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IL-7 Receptor Expression Provides the Potential for Long-Term Survival of Both CD62L^{high} Central Memory T Cells and Th1 Effector Cells during *Leishmania major* Infection[1]

Sara L. Colpitts, Nicole M. Dalton, and Phillip Scott[2]

Infection with the intracellular protozoan parasite *Leishmania major* induces a state of concomitant immunity wherein secondary immunity is dependent upon the persistence of the original pathogen. Our laboratory has described two populations of *Leishmania*-induced CD4$^+$ T cells that contribute to immunity: CD62L^{high} central memory T (T_{CM}) cells and CD62L^{low} effector T cells. To determine whether the prosurvival cytokine IL-7 contributes to maintaining these T cells, we examined expression of the IL7R on CD4$^+$ T cells activated during *L. major* infection. We found that T_{CM} cells present in chronically infected mice expressed high levels of the IL7R. However, in addition to the expression of the IL7R by T_{CM} cells, CD62L^{low} cells responding to *L. major* infection expressed the IL7R. Additional experiments revealed that a large percentage of the IL7R^{high}CD62L^{low} cells were Th1 cells, based on transcription at the IFN-γ locus and up-regulation of the Th1-promoting transcription factor T-bet. The up-regulation of T-bet did not prevent IL7R expression by *L. major*-responding CD4$^+$ T cells, nor did the absence of T-bet result in increased IL7R expression. Finally, blockade of IL7R signaling decreased the number of T-bet$^+$CD4$^+$ T cells, reduced IFN-γ production, and inhibited delayed-type hypersensitivity responses in immune mice challenged with *L. major*, indicating that IL7R signaling contributes to the maintenance of Th1 effector cells. Thus, both T_{CM} and Th1 effector cells can express the IL7R during chronic *L. major* infection, which provides a potential means for their long-term survival in addition to the presence of persisting parasites. ***The Journal of Immunology,*** **2009, 182: 5702–5711.**

Concomitant immunity refers to an immune state in which resistance to a pathogen is dependent upon the persistence of the original pathogen. Such is the case following the infection of C57BL/6 mice with the intracellular protozoan parasite *Leishmania major* (1). During a primary infection, IL-12 promotes the generation of a protective Th1 response that leads to a reduction in the parasite burden over the course of 10–12 wk and long-term immunity, although parasites persist in these animals for the lifetime of the host (2, 3). Similarly, individuals who have been naturally infected or immunized by a procedure referred to as leishmanization, where small numbers of live parasites are injected at an inconspicuous location, obtain life-long immunity to reinfection, but are believed to continue to harbor low levels of parasites (4). Although our extensive knowledge of the immune response to *L. major* indicates that Th1 effector cells are critical for immunity (5), how they are maintained once the disease is resolved is not well understood.

Our laboratory has shown that two populations of CD4$^+$ T cells are present in mice that have resolved an infection with *L. major* (6). One is a population of effector T (T_{EFF})[3] cells that express low levels of the lymph node (LN)-homing molecule CD62L and rapidly produce IFN-γ following Ag restimulation. Once generated in the LNs, these T_{EFF} cells migrate to the site of infection. We also found that central memory T (T_{CM}) cells, which express CD62L and circulate through lymphoid tissues, are present in chronically infected mice. These cells are long lived, as they are able to survive in the absence of parasites but can give rise to CD62L^{low} T_{EFF} cells following secondary challenge (6). We have also shown that the ability of T_{CM} cells to differentiate into IFN-γ-producing Th1 effector cells following transfer is dependent upon IL-12 production by the recipient (7). Therefore, one model to account for the maintenance of Th1 effector cells in mice that have resolved a primary infection with *L. major* might be that persistent parasites continually activate some of the long-lived T_{CM} cells to differentiate into short-lived T_{EFF} cells that then mediate concomitant immunity (5).

One way to monitor the potential for the long-term survival of T cells is to assess their expression of the IL7R (CD127). T cells are dependent upon IL-7 for their survival, because signaling through the IL7R promotes cell survival via the up-regulation of antiapoptotic proteins such as Bcl-2 and the glucose transporter Glut1 (8–12). The IL7R is expressed on naive T cells and is down-regulated following TCR engagement (8, 9, 13–21). T cells then

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[3] Abbreviations used in this paper: T_{EFF}, effector T; T_{CM}, central memory T; DTH, delayed-type hypersensitivity; eYFP, enhanced yellow fluorescence protein; FTAg, freeze-thawed Ag; LN, lymph node; dLN, draining lymph node; pi, postinfection; WT, wild type; KO, knockout; *dhfr-ts*, dihydrofolate reductase-thymidylate synthase.

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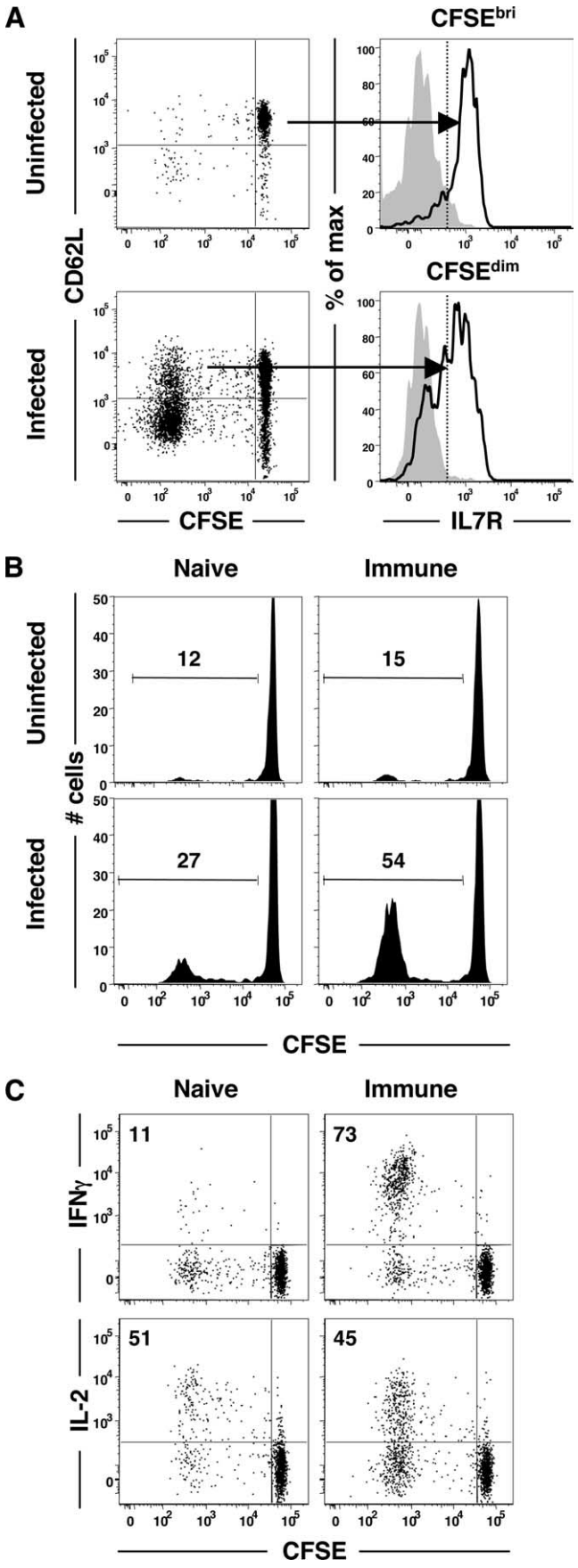

FIGURE 1. CD62L^{high} T_{CM} cells generated in response to *L. major* infection express high levels of the IL7R. *A*, CD4$^+$ T cells were enriched using MACS columns from the lymphoid tissue of C57BL/6 mice (CD45.2) that had been infected in the footpad with *L. major* 12 wk before harvest. The cells were CFSE labeled and transferred to naive CD45.1 recipients. Recipient mice were subsequently infected in the ear with *L. major*. Control mice were left uninfected for the duration of the experiment. Cells were isolated from the auricular dLN after 14 days. Plots have been gated on the CD4$^+$CD45.2$^+$CD45.1$^-$ lymphocytes and are representative of eight mice in three separate experiments. IL7R expression (black line) on the CFSE^{bright}CD62L^{high} cells of uninfected controls (*top*) and CFSE^{dim}CD62L^{high} cells of infected mice (*bottom*) is shown relative to isotype controls (shaded histogram). *B*, CD4$^+$CD62L^{high}IL7R^{high} cells from naive or immune mice (>12 wk pi) were sorted on a FACSAria cytometer with purity of >80%. The cells were CFSE labeled, and 1×10^6 cells were transferred to congenic recipients as described above 1 day before infection with *L. major*. Plots are gated on CD45.2$^+$CD45.1$^-$ lymphocytes and represent 5–6 mice per group in two independent experiments. Numbers indicate the percentages of CFSE^{dim} of the donor cell populations. *C*, Cytokine production by the donor cells from infected mice in *B* was assessed by intracellular cytokine staining. Numbers represent the percentages of cytokine positive cells of the CFSE^{dim} donor population.

have the capacity to up-regulate the IL7R for their continued maintenance (22–24). However, when T cells are continuously stimulated they fail to re-express the IL7R (15). For example, in lymphocytic choriomeningitis virus clone 13 and HIV infections, virus-specific CD8$^+$ T cells that persist during chronic infection exhibit low levels of IL7R expression in addition to impaired function (25–28). In the case of leishmaniasis, one might similarly predict that the pool of Th1 effector cells maintained by continual stimulation would also express low levels of the IL7R.

To determine the potential for long-term survival of the CD4$^+$ T cells activated by *L. major* infection, we have characterized their expression of the IL7R. We found that T_{CM} cells express high levels of the IL7R during chronic infection, which is consistent with their ability to survive long-term. Moreover, a population of CD4$^+$ IL7R^{high} T cells emerged within the first 2 wk despite the continued presence of parasites. Additional studies revealed that both CD62L^{high} and CD62L^{low} CD4$^+$ T cells expressed the IL7R. The presence of *Leishmania*-responsive CD62L^{low} T cells expressing high levels of the IL7R was unexpected and led us to further characterize these cells. By using IFN-γ reporter mice, we found that almost half of the Th1 cells expressed the IL7R. We also showed that the ability of CD62L^{low} CD4$^+$ T cells to express the IL7R was not inhibited by the up-regulation of the Th1-promoting transcription factor T-bet, nor did the absence of T-bet promote elevated IL7R expression. Lastly, we observed a significant decrease in the number of T-bet$^+$CD4$^+$ T cells in immune mice treated with Abs that block IL7R signaling, as well as a reduction in *Leishmania*-specific IFN-γ production and delayed-type hypersensitivity (DTH) responses. Taken together, these results indicate that concomitant immunity to *L. major* may be maintained not only by a population of T_{CM} cells that can differentiate into short-lived effector cells, but also by a pool of resting Th1 effector cells with the ability to access IL-7 to promote their survival.

Materials and Methods

Mice

C57BL/6J, B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1), and B6.129S6-*Tbx21*^{tm1Glm}/J (T-bet knockout (KO)) mice were purchased from The Jackson Laboratory or the National Cancer Institute (Fredricksburg, MD). IFN-γ reporter (*Yeti*) mice were provided by M. Mohrs (Trudeau Institute, Saranac Lake, NY) (29). Animals were maintained in a pathogen-free environment, and experiments were performed in accordance with the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.

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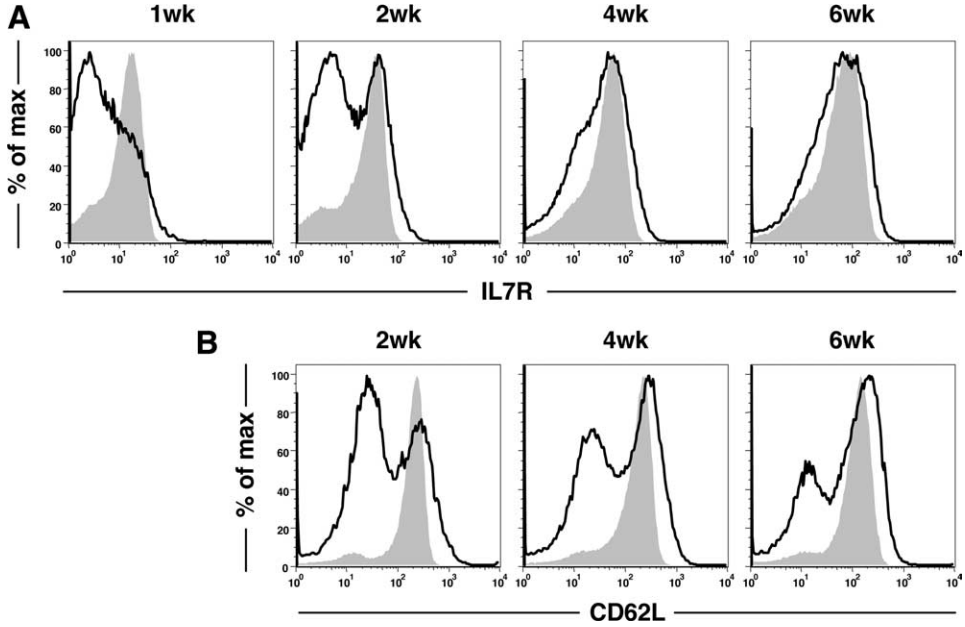

FIGURE 2. The population of activated CD4$^+$ T cells only transiently down-regulates IL7R expression, revealing two populations of IL7R-expressing cells during the primary immune response to infection. *A*, C57BL/6 mice were infected in the footpad with *L. major* and, at the indicated weeks pi, the draining popliteal LN was isolated for flow cytometry. Plots have been gated on CD4$^+$ cells, and the relative levels of IL7R expression are shown on CD44^{low} (shaded histograms) and CD44^{high} (black line) cells. *B*, CD62L expression is shown on the CD44^{high} IL7R^{high} cells (black line) in *A*. CD62L expression on the naive CD44^{low} IL7R^{high} cells (shaded histograms) is shown for comparison. Data are representative of six mice in two independent experiments.

Parasites and infections

L. major (MHOM/IL/80/Friedlin) parasites were grown in Schneider's insect medium supplemented with 20% heat-inactivated FBS and 2 mM glutamine. Infectious stage metacyclic parasites were enriched using density gradient centrifugation (30). Mice were infected in the hind footpad or the ear dermis with 1–2 $\times$ 10^6 parasites with similar results. For IL-12 treatments, IL-12 (0.5 μg) was administered with the parasites and additional IL-12 treatments were given on days 3 and 7 i.p. For secondary challenge, mice were infected in the contralateral footpad. DTH was determined by measuring footpad swelling 48 h postchallenge with a digital caliper (Mitutoyo) and subtracting the average size of the footpad before infection. To quantify parasite burden in the lesion, single cell suspensions of parasites were prepared from the tissue and plated in serial 10-fold dilutions. Each sample was plated in quadruplicate and the mean of the negative log parasite titer was determined after 7 days of culture at 26°C.

Adoptive transfers

CD4$^+$ T cells were enriched from the lymphoid tissue of donor mice before transfer either by using MACS (Miltenyi Biotec) or a FACSAria flow cytometer (BD Biosciences). All cells (except those isolated from *Yeti* mice) were CFSE labeled (1.25 μM) (Molecular Probes), and 5 $\times$ 10^6 cells were transferred to congenic recipients unless noted otherwise as in the Fig. 1 and Fig. 3 legends.

In vivo Ab treatment

Mice were treated with 200 μg of A7R34 (anti-IL7R; Bio X Cell) i.p. every 2–3 days over a 2-wk period. This treatment resulted in a loss of IL-7-dependent B cell precursors in the bone marrow and an inability to stain for surface expression of the IL7R (data not shown).

Flow cytometry

The following Abs used to detect cell surface markers were purchased from eBioscience: CD4, CD45.2, CD45.1, CD44, CD127 (IL7Rα) (PE or allophycocyanin only), and CD62L (PE, allophycocyanin, or PerCP-Cy5.5 only). Before intracellular cytokine staining, cells were stimulated with PMA, ionomycin, and brefeldin A for 4 h in vitro and fixed with 2% paraformaldehyde in PBS. For intracellular detection of cytokines and T-bet, cells were permeabilized with 0.2% saponin and stained with IL-2-allophycocyanin and IFN-γ-PE-Cy7 or T-bet-Alexa Fluor 647 (eBioscience). Data were acquired on an LSR II or a FACSCanto flow cytometer (BD Biosciences). Analysis was performed using FlowJo software (Tree Star). For all samples, gating was established using a combination of isotype and fluorescence-minus-one controls (31).

Ag-specific cytokine production

Splenocytes from naive and *L. major*-infected mice were plated at 4 $\times$ 10^6 cells/well of a 24-well plate and cultured with medium or freeze-thawed parasite Ag (FTAg) for 72 h. FTAg was prepared from stationary phase promastigotes subjected to four cycles of freezing at $-$150°C and thawing at 37°C. Detection of IFN-γ in the supernatant was determined by sandwich ELISA.

Statistics

Statistical analysis was performed using the Student's *t* test with Prism (GraphPad Software), and a value of $p < 0.05$ was considered statistically significant.

Results

T_{CM} cells generated during L. major *infection express the IL7R*

We previously characterized the T cells responding to *L. major* infection by phenotyping proliferating T cells following the adoptive transfer of CFSE-labeled polyclonal CD4$^+$ T cells to naive congenic mice that were subsequently infected with *L. major* (6). We found that a population of T$_{CM}$ cells contributed to immunity to reinfection and could be maintained in the absence of persistent parasites. To determine whether those T$_{CM}$ cells could use IL-7 to survive, we characterized the IL7R expression of T$_{CM}$ cells in chronically infected mice. CD4$^+$ T cells purified from C57BL/6 mice that had resolved a primary infection (referred to as immune mice) were CFSE labeled and transferred to naive congenic recipients. Recipient mice were infected the following day and sacrificed 2 wk following challenge. Although some cells from immune mice that proliferated in response to infection expressed low levels of CD62L, a T cell population expressing CD62L was maintained that we have previously identified as T$_{CM}$ cells (Fig. 1*A*) (6). Many of these CD62L^{high} cells expressed the IL7R at a level equivalent to that of resting CD4$^+$ T cells from uninfected mice, suggesting that T$_{CM}$ cells have the ability to maintain or re-express the IL7R following secondary challenge.

To demonstrate that these IL7R-expressing T$_{CM}$ cells were able to give rise to cytokine-producing T$_{EFF}$ cells, CD4$^+$CD62L^{high} IL7R^{high} cells from immune or naive mice were transferred to naive recipients and subsequently infected as described above. Polyclonal CD62L^{high}IL7R^{high} cells from both naive and immune mice contained a population of *Leishmania*-specific cells that proliferated in response to infection and, as expected, the percentage of cells that were CFSE^{dim} was significantly greater when the cells

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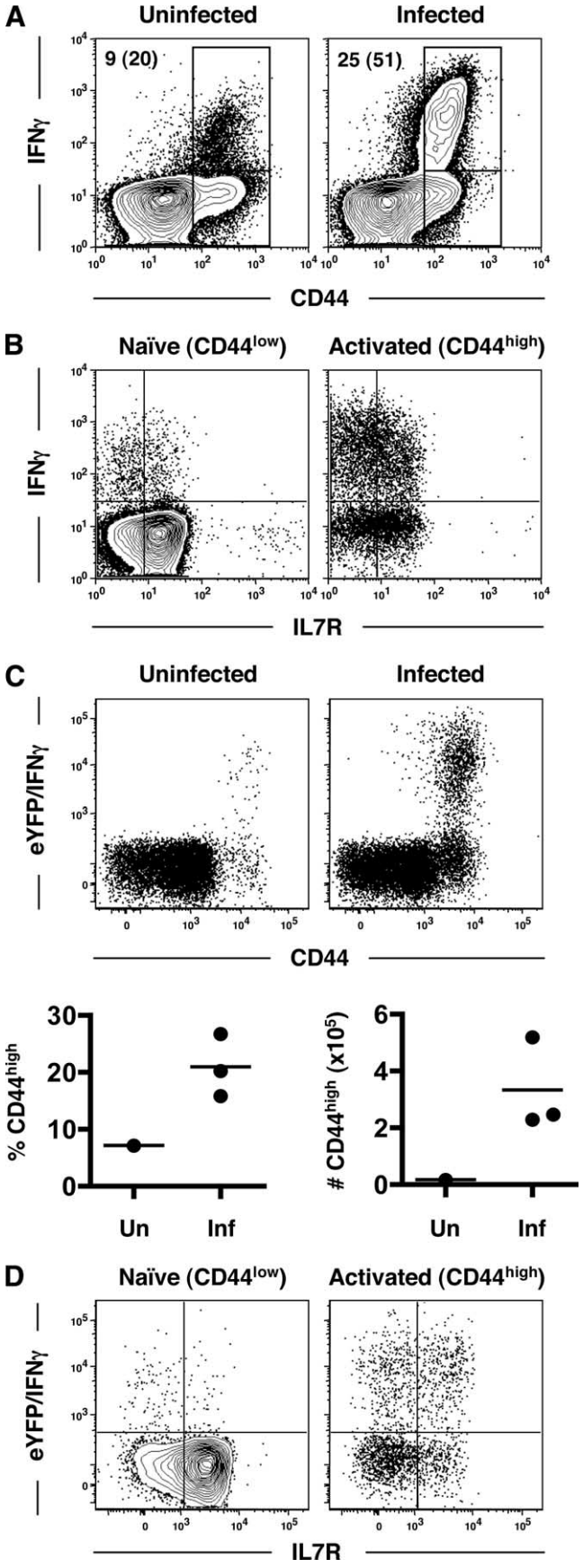

FIGURE 3. Th1-polarized cells can express the IL7R. *A*, C57BL/6 mice were infected in the footpad with *L. major*. After 2 wk, the draining popliteal LN was isolated from infected mice and peripheral LNs were also isolated from uninfected controls. Lymphocytes were examined for IFN-γ production by intracellular cytokine staining. Plots have been gated on $CD4^+$ T cells. Numbers indicate the percentages of $CD4^+$ T cells that are $CD44^{high}$, with the percentage of $CD44^{high}$ cells producing IFN-γ in parenthesis. *B*, IL7R expression vs IFN-γ production is shown in the $CD44^{low}$ vs $CD44^{high}$ cells from the infected mouse in *A*. *C*, Spleens and LNs were isolated from *Yeti* mice (CD45.2) and stained for CD4 and CD44 before cell sorting. Naive CD45.1 recipient mice received 4×10^6 $CD4^+CD44^{low}eYFP^-$ cells and were infected in the ear with *L. major* and then sacrificed after 2 wk. Plots have been gated on $CD45.2^+CD45.1^-$ donor cells from the auricular dLN of infected (Inf) mice and uninfected (Un) controls. *D*, IL7R expression vs eYFP/IFN-γ production is shown on donor $CD4^+$ cells from infected mice in *C* on the *Leishmania*-induced $CD44^{high}$ cells and naive controls ($CD44^{low}$). Plots are representative of three (uninfected) and seven (infected) mice in two independent experiments.

were derived from immune donor cells (Fig. 1*B*) (6). This difference most likely reflects an increase in the total number of *Leishmania*-specific cells within the donor pool of immune mice. Although cells from both categories of donor mice gave rise to IL-2-producing cells, $CD62L^{high}IL7R^{high}$ cells from immune mice gave rise to a significantly larger population of IFN-γ-producing cells than their naive counterparts (Fig. 1*C*). These results are consistent with our previous findings demonstrating that T_{CM} cells can proliferate, lose expression of CD62L, and become IFN-γ producers following reactivation (6). Thus, one way that concomitant immunity can be maintained is by sustaining a pool of T_{CM} cells that can become Th1 cells as needed.

Both CD62L^{low} and CD62L^{high} Leishmania*-activated CD4$^+$ T cells can express the IL7R during an ongoing infection*

A stereotypical response of T cells to TCR stimulation is the rapid down-regulation of the IL7R and, in the case of acute infections where the pathogen is cleared, this down-regulation is transient at the population level (8, 9, 13, 14, 16–20, 24). Although our data indicate that T_{CM} cells express the IL7R in *L. major* immune mice, T_{CM} cells represent a minority of the T cells responding to infection, and we predicted that a large percentage of the responding T cells would fail to re-express the IL7R due to continuous stimulation by persistent parasites. To characterize IL7R expression on $CD4^+$ T cells following in vivo infection with *L. major*, we first looked at the Ag-experienced $CD4^+$ T cells present in the LN draining the site of infection (dLN) by staining cells for the activation-induced adhesion molecule CD44. As an internal control, we compared the level of IL7R expression on the $CD44^{high}$ cells to that of the naive $CD44^{low}$ cells, which are known to be $IL7R^{high}$. We infected C57BL/6 mice with *L. major* and isolated the dLN at the indicated time points. At 1 wk postinfection (pi) we observed a global down-regulation of the IL7R on the pool of $CD44^{high}$ cells (Fig. 2*A*). However, as early as 2 wk pi, when the parasite burden is still increasing, a population of $IL7R^{high}$ cells was evident and gradually came to dominate the pool of activated cells over the next several weeks. These results illustrate that activated $CD4^+$ T cells in the dLN down-regulate the IL7R early following infection, similar to the findings in other viral and bacterial infection models. However, they begin to express increased levels of the IL7R soon after infection despite the ongoing inflammatory response and parasite burden.

Because we found that T_{CM} cells express the IL7R, we initially hypothesized that the $IL7R^{high}$ cells dominant in the $CD44^{high}$ population in the dLN as the infection progresses were developing T_{CM} cells. To ascertain whether this was the case, we examined CD62L expression on the IL7R-expressing cells seen at 2, 4, and 6 wk pi (Fig. 2*B*). The $CD44^{low}IL7R^{high}$ naive cells were largely

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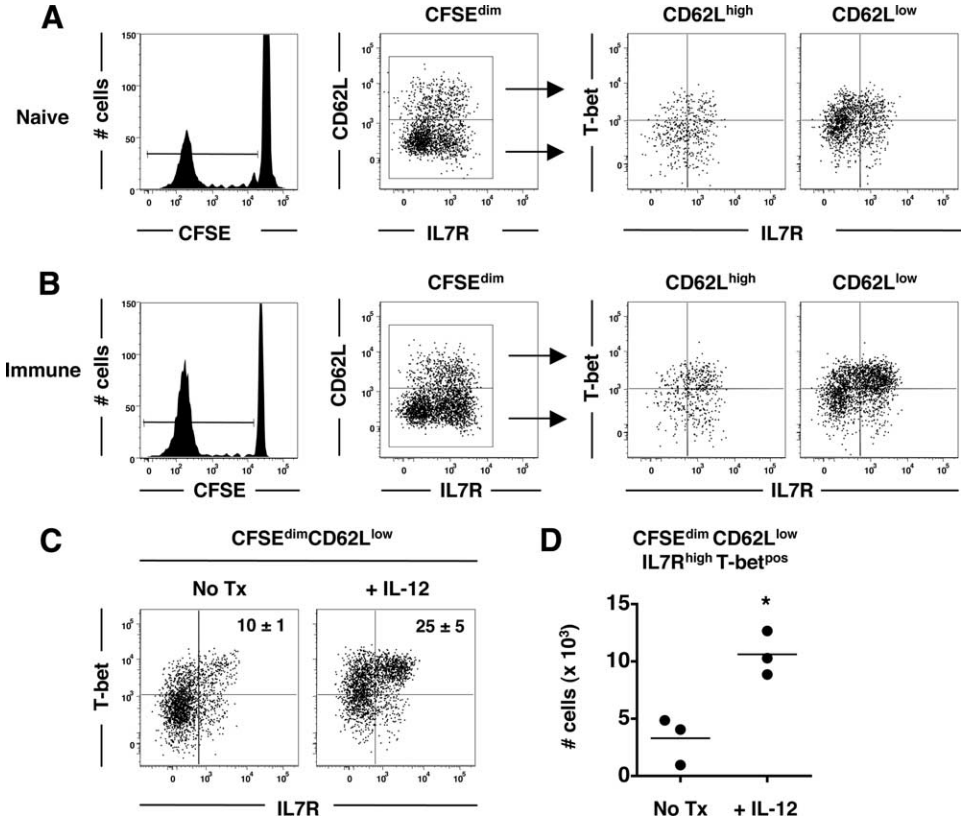

FIGURE 4. A population of IL7R-expressing cells has also up-regulated T-bet expression. *A* and *B*, $CD4^+$ T cells were MACS purified from either naive (*A*) or immune (*B*) donor mice CFSE labeled, and transferred to naive congenic recipients that were then infected in the ear with *L. major*. After 2 wk, dLNs (auricular) were isolated for flow cytometry. Plots have been gated on the donor $CD4^+$ $CD45.2^+CD45.1^-$ cells. Additional plots indicate expression of CD62L, IL7R, and T-bet on the $CFSE^{dim}$ cells. Data are representative of at least five mice in three independent experiments. *C* and *D*, Naive CFSE-labeled donor cells were transferred to naive congenic recipients as in *A*, and IL-12 was administered with the parasites at the time of infection and additionally on days 3 and 7 in the indicated mice. Numbers in *C* represent the percentage of $IL7R^{high}T\text{-}bet^+$ cells of the $CD62L^{low}$ population ± SEM. Asterisks (*) indicate significance of $p < 0.05$. No Tx, No treatment.

$CD62L^{high}$, and within the $CD44^{high}IL7R^{high}$ cells there was a population of $CD62L^{high}$ cells that most likely represents the *Leishmania*-specific T_{CM} population. However, there was also a large percentage of $CD44^{high}IL7R^{high}$ cells that were not expressing CD62L both at 2 wk and at later time points. Thus, our results not only indicate that the IL7R is down-regulated during the first few weeks of infection and relatively soon thereafter re-expressed, but that both T_{CM} cells ($CD62L^{high}$) and $CD62L^{low}$ cells express the IL7R.

Th1-polarized cells have an IL7R^{high} phenotype

Our data indicate that in addition to a population of T_{CM} cells, a population of $CD62L^{low}$ $CD4^+$ T cells activated by *L. major* infection express the IL7R as early as 2 wk after infection. To determine whether these $CD62L^{low}$ $IL7R^{high}$ T cells were effector cells, we asked whether they were able to make IFN-γ. We isolated the dLN from infected animals as in Fig. 2 to assess the capacity of the activated cells to produce IFN-γ ex vivo. In comparison to the LN of an uninfected mouse, there was an increase in both the percent $CD44^{high}$ and also the percent $IFN\text{-}\gamma^+CD4^+$ T cells following *L. major* infection (Fig. 3*A*). We then compared IL7R expression on the activated ($CD44^{high}$) cells vs the naive ($CD44^{low}$) cells of infected mice. We once again observed both $IL7R^{high}$ and $IL7R^{low}$ cells 2 wk pi, but we were surprised to find a percentage of $IL7R^{high}$ cells that were capable of producing IFN-γ (Fig. 3*B*). As a second approach to address this question without having to restimulate the cells in vitro, which can influence IL7R expression, we used the *Yeti* IFN-γ reporter mouse (29). By using this approach, we could also ensure that all eYFP/IFN-γ expression was the result of *L. major* infection. We enriched for naive T cells by sorting for $CD4^+CD44^{low}eYFP^-$ cells from *Yeti* mice and transferred the cells to congenic recipients before infection with *L. major*. Two weeks following infection, we assessed CD44 up-regulation and eYFP/IFN-γ expression within the donor cell population in the dLN. Similar to the dilution of CFSE in the above experiments, little CD44 up-regulation was observed in the donor T cell population of uninfected mice over the 2-wk period. However, there was a large increase in the percentage and total number of $CD44^{high}$ cells in *L. major*-infected mice (Fig. 3*C*). Analysis of the $CD44^{high}$ cells from infected mice showed at least four distinct populations of cells, including one group of cells that expressed eYFP and also expressed high levels of the IL7R (Fig. 3*D*). This indicates that a population of Th1-polarized cells express the IL7R relatively soon after infection.

L. major*-specific $CD4^+$ T cells coexpress T-bet and the IL7R*

As an alternative approach to test for the polarization of IL7R-expressing cells following *L. major* infection, we asked whether the Th1-promoting transcription factor T-bet, which is required for IFN-γ production by $CD4^+$ T cells, was up-regulated in $IL7R^{high}CD4^+$ T cells (32). We analyzed the response of both naive and immune $CD4^+$ T cells to *L. major* by transferring CFSE-labeled cells into naive congenic recipients that were subsequently challenged with *L. major* as in Fig. 1. After 2 wk, we sacrificed the mice and analyzed the donor T cells within the dLN. We gated on the $CFSE^{dim}$ cells and found that $CD62L^{low}$ cells were both $IL7R^{high}$ and $IL7R^{low}$ (Fig. 4*A*). This was consistent with our finding that the IL7R was expressed on $CD44^{high}$ cells directly ex vivo following infection (Figs. 2*B* and 3*B*). Next, by analyzing both the $CD62L^{high}$ and $CD62L^{low}$ cells for expression of T-bet and the IL7R, we observed a small but definable population of cells that coexpressed T-bet and the IL7R 2 wk following *L. major* infection (Fig. 4*A*). These results were somewhat surprising, as recent studies have suggested that the up-regulation of T-bet induces a down-regulation of the IL7R (33, 34). One possible explanation for this discrepancy was that the up-regulation of T-bet that we observed following a primary *L. major* infection was not high enough to

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induce IL7R down-regulation (34). However, cells from immune mice that have resolved a primary infection also coexpressed T-bet and the IL7R following a secondary *L. major* challenge (Fig. 4*B*). To further test whether enhanced T-bet expression might down-regulate the IL7R, we treated mice with IL-12 at the time of infection to induce a strong polarization of the primary response (Fig. 4*C*). Under these conditions, there was a significant increase in both the percentage and total number of IL7R^{high} T-bet-expressing cells (Fig. 4, *C* and *D*). Therefore, we found that T-bet up-regulation, even under IL-12-induced inflammatory conditions, was not associated with the down-regulation of the IL7R. Together, these findings suggest that Th1 cells generated during *L. major* infection have the potential to use IL-7-IL7R signaling to promote their long-term survival rather than face the eminent cell death typically associated with the acquisition of effector function.

IL7R down-regulation is independent of T-bet expression

The experiments described above demonstrate that in the presence of physiological levels of T-bet, the IL7R can be expressed by CD4$^+$ T cells. However, this did not exclude the possibility that higher levels of the IL7R might be expressed in the absence of T-bet. To test this possibility, we performed adoptive transfer experiments as described above. CD4$^+$ T cells from either wild-type (WT) or T-bet KO donors were CFSE labeled, transferred to naive congenic recipients, and infected with *L. major* following transfer. At 2 wk pi proliferation was not significantly different between WT- and KO-derived CD4$^+$ T cells, but in contrast to the WT donor cells, none of the proliferating T-bet KO cells made IFN-γ (Fig. 5*A*). IL7R expression was identical in both the WT- and KO-derived cells, suggesting not only that the IL7R can be expressed concurrently with T-bet in *L. major* infection, but also that no increase in IL7R expression is evident when T-bet is absent (Fig. 5, *B* and *C*). Taken together, these data suggest that T-bet has little influence on the IL7R expression of CD4$^+$ T cells responding to an *L. major* infection.

IL-7 maintains a population of CD4$^+$ effector T cells in immune mice

Our data suggest that a population of Th1 effector cells may be dependent upon IL-7 for their survival. To test this hypothesis, we treated immune mice for 2 wk with Abs that block signaling through the IL7R (anti-IL7R; A7R34). These mice exhibited a decrease in the total T cell pool as well as a decrease in both the naive CD44^{low} and activated CD44^{high} CD4$^+$ T cell populations (Fig. 6*A*). Within the population of activated T cells, there was a significant decrease in the CD62L^{high} T_{CM} cells and the T-bet$^+$ Th1 effector cells (Fig. 6, *B* and *C*). To determine whether the absence of IL7R signaling had a negative effect on the function of Th1 effector cells, we first assayed for the production of parasite-specific IFN-γ following in vitro restimulation with FTAg. When we normalized for the overall reduction in spleen size, we found a significant decrease in the amount of *Leishmania*-specific IFN-γ produced per spleen of those mice treated with the anti-IL7R mAb (Fig. 6*D*). To determine whether there was a decrease in Th1 effector cell function in vivo, we measured the DTH response in immune mice where IL-7R signaling was blocked and found a significant reduction in the DTH response of treated mice (Fig. 6*E*). As expected, because not all of the CD62L^{low} or IFN-γ$^+$ CD4$^+$ T cells expressed the IL-7R, the response was not totally ablated. In addition, there was no reduction in the ability of treated mice to mediate resistance to a secondary challenge (Fig. 6*F*). This was consistent not only with the presence of IL7R$^-$ Th1 effector cells but also with previous data suggesting that only a low number of T_{EFF} cells may be required to mediate protection (35). Nevertheless, these data indicate that, in agreement with its expression of the IL7R, a population of Th1 effector cells uses IL-7 to survive during *L. major* infection.

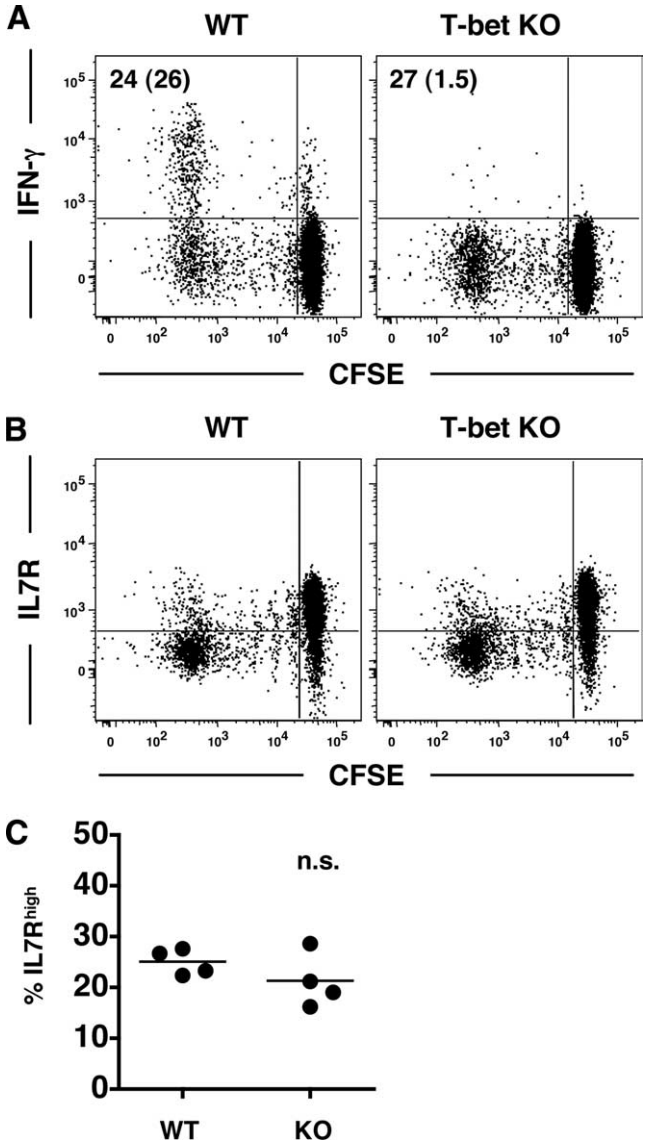

FIGURE 5. *Leishmania*-responding CD4$^+$ T cells express similar levels of the IL7R in the absence of T-bet expression. *A* and *B*, CD4$^+$ T cells were MACS-purified from naive WT or T-bet KO donors. Cells were CFSE labeled and transferred to naive CD45.1 congenic recipients that were infected in the ear with *L. major* the following day. At 2 wk pi, auricular dLNs were analyzed for CFSE dilution and IFN-γ production (*A*) or IL7R expression (*B*). Plots are gated on CD45.2$^+$CD45.1$^-$ cells and are representative of eight mice in two independent experiments. Numbers in *A* indicate the percentage of donor cells that are CFSE^{dim} with the percentages of IFN-γ$^+$ cells of the CFSE^{dim} population in parenthesis. *C*, The percentage of CFSE^{dim} cells expressing the IL7R is shown and was not significant (n.s.) between the two groups.

Discussion

Concomitant immunity is responsible for the substantial resistance to reinfection seen following the resolution of a primary infection with *Leishmania major*, but how this immunity is maintained is not well understood. Our previous data indicate that there is a pool of Ag-reactive CD4$^+$CD62L^{high} T_{CM} cells in immune mice that contribute to concomitant immunity (6). In this study we show that these CD4$^+$ T_{CM} cells express high levels of the IL7R and are able

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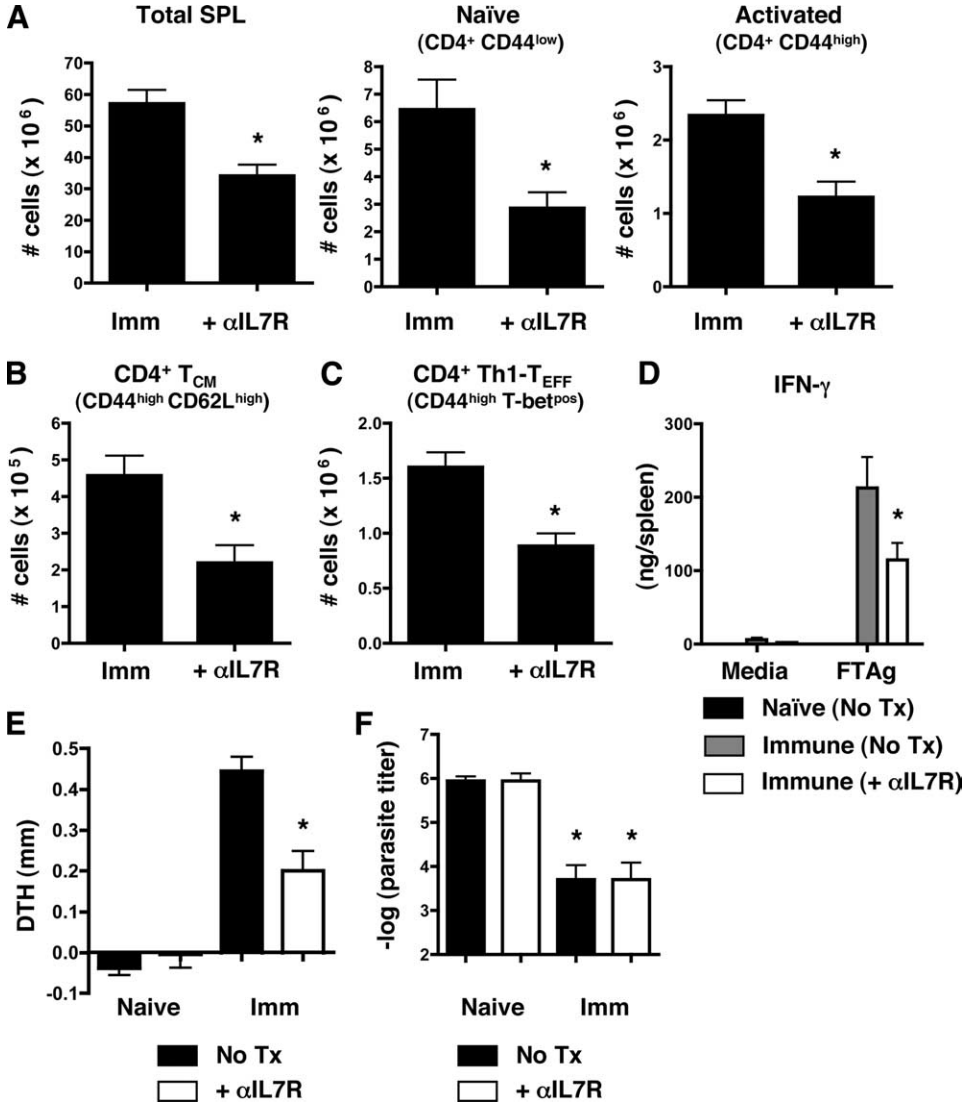

FIGURE 6. $CD4^+$ effector T cells use IL-7 in immune mice. *A–C*, Immune C57BL/6 mice were either treated with blocking Ab (+αIL7R) against the IL7R (anti-IL7R (A7R34); 200 μg every 2–3 days) or left untreated (Imm) as controls. After 2 wk of treatment the animals were sacrificed and the total numbers of cells in each of the indicated populations were calculated. Data are presented as the mean ± the SEM. SPL, Splenocytes. *D*, Splenocytes were also stimulated with leishmanial FTAg, and the supernatants collected after 72 h of culture were assayed for IFN-γ production. *E*, A second group of Ab-treated mice was challenged with *L. major* in the contralateral footpad, and DTH was measured by footpad swelling 48 h after secondary challenge. *F*, Parasite burden was determined by limiting dilution assay 2 wk after challenge, during which time no anti-IL7R treatment (No Tx) was administered. DTH and parasite burden in naive mice are shown as a control. Asterisks (*) indicate significance of $p < 0.05$.

to give rise to IFN-γ-producing Th1 cells after secondary challenge. Because IL-7 and IL7R signaling are required for T cell survival (8–10), these results are consistent with the ability of T_{CM} cells to persist long term in the absence of parasites (6). In contrast, because Th1 effector cells generated following *L. major* infection are believed to be short lived (6, 36, 37), we anticipated that Th1 cells generated during infection would not express the IL7R. This result would be consistent with studies in other chronic infections, where the presence of persisting pathogens is associated with Ag-specific T cells that do not express the IL7R (15, 25–28). However, we identified a population of infection-induced $CD4^+$ cells in *L. major*-infected mice that were $CD62L^{low}$ and expressed high levels of the IL7R. We characterized these T cells and found that a percentage of them were Th1 cells, based on their ability to make IFN-γ and express T-bet. Finally, by treating infected mice with blocking Abs against the IL7R, we found that both T_{CM} and Th1 effector cells are reduced in number in the absence of IL7R signaling, and this reduction correlated with a reduction in the magnitude of the immune response as measured by IFN-γ production and a DTH response. Thus, these results describe a population of $CD4^+IL7R^{high}CD62L^{low}T\text{-}bet^+$ T cells that we believe can not only contribute to concomitant immunity, but have the potential to be maintained by IL7R signaling.

One of the principal roles of IL-7 is to promote the survival of both naive and memory T cells, and expression of the IL7R following infection is thought to contribute to the dynamic changes in the quantitative T cell response during the expansion and contraction phases of an immune response (8, 38). For example, at the peak of the expansion phase to an acute viral or bacterial infection, the majority of the Ag-specific cells are $IL7R^{low}$ and are destined to be a short-lived population that can execute effector function and need not be maintained after the pathogen is cleared (8, 17–19, 21). In contrast, after contraction occurs the remaining Ag-specific T cells express high levels of the IL7R, which promotes their continued maintenance. Although these results suggest that IL7R expression plays a role in T cell contraction, recent studies have shown that while expression of the IL7R may be necessary for T cell survival, it does not guarantee the long-term survival of T cells (39, 40). This may in part be due to the fact that the amount of IL-7 available to cells is limited. However, our findings that both T_{CM} and Th1 cells express the IL7R suggest that both cell types have the potential to be maintained by IL-7. In support of this hypothesis, we found a global decrease in the $CD4^+$ T cell populations of immune mice subjected to IL7R-signaling blockade, which correlated with a decrease in IFN-γ production and the DTH response to a secondary challenge. A practical consequence of IL7R

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expression on these infection-induced T cell populations is that they could be expanded by exogenous treatment with IL-7, which has been shown to enhance the maintenance of memory T cells in other systems (16, 41–43).

One of the unexpected findings of this study was that despite the presence of persistent *L. major* parasites, immune mice contain *L. major*-responsive $CD4^+$ T cells that express the IL7R. This is in contrast to certain chronic viral infections such as lymphocytic choriomeningitis virus clone 13 and HIV, where T cells become functionally exhausted, are impaired in their ability to survive, proliferate, or secrete effector cytokines, and fail to express the IL7R (25–28, 44–46). However, the maintenance of IL7R-expressing T cells under conditions of concomitant immunity is not unique to *L. major*. For example, following infection with the related protozoan parasite *Trypanosoma cruzi*, where parasites persist at low levels in multiple tissues, a small percentage of T cells express the IL7R (23, 47, 48). Similarly, latent γ-herpesvirus persists for the life of the host under T cell-mediated control, and a percentage of the virus-specific $CD8^+$ T cells express high levels of the IL7R and provide functional secondary immunity (49, 50). The factors that determine whether chronic infections lead to the generation of functional IL7R-expressing memory T cells vs an exhausted T cell population that fails to express the IL7R are unknown. However, a likely difference may be the degree to which the Ag-responsive T cells are continuously stimulated (51). The fact that the IL7R is down-regulated upon TCR engagement suggests that many of the responding T cells present in *L. major*-infected mice were not recently stimulated through their TCR. Indeed, although *L. major*-infected mice harbor parasites following resolution of the disease, the number of parasites is extremely low, and many Ag-specific T cells may not be coming in contact with parasites. Moreover, the ability of T cells to be stimulated by parasites may be even further reduced due to the fact that *L. major*-infected macrophages have been shown to function poorly as APCs compared with noninfected cells (52). Thus, our data may suggest that many of the parasites are sequestered in a way that mimics pathogen clearance, allowing for the emergence of a population of IL-7R-expressing Th1 cells.

The optimal Th1 immune response would include the generation of sufficient effector T cells to control the infection while also generating memory T cells. One way that this might occur is by asymmetrical cell division at the initiation of the infection, whereby one daughter cell becomes a T_{EFF} cell while the other adopts memory characteristics (53). This model could explain how T_{CM} cells generated in leishmanial infection develop. However, the factors promoting the strongest Th1 response might be predicted to preclude the development of Th1 effector memory cells. In addition to the fact that IFN-γ and IL-12 have detrimental effects on T cell survival and/or memory T cell development (54–57), it has recently been demonstrated that the transcription factor required for commitment to the Th1 lineage, T-bet, inhibits IL7R expression (33, 34). Thus, in $CD8^+$ T cells, high levels of T-bet were associated with low levels of IL7R expression and the generation of short-lived effector cells; similarly, overexpression of T-bet inhibited IL7R expression in $CD4^+$ T cells (33, 34). Nevertheless, we found that $CD4^+$ T cells that have up-regulated T-bet can also express high levels of the IL7R during both a primary and secondary challenge with *L. major*. Moreover, even when we administered IL-12 during infection to further boost T-bet expression, IL7R expression was maintained. Why we failed to observe an inverse correlation between T-bet and IL7R is unknown, although one possibility is that IL7R expression is regulated differently in $CD4^+$ and $CD8^+$ T cells (58, 59). However, regardless of the reasons, our data clearly indicate that T-bet expression does not prohibit the development of IL7R-expressing Th1 cells. Thus, while these and previous studies indicate that $CD4^+$ T_{CM} cells in *L. major* infections may develop before the effector stage (7), our current results are consistent with studies indicating that T_{EFF} cells can also develop the capacity to survive long term (22, 23, 60, 61).

There is no successful vaccine for human leishmaniasis even though it is well known that individuals who have resolved a primary infection are resistant to reinfection. Indeed, for centuries people in regions endemic for cutaneous leishmaniasis have protected themselves from disfiguring lesions by intentionally infecting themselves with live parasites in an inconspicuous site, a process known as leishmanization (4). The goal for a leishmanial vaccine is to mimic the quality of protective immunity elicited by infection with WT parasites without having to resort to infections with virulent parasites; defining the T cells involved in this protection is one step in that direction. In our previous studies we showed that mice immunized with a thymidine auxotrophic *L. major* lacking dihydrofolate reductase-thymidylate synthase (*dhfr-ts*$^-$) generated a long-lived $CD4^+$ T_{CM} cell population and were protected against *L. major* challenge (6, 62). However, the resistance was not as effective as that associated with the resolution of a primary infection with virulent *L. major*, which was attributed to the absence of $CD4^+CD62L^{low}$ Th1 effector cells in the *dhfr-ts*$^-$-immunized mice (6). We hypothesized that $CD62L^{low}$ cells required persisting parasites to be maintained, although given our new results it is tempting to speculate that the $CD4^+CD62L^{low}IL7R^{high}T\text{-}bet^+$ T cell population we have identified in immune mice fails to be generated following immunization with *dhfr-ts*$^-$, an issue we are currently investigating. Nevertheless, a key to developing a better vaccine may require increasing the frequency of $IL7R^{high}T\text{-}bet^+$ T cells over a yet undefined threshold (63). Additionally, while we have focused on IL7R expression in this study, other characteristics of the $CD4^+CD62L^{low}T\text{-}bet^+$ T cell population may be important, such as the ability of these T cells to produce multiple cytokines such as IL-2, TNF-α, and IFN-γ (64, 65). The challenge of future studies is to identify various adjuvants and boosting regimes, including the administration of IL-7, that enhance the generation of both T_{CM} cells and $CD4^+CD62L^{low}IL7R^{high}T\text{-}bet^+$ cells and thus promote more robust immunity from a vaccine.

In summary, we have demonstrated for the first time that the IL7R is expressed on several populations of $CD4^+$ T cells over the course of infection with *L. major*. Expression of the IL7R on the population of T_{CM} cells that is maintained in chronically infected mice is consistent with previous studies showing that T_{CM} cells can use IL-7 to promote their long-term survival. However, in the current study we show that the presence of low levels of *L. major* parasites does not hinder the ability of these cells to express the IL7R. Moreover, the emergence of an IL7R-expressing population of Th1-polarized effector cells was observed early following infection, during the period of high parasite burden concurrent with the onset of the inflammatory response. Our results show that Th1 cells with the potential to signal through the IL7R contribute to concomitant immunity and suggest that the elicitation of this population may improve the efficacy of future vaccines to protect against cutaneous leishmaniasis.

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Disclosures

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