplast DNA (forward, 5′-CCTATTTTACACCAACCCCCAGT-3′; reverse, 5′-GGGTAGGGGCGTTCTGCGAAA-3′). After an initial 5-min denature at 95°C, 30 cycles of denaturing, annealing, and extension were performed at 95, 62, and 72°C, respectively, and products were visualized on a 1% agarose gel. Alternatively, cells from ear or flank skin were harvested and RNA was isolated using the RNeasy Plus kit (QIAGEN), and then converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). Parasites were detected by quantitative PCR using SYBR green technology on a ViiA7 real-time PCR system (Thermo Fisher Scientific) using primers for parasite ribosomal RNA (forward, 5′-TACTGGGGCGTCAGAG-3′; reverse, 5′-GGGTGTCATCGTTTGC-3′), and quantified by comparison to a standard curve of known parasite numbers with a limit of detection between 10^1 and 10^2 parasites.

Skin preparation and detection of *L. major*-specific cells. For ear preparation, dorsal and ventral layers of the ear were separated and incubated in RPMI (Invitrogen) with 250 µg/ml Liberase TL (Roche) for 90 min at 37°C in 5% CO_2. Ears were dissociated using a 40-µm cell strainer (BD) and resuspended in RPMI media containing 10% FBS. For flank preparation, flank skin was shaved using an electric trimmer equipped with a two-hole precision blade (Wahl) and treated with depilating agent (Nair) for 1 min. A section of dermis was excised, and then minced with a sterile scalpel blade into ∼2-mm sections. Flank sections were incubated in RPMI containing 250 µg/ml Liberase TL for 120 min, with vortexing every 30 min. The resulting solution was passed through a 40-µm cell strainer and resuspended in complete RPMI (cRPMI), which is supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 55 µM 2-mercaptoethanol. BM-derived dendritic cells were generated by culturing C57BL/6 bone marrow in GM-CSF supplemented cRPMI for 7–11 d. BMDCs were then harvested and infected for 5–8 h with stationary phase *L. major* at a ratio of 10:1 in the presence of 1 µg/ml CpG and LPS. Infected BMDCs were incubated at a ratio of 1:5 with 10^6 skin cells in 24-well plates for 12–16 h. Cells (*Ifng/Thy1.1* knock-in mice) were then stained for Thy1.1, or for the last 4 h the cells were incubated with 5 µg/ml BFA (eBioscience), stained for IFN-γ, and analyzed by flow cytometry.

Antibodies and flow cytometry. For flow cytometry analysis anti-CD45 AF700, anti-CD45.2 APC-eF780, anti-CD45.1 eF480, anti-CD90.2 APC, anti-CD69 FITC, anti-CD90.1 PE-Cy7, anti-CCR7 PE (eBioscience), anti-CD4 PE TexasRed (Invitrogen), anti-CD8b PerCp/Cy5.5, and anti–IFN-γ PE-Cy7 (BioLegend) were used. For in vivo CD4 depletion mice received i.p. injections of 250 µg GK1.5 on days −5 and −2 before challenge. For in vivo CXCR3 blockade mice received i.p. injections of 250 µg anit-CXCR3-173 mAb (BioXcell) one day before challenge. For intravascular staining mice were injected with 3 µg FITC anti-CD45.2 (eBioscience) 3 min before they were euthanized by CO_2. The data from flow cytometry were collected using LSRII flow cytometer (BD) and analyzed using FlowJo software (Tree Star).

Transcriptional profiling. For microarray experiments, flank tissue sections were homogenized in RLT buffer (QIAGEN) supplemented with 1% 2-ME using a bead-based homogenizer (MP FastPrep-24). RNA was isolated using the RNeasy Plus kit (QIAGEN). Biotin-labeled complementary RNA

(cDNA) was generated using the TargetAmp-Nano amplification kit (Epicentre). RNA and cDNA quality were assessed on a BioAnalyzer (Agilent). MouseRef-8 v2.0 Expression BeadChips (Illumina) were then hybridized with cDNA from 4 immune and 4 naive mice challenged in the flank with *L. major*. Analysis was performed using the statistical computing environment R (v3.0.2), with RStudio and Bioconductor suite packages (v0.97). Data has been deposited in the Gene Expresion Omnibus database for public access (under accession no. GSE69998). GSEA (Subramanian et al., 2005) was performed with the Broad Institute's MSigDB (v4.0) to query the C2: Canonical Pathways collection, which contains 1,320 gene sets. For in vitro experiments, skin cells from flank skin (2×10^6 cells) were incubated with metacyclic *L. major* at a ratio of 10:1 and cultured overnight in a 24-well plate at 37°C. RNA was isolated from cells using the RNeasy Plus kit (QIAGEN) and converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific), and then the relative abundance was calculated by quantitative PCR using SYBR Green technology on a ViiA7 Real time PCR system (Thermo Fisher Scientific) with primers for IFN-γ (forward, 5′-GCTGTTACTGCCACGGCACAGT-3′; reverse, 5′-TCACCATCCTTTTGCCAGTTCCTCC-3′), CXCL9 (forward, 5′-TCCTTTTGGGCATCATCTTGC-3′; reverse, 5′-TTTGTAGTGGATCGTGCCTGG-3′), CXCL10 (forward, 5′-CCAAGTGCTGCCGTCATTTTC-3′; reverse, 5′-GGCTCGCAGGGATGATTTGAA-3′), GBP1 (forward, 5′-ACCTGGAGACTTCACTGGCT-3′; reverse, 5′-TTTATTCAGCTGGTCCTCCTGTATCC-3′), and GBP2 (forward, 5′-ACCTGGAACATTCCCTGACC-3′; reverse, 5′-ACAGCTCCTCCTCCCGCAGAG-3′) normalized to RPS11 (forward, 5′-CGTGACGAAGATGAAGATGC-3′; reverse, 5′-GCACATTGAATCGCACAGTC-3′) RNA.

Skin grafts. Donor skin was prepared under sterile conditions from naive and immune mouse flank skin by shaving, depilating, cleaning with chlorhexidine (Vetoquinol), and then excising the dermal layer using sterile instruments. Donor mice for immune grafts were 14–20 wk after infection. 8-mm biopsy punches (Miltex) were used to size donor grafts, which were then placed onto a graft bed generated by a 6-mm biopsy punch of mice anesthetized with isoflurane and treated with 0.1 mg/kg buprenorphine as a preemptive analgesic. Grafts were covered with nonadherent dressing (Adaptec) and held in place with Tegaderm (3M) wrapped circumferentially around the body of the mouse to provide protection against loss and trauma. Mice were monitored twice daily for the first 48 h and daily for 7 d after grafting, given buprenorphine, and rewrapped where necessary. In challenge experiments, graft skin was injected intradermally with 2×10^6 metacyclic *L. major* 7 or 30 d after grafting.

Adoptive transfer. For recruitment and protection studies, single-cell suspensions from the spleen of *L. major* or LCMV immune mice were CFSE labeled as previously described and resuspended at $5–10 \times 10^7$ cells/ml in PBS, and then the cells were transferred i.v. in 400-µl vol into recipient mice.

Statistical analysis. Statistical analysis was performed with the Mann-Whitney test (two-sided Student's *t* test, paired or unpaired where applicable) in Prism software (GraphPad). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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