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Central memory T cells mediate long-term immunity to *Leishmania major* in the absence of persistent parasites

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Infection with *Leishmania major* induces a protective immune response and long-term resistance to reinfection, which is thought to depend upon persistent parasites. Here we demonstrate that although effector CD4$^+$ T cells are lost in the absence of parasites, central memory CD4$^+$ T cells are maintained. Upon secondary infection, these central memory T cells become tissue-homing effector T cells and mediate protection. Thus, immunity to *L. major* is mediated by at least two distinct populations of CD4$^+$ T cells: short-lived pathogen-dependent effector cells and long-lived pathogen-independent central memory cells. These data suggest that central memory T cells should be the targets for nonlive vaccines against infectious diseases requiring cell-mediated immunity.

Experimental infections with *Leishmania major* have helped define the requirements for the development of T helper type 1 (T_H1) cells *in vivo*[1,2]. Yet how *Leishmania*-specific memory CD4$^+$ T cells develop and are maintained is not understood. This knowledge is critical for the development of leishmanial vaccines. Indeed, although many studies have been done with experimental vaccines against *Leishmania*[3–7], currently there is no vaccine for human leishmaniasis, a disease that causes considerable morbidity and mortality throughout the world[8].

Resolution of a Leishmania infection results in lifelong immunity in both mice and humans[2]. Control of a primary infection does not eliminate all parasites, and the persistent parasites may contribute to the ability of healed animals to maintain immunity. For example, very low doses of *L. major* induce protection in BALB/c mice, but immunity is lost in animals that eliminate all of the parasites[9]. Similarly, *L. major*–infected interleukin (IL)-10–deficient mice show sterile cure (no persistence of pathogenic organisms) and lose their resistance to reinfection[10]. These results could suggest that leishmanial infections—and possibly other chronic infectious diseases—do not induce a true memory response, and that resistance in leishmaniasis reflects the continued presence of effector T (T_{EFF}) cells resulting from the persistent existence of pathogenic organisms. If leishmanial infections fail to generate memory T cells, it may be difficult to develop a nonlive vaccine against leishmaniasis. This has prompted us to characterize the CD4$^+$ T cells that provide immunity to reinfection with *L. major*.

Recent studies have shown that memory T cells are heterogeneous, containing subsets that migrate through lymph nodes—termed central memory T (T_{CM}) cells—and others that migrate to tissues and make effector cytokines (effector memory T (T_{EM}) cells)[11–15]. Experiments with several pathogens indicate that CD8$^+$ T_{CM} cells are derived from a T_{EM} cell population, develop once the pathogen has been cleared, and mediate protective immunity[16,17]. Much less is known about CD4$^+$ T-cell memory, and sufficient differences with memory CD8$^+$ T cells have been noted to suggest that CD4$^+$ and CD8$^+$ T-cell–mediated immunologic memory may be distinct[15].

Here we characterize the CD4$^+$ T cells that mediate infection-induced immunity to *L. major* and directly address the role of parasite persistence in the development and maintenance of CD4$^+$ T-cell memory. Using adoptive transfer of polyclonal T cells from immune mice, we demonstrate that *Leishmania*-specific T_{CM} cells develop in the presence of parasites and can provide protection to challenge infection. We also show that whereas effector T cell responses are lost if parasites are eliminated, T_{CM} cells are maintained and protect mice against challenge infection. These data suggest that expansion of the T_{CM} cell population, rather than targeting short-lived effector T cells, should be the goal for vaccines against leishmaniasis and possibly other infectious diseases requiring cell-mediated immunity.

RESULTS

Immune mice maintain lymph node–homing CD4$^+$ T cells

Resistance to reinfection is thought to be controlled in part by the presence of T_{EFF} cells that migrate to the challenge site and mediate delayed-type hypersensitivity (DTH). This response does not require homing of T cells to the lymph nodes, as treatment of immune C57BL/6 (B6) mice during a secondary infection with a blocking antibody against CD62L, a molecule that allows T cells to enter lymph nodes from the blood,[18] abrogated homing to lymph nodes, but had no effect on DTH or immunity (see **Supplementary Fig. 1** online). Because another critical component of memory may be the ability of T cells to expand upon reinfection, we asked whether a second population of T cells from immune mice—analogous to T_{CM} cells—preferentially homes to the lymph nodes

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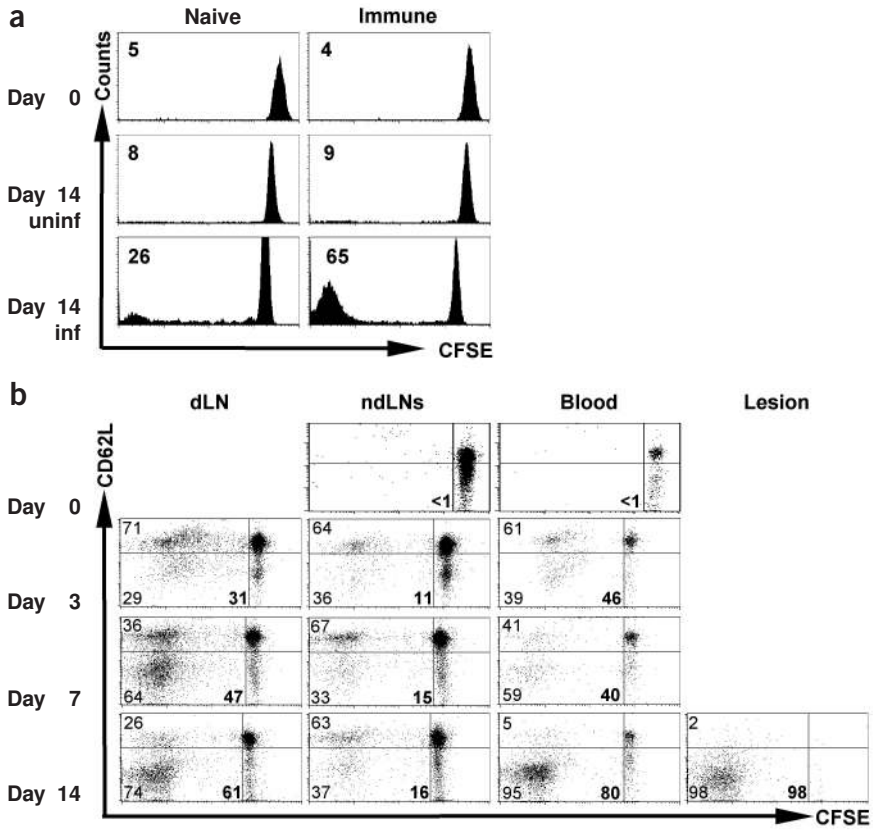

Figure 1 Central memory CD4+ T cells develop during *L. major* infection. (**a**) Immune cells proliferate in the dLN. Mice (Thy1.2) were challenged with *L. major* after receiving CFSE-labeled CD4+ T cells (Thy1.1) from naive or immune mice, and histograms gated on donor cells from the dLNs are shown; numbers represent percentage of donor $CFSE^{dim}$ T cells. (**b**) T_{CM} cells downregulate CD62L and migrate to lesions. Immune CD4+ T cells were transferred as in **a**. Bold numbers represent percentage of donor $CFSE^{dim}$ T cells; numbers in corners represent percentage of $CD62L^{high}$ or $CD62L^{low}$ $CFSE^{dim}$ cells. Data are representative of three or more experiments. Uninf, uninfected; inf, infected.

draining the site of challenge and proliferates. To address this question, we transferred carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4+ T cells from naive or immune B6 mice into naive Thy1-disparate B6 recipients; transfer of purified CD4+ T cells from immune mice provides protection in naive recipients (**Supplementary Fig. 2** online). These mice were subsequently challenged with *L. major*, and we assessed the proliferative response of the donor T cells in the draining popliteal lymph nodes (dLN).

We found no evidence of spontaneous proliferation by donor T cells from either immune or naive mice after transfer into mice that were not challenged with *L. major*. In contrast, donor T cells from both naive and immune mice were found to proliferate in the dLN after challenge (**Fig. 1a**). Proliferation by immune T cells was substantially greater than that by naive T cells. We observed proliferation, which increased over time, of donor T cells from immune mice in the dLN as early as 3 d after challenge infection (**Fig. 1b**). These data indicate that an expanded population of antigen-reactive CD4+ T cells persists in immune mice that, as indicated by their lymph node–homing capacity, possess the primary attribute of T_{CM} cells.

T_{CM} cells downregulate CD62L and migrate to lesions

The lymph node–homing molecule CD62L is downregulated after activation, and loss of CD62L expression is coupled to the development of tissue-homing T_{EFF} cells during an immune response[19–21]. Before adoptive transfer, CD4+ T cells from immune mice contained a higher percentage of $CD62L^{low}$ and $CD44^{high}$ cells than those from naive animals (**Supplementary Fig. 3** online). At day 3 after challenge, 71% of the cells in the dLN that responded to infection by proliferating expressed high levels of CD62L, whereas by day 14 only 26% of the proliferating cells still maintained expression of CD62L (**Fig. 1b**). Moreover, by 2 weeks after infection, 80% of the donor T cells in the blood were $CFSE^{dim}$, indicating they had proliferated, and most had downregulated CD62L. We were unable to isolate measurable numbers of donor T cells at the site of infection at 3 or 7 d, but by 2 weeks donor T cells that had divided and lost expression of CD62L—most likely tissue-homing emigrants from the dLN—were present in the lesions.

Although the majority of proliferating donor T cells in the dLN downregulated expression of CD62L, some maintained high levels of CD62L. Because we found a population of $CD62L^{high}$ $CFSE^{dim}$ CD4+ T cells in the nondraining lymph nodes (ndLN) (**Fig. 1b**), we believe that after proliferation in the dLN, the subset of donor T cells that maintained or re-expressed CD62L migrated to and through the ndLN. This would allow the maintenance of a population of antigen-experienced, lymph node–homing T cells with migratory and phenotypic characteristics of T_{CM} cells, even as the majority of the T_{CM} cells differentiate into T_{EFF} cells.

CD62L defines distinct *Leishmania*-specific CD4+ T cells

We next asked whether differential expression of CD62L would define not only the migratory potential of *Leishmania*-reactive T cells, but also their cytokine profiles. When T cells from immune mice were stimulated *in vitro* with soluble leishmanial antigen (SLA), two distinct populations of responding cells could be identified based on CD62L expression (**Fig. 2a**). We found that interferon-γ (IFN-γ) production was restricted primarily to the $CD62L^{low}$ cells, whereas an equivalent percentage of $CD62L^{high}$ and $CD62L^{low}$ cells produced IL-2. Despite the similar percentages of IL-2–producing cells, the mean fluorescence intensity (MFI) was twofold higher in $CD62L^{low}$ CD4+ T cells. To confirm that antigen-specific IFN-γ production was confined to $CD62L^{low}$ CD4+ T cells, we purified $CD62L^{high}$ and $CD62L^{low}$ fractions from immune mice and stimulated them with SLA. Although both $CD62L^{high}$ and $CD62L^{low}$ CD4+ T cells responded to SLA by proliferating, the production of IFN-γ was restricted to the $CD62L^{low}$ population (**Fig. 2b**). Consistent with these results, the accumulation of IFN-γ in the supernatants was significantly higher in the $CD62L^{low}$ population (**Supplementary Fig. 4** online).

To determine whether CD62L expression defined functionally distinct T cell subsets *in vivo*, we analyzed the cytokine responses of transferred immune cells after infection. Only cells that had proliferated in response to infection produced substantial levels of IFN-γ (**Fig. 2c**).

A small population of donor T cells from naive mice produced IFN-γ in the dLN 2 weeks after infection. But as early as 1 week after infection, a large percentage of immune donor T cells produced IFN-γ in the dLN, which increased by 2 weeks after infection. Consistent with our *in vitro* results, the overwhelming majority of the IFN-γ–producing cells in the dLN were contained in the $CD62L^{low}$ population (**Fig. 2d**). Furthermore, the donor T cells present in the lesion expressed low levels of CD62L and >90% of the cells produced IFN-γ. The higher percentage of IFN-γ^{pos} $CD62L^{low}$ cells in the lesion compared with the dLN may reflect an additional differentiation of the effector T cell population, such as the acquisition of P- and E-selectin ligands[21]. Although there was a measurable population of proliferating donor T cells in the ndLNs, the cells were predominantly IFN-γ^{neg} and $CD62L^{high}$ (**Fig. 2c,d**). These results demonstrate a phenotypic and functional dichotomy of antigen-experienced $CD4^+$ T cells that develop after infection with *L. major*—lymph node–homing, $CD62L^{high}$, IFN-γ^{neg} T_{CM} cells and tissue-homing, $CD62L^{low}$, IFN-γ^{pos} T_{EFF} cells.

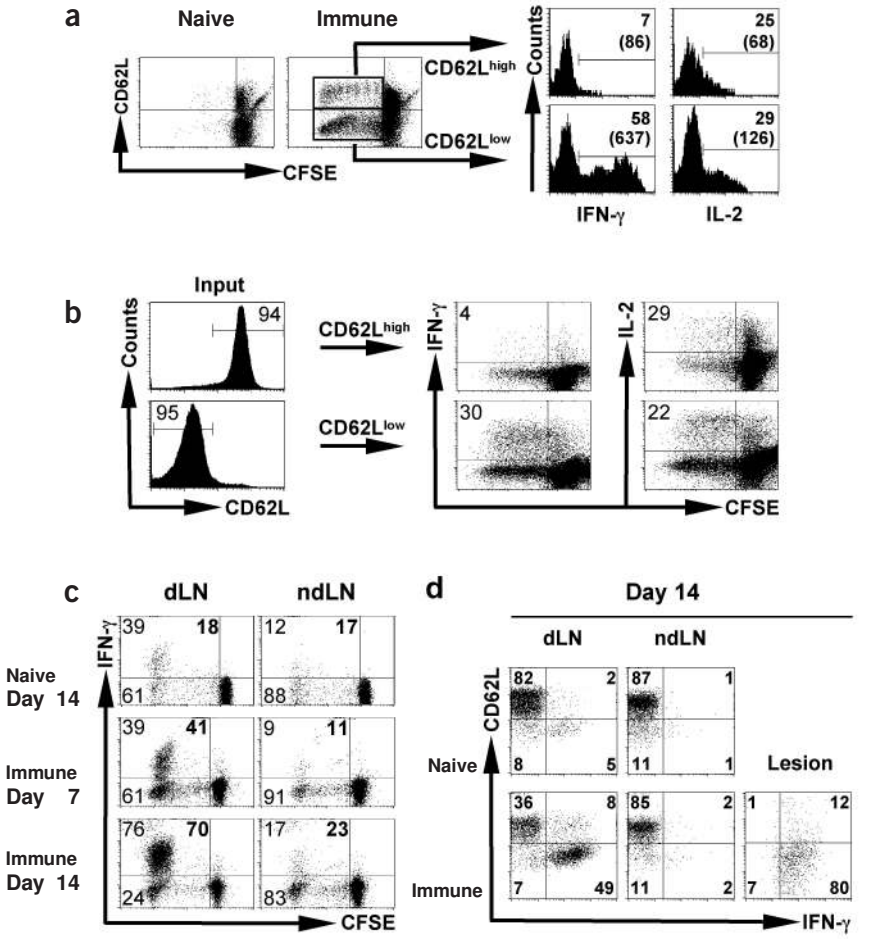

Figure 2 CD62L defines functionally distinct populations of $CD4^+$ T cells. (**a**,**b**) Proliferating $CD62L^{high}$ $CD4^+$ T cells do not produce IFN-γ. Total (**a**) or sorted (**b**) immune cells were stimulated *in vitro* with SLA. Numbers represent percentage of IFN-γ^{pos} or IL-2^{pos} $CFSE^{dim}$ T cells or (MFI). (**c**,**d**) Production of IFN-γ by immune $CD4^+$ T cells *in vivo*. Mice were challenged with *L. major* after receiving CFSE-labeled $CD4^+$ T cells from naive or immune mice. (**c**) Bold numbers represent percentage of donor $CFSE^{dim}$ T cells; numbers in the upper left corner represent percentage of IFN-γ^{pos} $CFSE^{dim}$ cells. (**d**) Numbers represent percentage of donor T cells in each quadrant. Data are representative of two or more experiments.

T_{CM} cells mediate protective immunity

To directly address whether the T_{CM} cells present in immune mice could differentiate into T_{EFF} cells and mediate protection, we purified $CD4^+$ $CD62L^{high}$ T cells from immune mice and tested their protective capacity. Immunity was evident in mice that received $CD4^+$ $CD62L^{low}$ T cells 3 weeks after challenge, consistent with their ability to rapidly produce IFN-γ (**Fig. 3a**). Despite the lack of antigen-specific IFN-γ production by $CD62L^{high}$ $CD4^+$ T cells (**Fig. 2**), by 6 weeks mice that received either $CD62L^{low}$ or $CD62L^{high}$ $CD4^+$ T cells were protected against challenge infection (**Fig. 3b**). We hypothesized that the $CD62L^{high}$ $CD4^+$ T cells provided protection to *L. major* challenge by differentiating into tissue-homing T_{EFF} cells. To test this, we transferred purified CFSE-labeled immune $CD62L^{high}$ $CD4^+$ T cells into naive mice and challenged with *L. major*. By 2 weeks after challenge, there was equivalent proliferation in the dLN of mice that received total $CD4^+$ T cells and purified $CD62L^{high}$ cells (**Fig. 3c**). Critically, in mice that received purified $CD62L^{high}$ T cells, a population of donor $CD4^+$ T cells was isolated from the site of infection at 2 weeks that was uniformly $CFSE^{dim}$ and expressed low levels of CD62L (**Fig. 3c**). These results provide direct evidence that lymph node–homing T_{CM} cells can mediate protective immunity against *L. major* infection by differentiating into tissue-homing $CD62L^{low}$ T_{EFF} cells after challenge.

T_{CM} cells in the ndLNs mediate protective immunity

If T_{CM} cells represent an expanded population of antigen-specific T cells migrating though lymph nodes, then ndLN from immune mice should contain T_{CM} cells that could mediate protection. To test this, we assessed the ability of cells from the ndLN of immune mice to provide protection after adoptive transfer. Cells isolated from ndLN of immune B6 mice responded to leishmanial antigen *in vitro* by proliferating (**Fig. 4a**), and secreting IL-2 but not IFN-γ (**Fig. 4b**), demonstrating that a population of antigen-reactive T cells, but not T_{EFF} cells, were present in the ndLN. In contrast, cells from the dLN of immune mice proliferated and produced both IL-2 and IFN-γ in response to antigen stimulation. Nevertheless, despite the lack of an effector response by the cells isolated from ndLN, these cells were able to confer considerable protection to challenge infection at levels that were comparable to cells from the dLN (**Fig. 4c**). These data demonstrate that the T_{CM} cells found in the ndLN provide a pool of T cells that can mediate protective immunity.

T_{CM} cells persist in the absence of parasites

One of the most important questions regarding immunologic memory is the role of antigen persistence in the maintenance of functional memory T cells. To directly address whether T_{CM} cells were maintained if parasites were eliminated, we used a thymidine

auxotrophic mutant of *L. major* (*dhfr-ts*$^-$; parasites designated as such lack the gene for dihydrofolate reductase-thymidylate synthase) that is unable to cause pathology or persist *in vivo*[22]. We infected B6 mice with either wild-type *L. major* or *dhfr-ts*$^-$; at 4 weeks after infection, when parasites were detected in both groups of mice, the immune response—as measured by antigen-specific IFN-γ production and DTH—was equivalent (**Fig. 5a**,**b**). As expected, by 15 weeks after infection, mice infected with wild-type *L. major* contained low levels of parasites (10^2–10^3 parasites) in the lesion and the dLN, whereas mice infected with *dhfr-ts*$^-$ had no detectable parasites (data not shown)[22,23]. It has been reported that immune responses to *L. major* are not maintained in the absence of persistent parasites[9,10]. Similarly, we found that mice infected with wild-type *L. major* mice had DTH and antigen-specific IFN-γ recall responses, whereas mice infected for 25 weeks with *dhfr-ts*$^-$ did not show *Leishmania*-specific DTH or IFN-γ responses (**Fig. 5c**,**d**), indicating that *Leishmania*-specific T_{EFF} cells were lost in the absence of persistent parasites. To determine whether T_{CM} cells are present in animals infected for 25 weeks with *dhfr-ts*$^-$, CFSE-labeled CD4$^+$ T cells from these mice were transferred into naive Thy1-disparate B6 hosts that were subsequently challenged with *L. major*. Infection-induced proliferation of CD4$^+$ T cells from *dhfr-ts*$^-$-infected mice was evident in the dLN by 2 weeks after challenge, with a much smaller response by donor T cells from naive mice (**Fig. 5e**). Thus, T_{CM} cells can be maintained once parasites are no longer present.

Persistent parasites are not required to maintain immunity

To test whether the presence of T_{CM} cells in mice that no longer contain parasites is associated with long-term immunity and resistance to reinfection, we challenged the mice with virulent *L. major* 25 weeks after they had resolved a primary infection with wild-type *L. major* or *dhfr-ts*$^-$ parasites. As mentioned above, at this time no parasites could be detected in the *dhfr-ts*$^-$–infected mice. Although the protection evident in mice that had resolved a primary infection with wild-type *L. major* was greater, we observed significant ($P < 0.01$) protection in mice that we had infected with *dhfr-ts*$^-$ parasites (**Fig. 5f**). These results indicate that in the absence of an effector response or persistent parasites, substantial protective immunity is maintained.

Our data show that T_{CM} cells—but not T_{EFF} cells—persist in the absence of parasites, and thus that the protective capacity of CD4$^+$ T cells isolated from mice that had resolved infection with *dhfr-ts*$^-$ should be found exclusively in the CD62L^{high} compartment. To directly address this, we sorted CD4$^+$ T cells based upon CD62L expression from mice that had resolved infection with *dhfr-ts*$^-$ parasites. CD62L^{high}, but not CD62L^{low}, CD4$^+$ T cells mediated protection after transfer into naive hosts (**Fig. 5g**), further demonstrating that in the absence of persistent parasites, T_{EFF} cells are lost while T_{CM} cells are maintained.

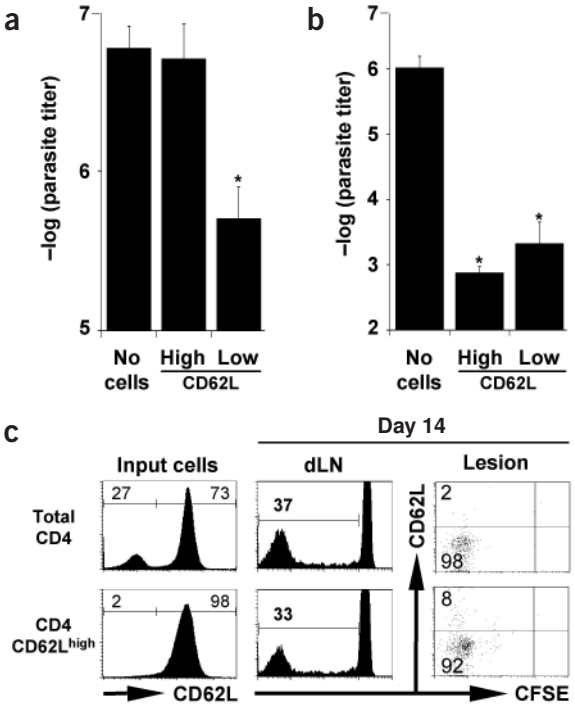

Figure 3 T_{CM} cells mediate protective immunity. (**a**,**b**) Protection by immune T cell populations. Mice were challenged with *L. major* after receiving immune CD62L^{high} or CD62L^{low} cells, and parasite burdens in lesions assessed after 3 (**a**) or 6 weeks (**b**). (**c**) CD62L^{high} CD4$^+$ T cells transferred as in **a** acquire the ability to home to infected tissues. Bold numbers represent percentage of donor CFSE^{dim} T cells. Numbers in dot plots represent percentage of CD62L^{high} or CD62L^{low} CFSE^{dim} cells. Data are representative of two or more experiments. *$P < 0.01$ compared to controls.

DISCUSSION

We have found that two CD4$^+$ T cell populations participate in maintaining immunity to *L. major*, only one of which requires persistent parasites. The CD4$^+$ T cells with the characteristics of effector T cells—tissue homing, CD62L^{low}, IFN-γ^{pos}—mediate DTH responses, are protective and depend on the continued presence of parasites. By adoptively transferring CFSE-labeled CD4$^+$ T cells from Thy1-disparate immune mice, we were able to characterize another polyclonal CD4$^+$ T cell population associated with immunity. These cells have the characteristics of T_{CM} cells: they home to lymph nodes and do not produce IFN-γ, but upon challenge they

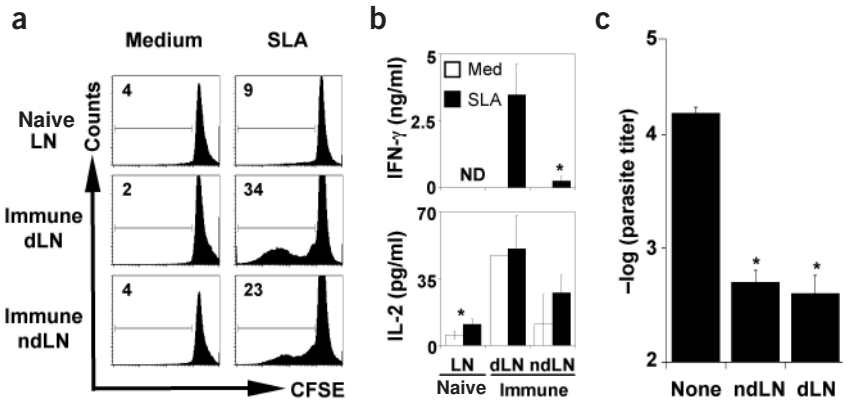

Figure 4 Protective T_{CM} cells are present in ndLNs. (**a**) Proliferative response by CD4$^+$ T cells from lymph nodes after stimulation *in vitro*. Histograms are gated on CD4$^+$ T cells. (**b**) *Leishmania*-specific IFN-γ and IL-2 production by lymph node cells stimulated *in vitro*. (**c**) Mice were challenged with *L. major* after receiving immune cells, and parasite burdens in lesions assessed after 6 weeks. Data are representative of two or more experiments. *$P < 0.01$ compared to controls.

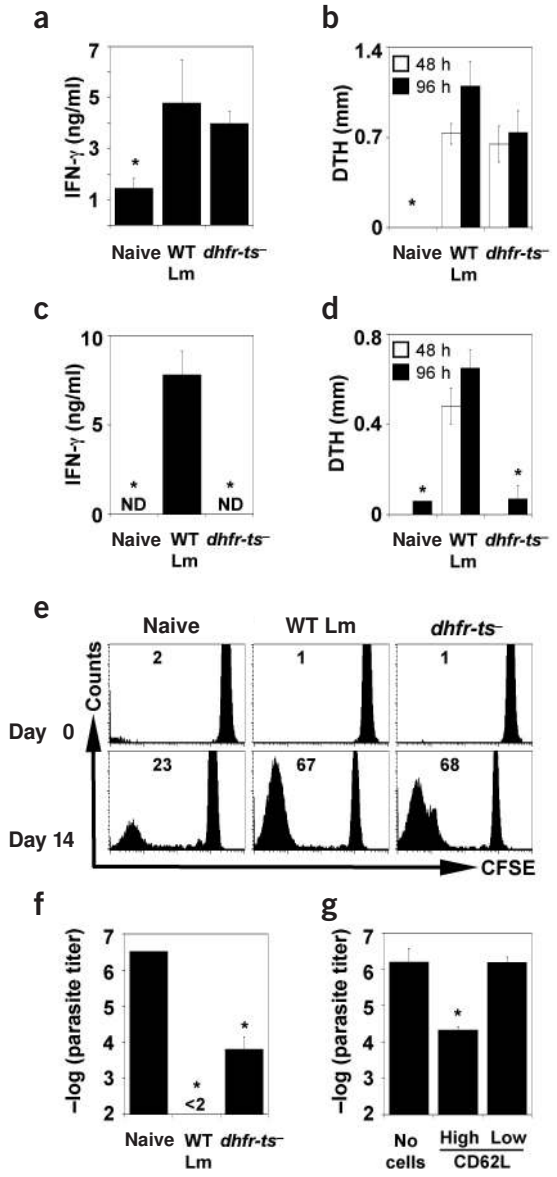

Figure 5 Parasite persistence is required for the maintenance of T_{EFF} cells, but not T_{CM} cells and protective immunity. (**a**–**d**) Equivalent immune responses in mice infected for 4 weeks with wild-type *L. major* or *dhfr-ts*⁻, but loss of effector responses in the absence of persistent parasites. (**a**,**c**) *Leishmania*-specific IFN-γ responses by dLN cells from infected mice at 4 (**a**) or 25 weeks (**c**). (**b**,**d**) DTH in infected mice at 4 (**b**) or 25 weeks (**d**). (**e**) T_{CM} cells are maintained in the absence of persistent parasites. Mice were challenged with wild-type *L. major* after receiving CFSE-labeled $CD4^+$ T cells from 25-week infected mice. Donor T cells from the dLN are shown; bold numbers represent percentage of donor $CFSE^{dim}$ T cells. (**f**) Protective immunity in the absence of persistent parasites. Infected mice (25 weeks) were challenged with wild-type *L. major* and parasites in lesions assessed after 6 weeks. (**g**) $CD62L^{high}$, but not $CD62L^{low}$, $CD4^+$ T cells from *dhfr-ts*⁻-infected B6 mice are protective. Mice were challenged with *L. major* after receiving sorted cells from *dhfr-ts*⁻-infected mice (25 weeks), and parasite burdens assessed after 6 weeks. Data are representative of two or more experiments. $^*P < 0.01$ compared to controls. WT Lm, wild-type *Leishmania major.*

proliferate in the dLN, adopt an effector phenotype, migrate to the site of infection and mediate protective immunity. Critically, they do not require persistent parasites to be maintained. Because *Leishmania*-specific T_{CM} cells survive in the absence of parasites and mediate protective immunity, expansion of this T-cell population is an appropriate goal for vaccines against pathogens that require cell-mediated immunity.

How T_{CM} cells develop is not understood, although previous studies with viral and bacterial infections indicate that $CD8^+$ T_{CM} cells develop only after clearance of pathogens[16,17]. In contrast, our data show that $CD4^+$ T_{CM} cells develop in the presence of persistent parasites; this observation is consistent with studies demonstrating that mice infected with *L. major* contain an expanded population of antigen-reactive $CD62L^{high}$ T cells that can become T_H1 or T_H2 cells[24,25]. The discrepancy may reflect critical intrinsic differences between memory CD4 and CD8 T cells[15]. It has been suggested that $CD4^+$ T_{CM} cells are derived from T cells that fail to differentiate into T_{EFF} cells[26–28], although how a small percentage of cells maintain this phenotype is unknown. Cessation of proliferation due to CTLA-4 expression might be involved[29]; however, in our studies, proliferation by itself does not seem to dictate whether cells become T_{CM} cells or effector T cells.

Some have suggested that the maintenance of cell-mediated immunity requires the presence of antigen[9,10,30,31], but others have shown that memory T cells persist in the absence of antigenic stimulation[32–37]. Our results demonstrate that there are at least two distinct populations of $CD4^+$ T cells that develop after resolution of an infection with *L. major*, only one of which requires the presence of persistent parasites. Although we show that the pathogen-independent T_{CM} cells mediate protective immunity, optimal protection may require both subsets. Previous studies, in which mice were infected with low numbers of parasites and immunity was lost when parasites were eliminated, may indicate that sufficient expansion of the T_{CM} cell pool to provide protection depends on the initial overall T-cell response[9,10]. How parasite dose influences the development and efficacy of T_{CM} cells will be an important area for future study.

The findings presented here have relevance to the development of vaccines for diseases that require cell-mediated immunity, such as leishmaniasis, tuberculosis and AIDS[38,39]. Vaccines that expand the effector T cell pool may not lead to long-term protection, because these cells are short-lived in the absence of persistent antigen. However, the appropriate T cells to expand may be T_{CM} cells. The molecular mechanisms involved in maintaining T_{CM} cells *in vivo*, as well as the protocols necessary to optimally induce these cells, are still unknown; defining both will be critical for better understanding $CD4^+$ T cell memory and for the rational development of vaccines against a broad range of pathogens.

METHODS

Animals. C57BL/6J (B6) and B6.PL-*Thy1*^{a}/*Cy* (B6 Thy1.1) mice were obtained from the Jackson Laboratories. Animals were maintained in a specific pathogen–free environment and tested negative for pathogens in routine screening. All experiments were carried out following the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.

Parasites and antigens. We used wild-type *L. major* (WHO/MHOM/IL/80/Friedlin, wild-type *L. major*) and thymidine auxotrophic *L. major* (E10-5A3, *dhfr-ts*⁻)[22] for these studies. We grew parasites to stationary phase in Grace's insect cell culture medium (Life Technologies) with 20% fetal bovine serum (Hyclone; ≤0.125 EU/ml), 100 U/ml of penicillin, 100 μg/ml of streptomycin and 2 mM glutamine (Sigma). We added thymidine (10 μg/ml, Sigma) to cultures of *dhfr-ts*⁻ parasites. We harvested stationary-phase promastigotes and washed them three times in PBS. SLA was prepared as described previously[40].

Infections. For primary infections, we injected the hind footpads of mice with 2×10^6 stationary-phase wild-type *L. major* parasites or 10×10^6 stationary-phase *dhfr-ts*$^-$ parasites and then allowed the mice to completely resolve the inflammatory lesion. In some infections, a single dose of IL-12 (0.5 μg) was added to the *dhfr-ts*$^-$ parasites to ensure the induction of a strong primary T_H1 response. For secondary infections, immune mice (>12 weeks after primary infection) were rechallenged in the contralateral footpad with 2×10^6 stationary phase wild-type *L. major* parasites. Secondary lesion size was determined by subtracting the size of the footpad before infection from the size of the secondary lesion using digital calipers (Mitutoyo). To quantify parasites in lesions, single-cell suspensions were prepared and plated in twofold serial dilutions (initial dilution of 1:40) in Grace's insect culture medium. Each sample was plated in quadruplicate and the mean of the negative log parasite titer was determined after 7 d of culture at 26 °C.

CD4$^+$ T cell purification and adoptive transfer. Naive or immune B6 Thy1.1 mice were depleted of CD8$^+$ T cells by injection with 250 μg of H35 (Rat IgG2b) 1 and 3 d before they were killed (>95% effective). For *in vivo* studies, cells were isolated from draining popliteal lymph nodes, nondraining lymph nodes (contralateral popliteal and superficial inguinal, axial, brachial and cervical lymph nodes) and spleens, red blood cells were lysed in hypotonic solution, and in some cases lymphocytes were pooled. CD4$^+$ T cells were purified using a T-cell purification column (R&D Systems) according to the manufacturer's recommendations. In some experiments, CD4$^+$ T cells were further separated based on expression of CD62L by MACS columns (Miltenyi Biotec) with 95–98% purity of CD62L^{high} and CD62L^{low} fractions. CD4$^+$ T cells were stained with 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) as previously described[21,41]. Five to ten million CFSE-labeled CD4$^+$ T cells were transferred via the retro-orbital plexus into naive Thy1.2 B6 recipients. Mice were challenged 24 h later with *L. major* as described above. Protection was assessed at 3 or 6 weeks after challenge infection, and data shown are from 3–4 mice per group.

***In vitro* cell culture and cytokine analysis.** Lymph nodes and spleens were harvested from naive or immune B6 mice and single-cell suspensions prepared in complete tissue culture medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, and 5×10^{-5} M β-mercaptoethanol). Lymphocytes were isolated from lesions as previously described[21]. In some experiments draining and nondraining lymph nodes were not pooled. Cells were labeled with CFSE and plated at 2×10^5/well in round-bottom 96-well culture plates in medium alone or in the presence of SLA (50 μg/ml). Purified CD4$^+$ T cells were stimulated in the presence of irradiated syngeneic splenocytes (1×10^5/well). Supernatants were analyzed for cytokine production by sandwich ELISA using paired monoclonal antibodies to detect IL-2 (JES6-1A12 and JES6-5H4) and IFN-γ (R46A2 and polyclonal rabbit antibodies to IFN-γ)[21].

Flow cytometry and intracellular cytokine staining. Cells were stained with fluorochrome-conjugated monoclonal antibodies against CD4 (RM4-5), CD44 (IM7), CD62L (MEL14), Thy1.1 (OX-7), IL-2 (JES6-5H4) and IFN-γ (XMG1.2) or isotype-specific control antibodies (BD Pharmingen) before data acquisition on a FACSCalibur flow cytometer (BD Pharmingen). Analysis was carried out using CellQuest Pro software (Version 5.1, BD Pharmingen). Intracellular cytokine staining was performed as previously described[21]. To examine coexpression of CD62L and cytokines, the metalloproteinase inhibitor TAPI-2 (35 μg/ml; Calbiochem) was included for the final 4 h of stimulation to prevent activation-induced cleavage of CD62L. The concentration of TAPI-2 used neither activated T cells nonspecifically nor induced cell death. All plots are gated on CD4$^+$ T cells; in transfers, all plots are gated on donor (Thy1.1) CD4$^+$ T cells.

Statistics. Results represent the mean ± s.d.. Statistical significance was determined by Student's *t*-test and results were considered significant with a P value <0.01.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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