

IL-4 receptor expression on CD8⁺ T cells is required for the development of protective memory responses against liver stages of malaria parasites

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IL-4 receptor (IL-4R)-deficient CD8⁺ T cells specific for the circumsporozoite protein of *Plasmodium yoelii* develop a severely impaired memory response after priming with parasites. Memory CD8⁺ T cells lacking the IL-4R are unable to establish a stable population residing in nonlymphoid organs, although they develop normally in lymphoid organs. Because memory cells from nonlymphoid organs disappear shortly after immunization, the protective antiparasitic activity of this T cell response also is lost. These results demonstrate that IL-4/IL-4R interactions on CD8⁺ T cells play a critical role in modulating the development and tissue distribution of memory cells induced by parasite immunization. They also indicate that memory cells residing in nonlymphoid tissues are critical for protective immunity against malaria parasites.

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Abbreviations used: IL-4R, IL-4 receptor.

Naive CD8⁺ T cells undergo an intense process of differentiation and proliferation after recognizing antigenic peptides derived from viruses, bacteria, or parasites (1). During the primary response, CD8⁺ T cells develop antimicrobial effector mechanisms as early as 24–48 h after immunization, and acquire the capacity to circulate between lymphoid and nonlymphoid organs (2, 3). These activated cells establish memory populations within 20–25 d that ensure the persistence of efficient protective immunity against reinfection and disease (4–6). Memory CD8⁺ T cells consist of subpopulations, which may differ, in the expression of certain surface markers (4, 7), cytokine receptors (8), and anatomic location. Based on these criteria, at least two different memory subsets have been defined: “central” memory cells, which are found primarily in lymphoid organs, and “effector” memory cells, which reside in nonlymphoid organs (9, 10). Although there is consensus that memory CD8⁺ T cells are heterogeneous pop-

ulations, the precise molecular identity of the different subsets and their respective role in protective immunity has not been elucidated fully.

The mechanisms by which CD8⁺ T cells are activated and differentiated are controlled by members of the cytokine-receptor γ -chain family, particularly IL-2, IL-7, and IL-15. These cytokines seem to promote the proliferation and survival of activated CD8⁺ T cells, and ensure the maintenance of long-lived memory CD8⁺ T cell responses (11). IL-4, another member of this γ -chain cytokine family, also plays a major role in the induction of certain protective CD8⁺ T cell responses. Early studies indicated that IL-4 was necessary to activate CD8⁺ T cells in vitro (12), and to establish in vivo antitumor protective immunity mediated by CD8⁺ T cells (13). Studies with the rodent malaria parasite *Plasmodium yoelii*, using TCR transgenic CD8⁺ T cells specific for the malaria parasite epitope SYVPSAEQI, showed that CD8⁺ T cells transferred into IL-4-deficient mice cannot develop an antigen-specific CD8⁺ T cell response after immunization with parasites (14). In addition, a recent study demonstrated that the induction of CD8⁺ T cell-mediated protective immunity against *Leishmania* parasites also is strictly IL-4 dependent (15).

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These findings, which indicate a major effect of IL-4 on CD8⁺ T cell responses, raised basic questions regarding the nature of the mechanisms that are involved in the regulatory activity of IL-4. It is unknown whether IL-4 is needed during the initial processes of activation of naive CD8⁺ T cells, or whether it plays a role in the development and maintenance of memory populations. Moreover, it has not been determined whether IL-4 directly influences CD8⁺ T cell function through the IL-4 receptor (IL-4R) expressed in the surface of these cells or whether this cytokine effect is indirect—through dendritic or CD4⁺ T cells—both of which are known to exert strong regulatory effects on CD8⁺ T cell responses. Here we describe studies using the rodent malaria system *P. yoelii*, which indicate that IL-4/IL-4R interactions on CD8⁺ T cells are crucial to the development of memory CD8⁺ T cells residing in nonlymphoid tissues. We also establish that this IL-4R-dependent memory subset is critical to protection against malaria parasite infection.

RESULTS

IL-4R-deficient CD8⁺ T cells develop an abnormal response

To study the effect of IL-4 on CD8⁺ T cells, we crossed BALB/c IL-4R KO mice (16) and BALB/c transgenic mice expressing a TCR specific for the SYVPSAEQI epitope located in the circumsporozoite protein of *P. yoelii* (2). After the generation of F1 mice and subsequent backcross, we obtained mice harboring TCR transgenic CD8⁺ T cells lacking the IL-4R. Normal and IL-4R KO TCR transgenic CD8⁺ T cells were used in adoptive transfer experiments to compare their activation profile and homing behavior after *in vivo* activation. Mice receiving IL-4R KO or normal transgenic CD8⁺ T cells were immunized 24 h later, with radiation-attenuated *P. yoelii* sporozoites; the respective responses were evaluated at different time points. As determined by ELISPOT, the antigen-specific CD8⁺ T cell responses in spleen of mice receiving normal or IL-4R KO cells were similar 4 d after immunization. However, at days 6 and 10 after immunization, it became evident that the magnitude of the T cell response in mice receiving IL-4R KO cells was reduced severely compared with the response observed in mice receiving normal T cells (Fig. 1 A). FACS analysis of spleen cells after staining with SYVPSAEQI tetramers and anti-CD8 antibodies confirmed the results of the ELISPOT assay (Fig. 1 B). After day 13, mice receiving normal or IL-4R KO cells had comparable numbers of memory cells in spleen (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20042463/DC1>).

The estimation of the absolute number of epitope-specific CD8⁺ T cells present at different time points after immunization also indicated that between days 4 and 10 after immunization, the total number of IL-4R KO CD8⁺ T cells present in spleen had decreased significantly compared with the response of normal CD8⁺ T cells (Fig. S1). To evaluate the survival rate of these cells, we examined the expression of various anti- and proapoptotic markers. In three independent experiments, FACS analysis of tetramer⁺/CD8⁺ nor-

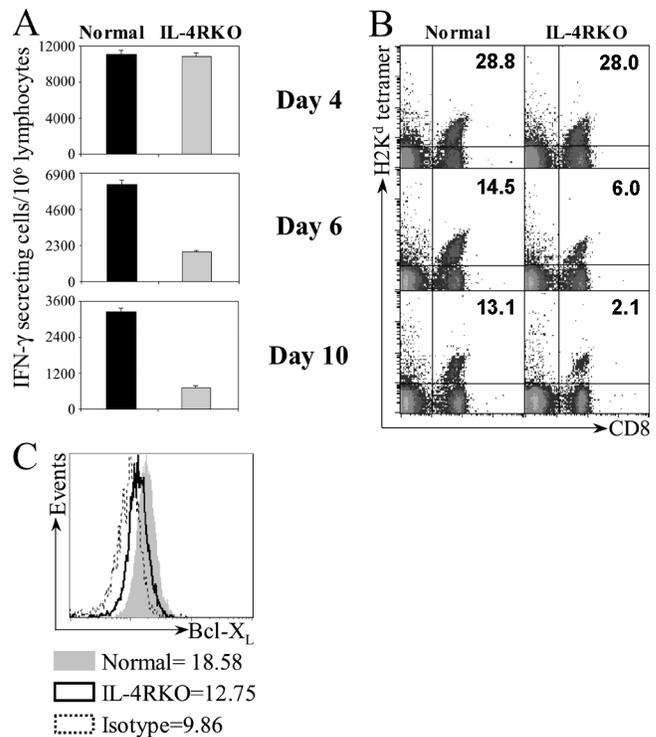


Figure 1. IL-4R KO CD8⁺ T cells develop a reduced response after immunization. Naive normal or IL-4R KO transgenic CD8⁺ T cells (5×10^5) were transferred into normal BALB/c mice which were subsequently immunized with 5×10^4 irradiation-attenuated sporozoites. The CD8⁺ T cell response of each was measured in pooled spleen cells, at different days after immunization. Each group consisted of four mice. (A) ELISPOT was used to determine the number of antigen-specific IFN- γ -secreting cells after incubation with antigen (14). Negligible numbers of IFN- γ spots were obtained when cells were incubated without antigen. The data are representative of four independent experiments that yielded the same results. Error bars represent SEM. (B) FACS analysis of this response in spleen was performed by staining with anti-CD8-APC antibodies and tetramer-PE (2). Plots were gated on lymphocytes. The values in the top right corner represent the mean percentages of tetramer⁺ cells within the total CD8⁺ T cell population. The data are representative of four independent experiments that yielded the same results. (C) Intracellular Bcl-X_L staining of tetramer⁺/CD8⁺ T cells at day 4 after immunization. The mean fluorescent intensity of Bcl-X_L expression is indicated. Gray line graph identifies normal CD8⁺ T cells; solid line represents IL-4R KO CD8⁺ T cells; and dotted line represents Ig isotype control. These data are representative of three independent experiments, using three to five mice per group. The values indicate the mean fluorescent intensity of Bcl-X_L expression, the decrease observed in the IL-4R KO cells as compared with the normal cells was statistically significant ($P = 0.002$; see Fig. S2).

mal and IL-4R KO cells indicated consistently that the level of expression of Bcl-X_L in IL-4R KO cells was reduced at day 4 after immunization (Fig. 1 C; and Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20042463/DC1>). Differences of similar magnitude in the expression of Bcl-X_L were reported among long-lived (IL-7R^{hi}) and short-lived (IL-7R^{lo}) activated CD8⁺ T cells (8). We did not detect differences in the expression levels of Bcl-2 or Bax.

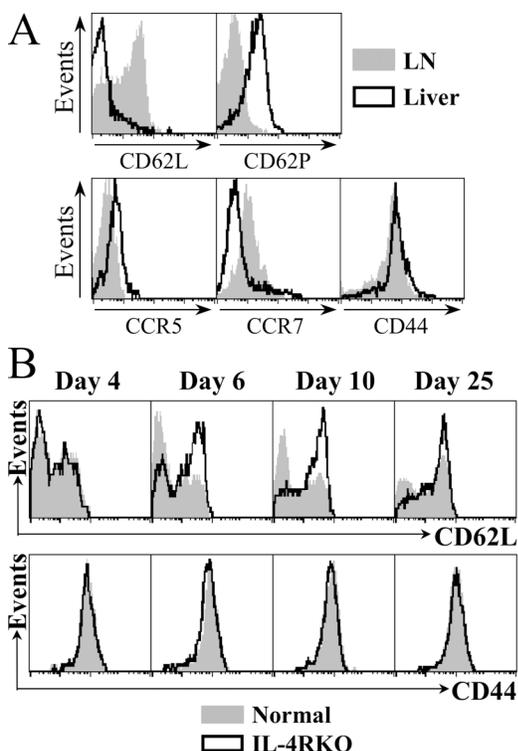


Figure 2. Characterization of memory CD8⁺ T cell subsets and activated IL-4R KO CD8⁺ T cells. Memory CD8⁺ T cells were obtained 20 d after immunization from liver and lymph nodes of mice that received normal transgenic CD8⁺ T cells and were immunized with parasites. (A) Phenotypic analysis of normal memory T cells obtained from liver or lymph nodes (LN) was performed by flow cytometry after staining the cells with tetramer-PE, CD8-APC, and FITC-labeled antibodies against CD62L, CD62P, CCR5, CCR7, and CD44. Line graphs are gated on tetramer⁺/CD8⁺ T cells. (B) Expression of CD44 and CD62L in activated CD8⁺ T cells were assessed using spleen lymphocytes obtained from mice that received normal or IL-4R KO CD8⁺ T cells, and were immunized with attenuated parasites, as described in the legend of Fig. 1. At specified days after immunization, FACS analysis was performed using anti-CD8-APC antibodies, tetramer-PE, and FITC-labeled antibodies against CD44 or CD62L. The line graphs represent data obtained after gating on tetramer⁺/CD8⁺ T cells. Solid gray graphs identify normal CD8⁺ T cells, and solid lines represent IL-4R KO CD8⁺ T cells. The increase in the mean fluorescent intensity of CD62L expression observed in the IL-4R KO as compared with the normal cells after day 6 was statistically significant ($P < 0.05$). These data are representative of four independent experiments, using three mice per group at each time point.

Annexin-V staining of spleen cells from immunized mice did not yield consistent differences between normal and IL-4R KO transgenic CD8⁺ T cells (unpublished data). However, this is not unexpected; the presence of apoptotic cells is difficult to evaluate *ex vivo* because they are cleared rapidly by phagocytes (17, 18).

Using multiparameter FACS analysis after surface staining with tetramer-PE and anti-CD8-APC, we analyzed the expression of several canonical cell surface markers that are known to undergo changes after *in vivo* activation of these T cells. First, we determined that memory responses gener-

ated by normal TCR transgenic CD8⁺ T cells developed memory populations displaying surface phenotypes that were consistent with those previously described for memory subsets residing in lymphoid and nonlymphoid organs, usually referred to as central and effector memory subsets, respectively. Memory cells obtained from the liver, compared with those from lymph nodes, express lower levels of CD62L and CCR7 and higher levels of CD62P and CCR5. As expected, these CD8⁺ T cell populations express comparable levels of CD44 (Fig. 2 A). Contrary to other reports (7, 19), we found no differences in the expression of CD27 and Granzyme B (unpublished data). We also determined that naive normal and IL-4R KO CD8⁺ T cells used in adoptive transfer experiments displayed an identical CD44^{lo} CD62L^{hi} phenotype, typical of naive cells (reference 2; Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20042463/DC1>). When comparing the *in vivo* activated normal and IL-4R KO cells in spleen at different time points after immunization, the surface markers CD11a, CD11b, CD11c, CD25, CD27, CD31, CD45RB, CD49d, CD69, CD71, CD122, CCR5, IL-7R, granzyme B, P-selectin (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20042463/DC1>), and CD44 (Fig. 2 B) were expressed at similar levels. However, there were significant differences in the expression of CD62L (Fig. 2 B). 4 d after immunization, normal and IL-4R KO CD8⁺ T cells showed a characteristic profile of CD62L expression with clearly identifiable CD62L^{hi} and CD62L^{lo} subpopulations. In the following days, IL-4R KO cells increased expression of this marker; by day 10 they become a homogeneous population comprising mostly CD62L^{hi} cells (Fig. 2 B).

IL-4R is necessary to develop memory cells in nonlymphoid organs

In view of these results suggesting that IL-4R KO CD8⁺ T cells undergo an abnormal development in spleen, we performed experiments to compare the homing of activated T cells to lymph nodes and nonlymphoid organs. In these experiments, normal or IL-4R KO transgenic CD8⁺ T cells were transferred to naive mice that were immunized with attenuated parasites 24 h later. The presence of antigen-specific CD8⁺ T cells in lymph nodes, liver, lung, kidneys, and spleen was evaluated by FACS at different time points. 4 d after immunization there were similar numbers of tetramer⁺/CD8⁺ T cells in the lymphoid and nonlymphoid organs of mice receiving normal or IL-4R KO cells (Fig. 3). A striking difference became apparent 10 and 25 d after immunization; the number of tetramer⁺/CD8⁺ cells resident in liver, kidney, and lung organs was reduced severely in mice receiving IL-4R KO cells.

Although memory IL-4R KO cells disappear from nonlymphoid organs shortly after immunization, almost identical numbers of tetramer⁺/CD8⁺ T cells were found at all times in the lymph nodes of mice receiving normal or IL-4R KO cells. The kinetics of these responses in spleen followed an intermediate pattern. 10 d after immunization the responses

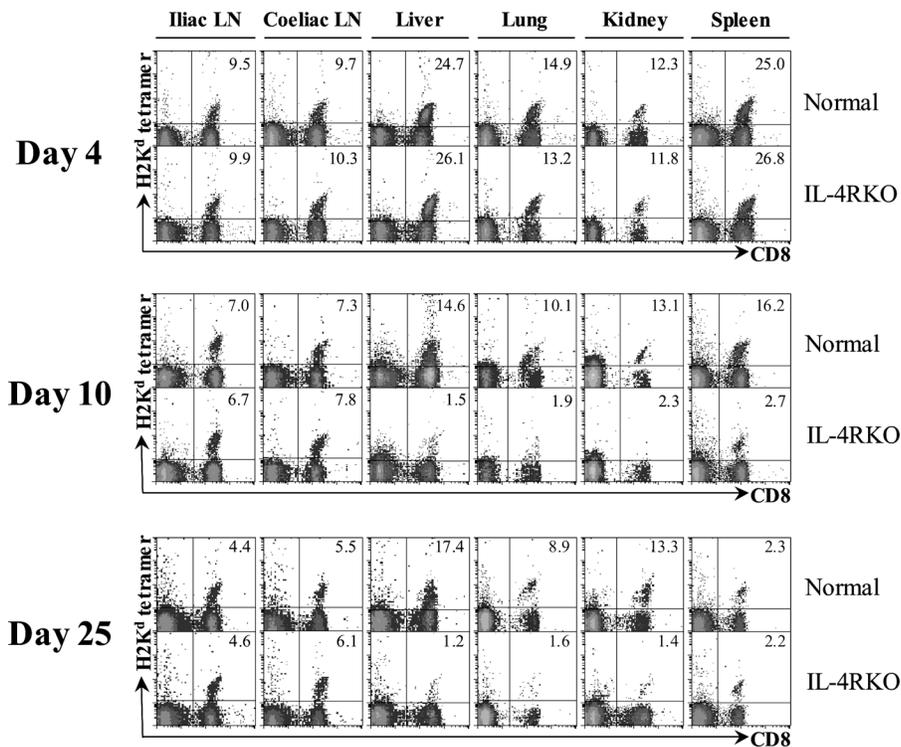


Figure 3. IL-4R KO CD8⁺ T cells do not develop peripheral memory. CD8⁺ T cell responses developed by normal or IL-4R KO CD8⁺ T cells were evaluated in different organs and at different time points after immunization with parasites. The adoptive transfer of transgenic CD8⁺ T cells and immunization with parasites was performed as described in the legend of Fig. 1. Mononuclear cells that were purified from lymphoid and nonlymphoid organs were analyzed by FACS after staining with tetramer-PE and

anti-CD8-APC. Plots represent tetramer⁺/CD8⁺ lymphocyte pools derived from four mice. The values in the top right corners represent mean percentages of tetramer⁺ cells within the total CD8⁺ T cell population. The decrease observed after 10 d in the percentages of IL-4R KO cells from liver, lung, and kidney was statistically significant ($P < 0.05$). These data are representative of four independent experiments, using three mice per group at each time point.

of normal and IL-4R KO T cells were different in magnitude, whereas after day 13 the magnitude of the responses was comparable (Figs. S1 and S3).

The estimation of the absolute number of memory cells in the liver and lymph nodes at days 6 and 10 after immunization confirmed the results suggested by FACS analysis shown in Fig. 3. The total number of memory IL-4R KO cells in liver—compared with normal memory CD8⁺ T cells—was reduced strongly at day 10 (Fig. 4 A), whereas no differences could be detected in lymph nodes (Fig. 4 B and Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20042463/DC1>). The homing of memory cells to lungs and kidneys at different time points after immunization followed a pattern similar to that observed in liver (Fig. S6, available at <http://www.jem.org/cgi/content/full/jem.20042463/DC1>).

This striking effect of IL-4R deficiency in the development of memory CD8⁺ T cells in the liver was confirmed further by IL-4 neutralization experiments. Mice that received normal transgenic CD8⁺ T cells and were immunized with parasites were treated with a monoclonal antibody against IL-4 at different time points. The development of memory populations was evaluated 16 d after immunization with parasites. The results indicated that treatment with

anti-IL-4 during the first 6 d after immunization induced a severe reduction of the memory CD8⁺ T cell population in the liver. In contrast, the memory populations residing in lymph nodes were not affected. Treatment with anti-IL-4 between days 7 and 14 after immunization does not have a major effect on the magnitude of any memory population (Fig. 5). These results clearly indicate that IL-4 is critical during the early events of the CD8⁺ T cell activation at a time when activated cells migrate to nonlymphoid organs and establish memory populations in these tissues.

Normal and IL-4R KO memory cells residing in lymphoid organs display identical phenotypic and functional properties

Although the previous experiments indicated that the numbers of normal and IL-4R KO memory cells residing in lymph nodes were similar, we evaluated additional phenotypic and functional features of these memory cells. Phenotypic analysis of memory CD8⁺ T cells residing in the lymph nodes 25 d after immunization indicated that both IL-4R KO and normal cells display an identical CD62L^{hi} CD44^{hi} phenotype profile (Fig. 6 A). In addition, we found that both cell types displayed a comparable capacity to produce IL-2 and IFN- γ (Fig. 6 B). Finally, we performed experi-

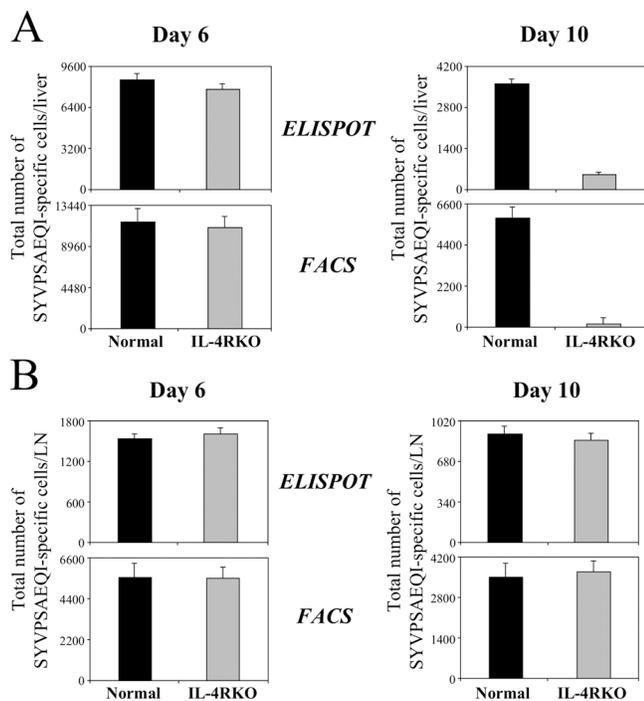


Figure 4. Quantification of CD8⁺ T cell responses in liver and lymph nodes. The total number of SYVPSAEQI-specific T cells present in the liver (A) and lymph nodes (B) of mice was determined 6 and 10 d after adoptive transfer of CD8⁺ T cells and immunization with 5×10^4 attenuated sporozoites. The estimation of the total number of epitope-specific cells was obtained by multiplying the percentage of tetramer⁺/CD8⁺ T cells (FACS) or IFN- γ spots (ELISPOT) by the total numbers of lymphocytes isolated from the respective organs. Each histogram represents data from pooled lymphocytes obtained from four mice. These data are representative of four independent experiments, using three mice per group at each time point. Error bars represent SEM.

ments to evaluate the *in vivo* proliferation of IL-4R KO and normal memory cells after immunization. IL-4R KO and normal memory cells isolated from lymph nodes 25 d after immunization were labeled with CFSE, and equal numbers of each cell type were transferred to naive mice. The recipient mice were immunized with sporozoites; the proliferative response of these cells was assessed by evaluating CFSE dilution of tetramer⁺/CD8⁺ T cells using three-color FACS analysis. The results indicated a nearly identical profile of proliferation in both cell types (Fig. 6 C). Taken together, the results of these experiments indicate that lymph node cells lacking the IL-4R develop phenotypic, functional, and proliferative characteristics that are identical to those of normal CD8⁺ T cells.

IL-4R is critical for the development of protection against parasite challenge

The findings suggesting an unequal development of memory subsets in the absence of the IL-4R raise the obvious question about the *in vivo* protective activity of CD8⁺ T cells that are generated by normal and IL-4R KO cells. To evalu-

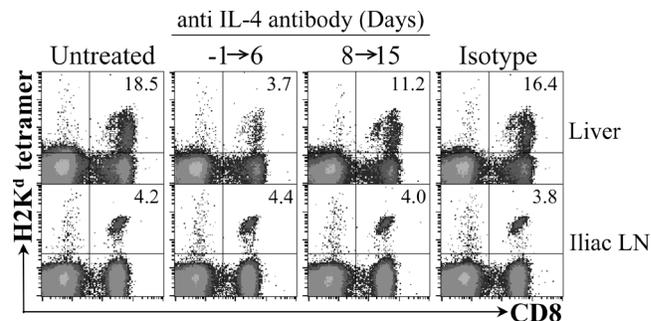


Figure 5. Treatment with anti-IL-4 antibody reduces the number of memory CD8⁺ T cells in the liver. Naive transgenic CD8⁺ T cells were transferred into normal mice, some of which received a daily i.p. injection of 0.1 mg of rat antibody against mouse IL-4 (11B11) at each of the indicated days. Control groups received no treatment or a similar amount of nonspecific rat IgG. These mice were immunized with 5×10^4 attenuated sporozoites; 16 d later the number of epitope-specific CD8⁺ T cells was evaluated by FACS in the liver and iliac lymph nodes of immunized mice. The data of each group were obtained from pooled lymphocytes that were taken from the lymph nodes or the liver. Each group consisted of four mice. Plots were gated on lymphocytes; the number in the upper right corner represents the frequency of tetramer⁺/CD8⁺ T cells in the CD8⁺ population. The data are representative of three independent experiments. The decrease observed in the percentages of the group that was treated with anti-IL-4 antibody during days -1 to 6, as compared with the untreated mice, was statistically significant ($P = 0.0196$).

ate the antiparasitic activity of these CD8⁺ T cell responses, we performed experiments in which mice received equal numbers of normal or IL-4R KO cells, and were immunized with a low dose of attenuated *P. yoelii* sporozoites (5×10^3). This parasite dose activates T cells but induces negligible amounts of antibodies. At different time points, immunized mice were challenged with viable sporozoites, and parasite development in the liver was evaluated 36 h after infection by using a real-time PCR assay (20). To ensure that the protective activity measured in this assay reflected only the effect of transferred CD8⁺ TCR transgenic cells, the endogenous T cells were depleted by treatment with anti-CD8 antibody before the transfer of transgenic cells, and with anti-CD4 4 d after sporozoite immunization after activation and expansion of CD8⁺ T cells had occurred. The results of these protection experiments indicated that mice receiving normal or IL-4R KO CD8⁺ T cells were equally capable of inhibiting parasite development if challenged 6 d after immunization (Fig. 7 A). However, when these mice were challenged 16 d after immunization, mice receiving IL-4R KO cells had lost their protective immunity because they no longer inhibited the development of the parasite liver stages (Fig. 7 B).

To gain new insights into the functional properties of memory cell populations, we performed experiments to assess the functional status of spleen memory cells after adoptive transfer into naive mice. It is known that transferred memory cells purified from different organs acquire the capacity to home to nonlymphoid organs, regardless of their tissue of origin or level of expression of CD62L (21–23).

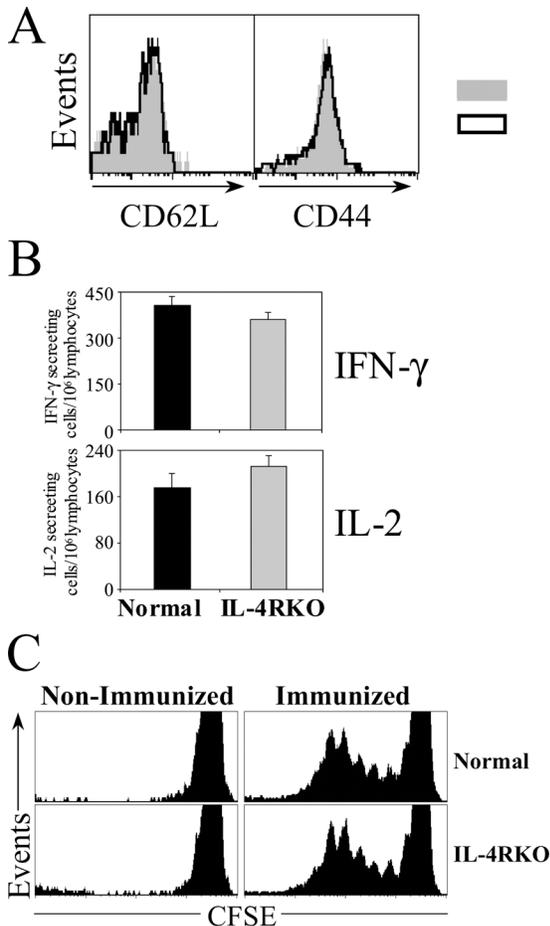


Figure 6. Phenotypic, functional, and proliferative characteristics of memory CD8⁺ T cells from lymph nodes. Memory CD8⁺ T cells were obtained from lymph nodes of mice that received normal or IL-4R KO CD8⁺ T cells and were immunized with parasites as described in the legend of Fig. 1. 25 d after immunization, these cells were analyzed by FACS. (A) Surface staining with anti-CD8-APC antibodies, tetramer-PE, and CD62L-FITC or CD44-FITC. Solid gray line graphs represent normal CD8⁺ T cells and solid lines represent IL-4R KO cells. (B) ELISPOT assay was performed to evaluate the capacity of these cells to produce IL-2 and IFN- γ , after antigen stimulation. Error bars represent SEM. (C) The *in vivo* proliferation of these memory cells also was assessed using normal and IL-4R KO memory CD8⁺ T cells obtained from lymph nodes 25 d after immunization. These memory cells were labeled with CFSE, and transferred into mice which were immunized with 5×10^4 irradiation-attenuated sporozoites. The proliferation of the transferred tetramer⁺/CD8⁺ memory T cells was evaluated by FACS 3 d after immunization, after staining cells with tetramer and anti-CD8. Line graphs show the CFSE dilution patterns of cells, obtained by FACS after gating on tetramer⁺/CD8⁺ T cells. These data are representative of two independent experiments, using three mice per group at each time point.

Thus, we evaluated the homing properties and protective capacity of transferred normal and IL-4R KO CD8⁺ T memory cells purified from spleen. These memory CD8⁺ T cells were obtained 25 d after immunization—a time point at which they are no longer dependent on IL-4. IL-4R KO

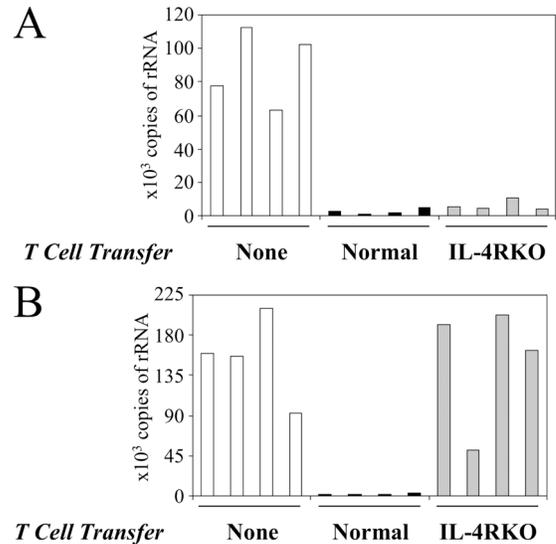


Figure 7. Memory IL-4R KO CD8⁺ T cells are not protective. An equal number of normal or IL-4R KO transgenic CD8⁺ T cells were transferred to mice which were immunized 24 h later with 5×10^3 irradiated sporozoites. These mice were challenged with viable sporozoites at 6 d (A) or 16 d (B) after immunization. 34 h after parasite challenge, the livers of infected mice were excised to measure the parasite load using real-time PCR (20). The endogenous CD8⁺ and CD4⁺ T cells were eliminated by treatment with specific antibodies. The histograms represent the number of plasmodial rRNA copies. The data are representative of two independent experiments, using four mice per group at each time point.

memory cells consists mostly of CD62L^{hi} cells, whereas normal memory cells were CD62L^{hi} and CD62L^{lo}. The number of tetramer⁺/CD8⁺ memory cells used for these adoptive transfer experiments (1.5×10^6) is the minimum necessary to inhibit parasite development. As assessed by ELISPOT, the results shown in Fig. 8 A indicate that normal and IL-4R KO memory CD8⁺ T cells were equally efficient at homing to the spleen and liver. Most importantly, the new homing profile of IL-4 R KO cells (Fig. 8 A) seemed to coincide with the capacity of these cells to inhibit the development of liver stage parasites as efficiently as normal memory cells (Fig. 8 B). The results of additional experiments aimed at evaluating the antiparasitic activity of CD62L^{hi} and CD62L^{lo} normal memory cells obtained from spleen were consistent with the previous findings. In these experiments, normal memory cells from spleen were stained with tetramer-PE, CD8-APC, and anti-CD62L-FITC. Subpopulations expressing CD62L^{hi} and CD62L^{low} were purified by FACS cell sorting, and equal numbers of memory cells were transferred to naive mice (Fig. 9 A). As shown in Fig. 9 B, these experiments indicated that CD62L^{hi} and CD62L^{lo} have a comparable capacity to inhibit parasite development. Identical results were obtained in experiments in which these memory CD8⁺ T cell subsets were purified based on staining with anti-Thy1.1 antibodies—instead of tetramer—after transfer of Thy1.1 CD8⁺ T cells into Thy1.2 recipient mice (unpublished data).

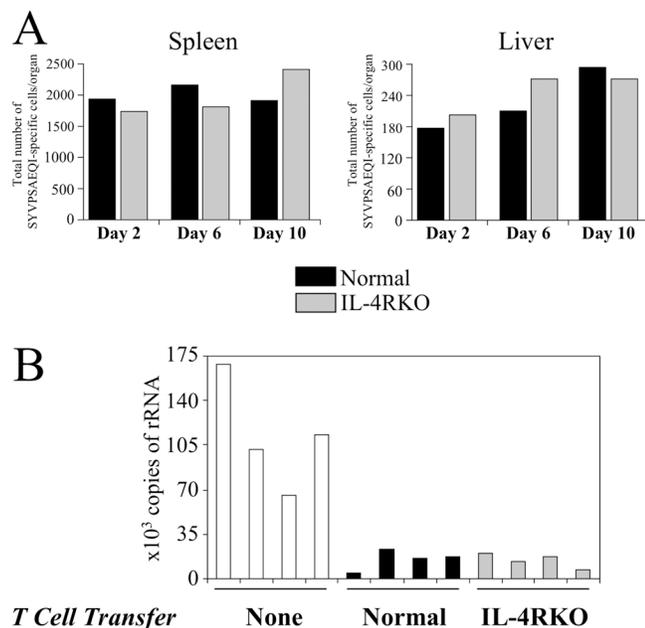


Figure 8. Homing properties and antiparasitic activity of adoptively transferred memory CD8⁺ T cells. SIVPSAEQI-specific memory CD8⁺ T cells were generated from mice that received equal numbers of IL-4R KO naive transgenic cells and were immunized with irradiated sporozoites. 20 d after immunization, tetramer⁺/CD8⁺ T cells were obtained from spleen. Equal numbers of normal or IL-4R KO tetramer-specific memory CD8⁺ T cells were transferred to naive mice. (A) Following adoptive transfer, the number of SIVPSAEQI-specific memory CD8⁺ T cells in the spleen and liver was measured by ELISPOT at the indicated times. To determine the protective capacity of adoptive transferred memory cells, the naive recipient mice were challenged with 3×10^4 viable sporozoites 10 d after transfer of memory cells. 34 h later, the livers of infected mice were excised to measure the parasite load using real-time PCR (20). (B) The histograms represent the number of plasmodial rRNA copies. These data are representative of two independent experiments, using four mice per group.

Taken together, these results indicate that normal and IL-4R KO CD8⁺ T cells are capable of generating memory cells with antiparasitic activity. However, their *in vivo* protective activity seems to be defined by their capacity to home to, and remain in, nonlymphoid tissues.

DISCUSSION

The regulatory effects of IL-15, IL-7, and IL-2 on the development of memory CD8⁺ T cell responses has been well-documented in studies using different viral systems. In contrast, the role of IL-4—which is critical to the development of CD8⁺ T cells responses against certain tumors and parasites—remains poorly characterized. In the present study we used TCR transgenic CD8⁺ T cells specific for the epitope SIVPSAEQI of *P. yoelii* to compare normal and IL-4R KO CD8⁺ T cells with regard to the induction and maintenance of memory responses. During the first 4 d after immunization, IL-4R KO CD8⁺ T cells developed an apparently normal proliferative activity and differentiation profile. How-

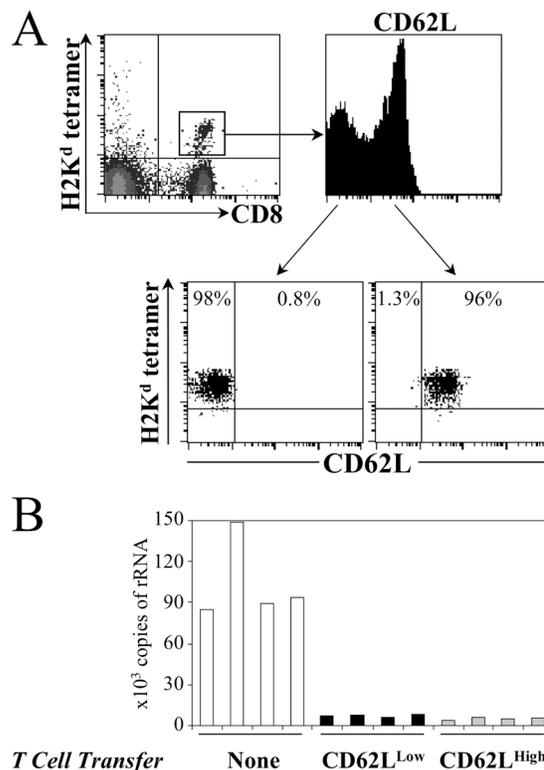


Figure 9. Antiparasitic activity of CD62L^{lo} and CD62L^{hi} memory CD8⁺ T cells. SIVPSAEQI-specific memory CD8⁺ T cells were generated from mice that received naive normal transgenic cells and were immunized with irradiated sporozoites. (A) 20 d after immunization, tetramer⁺/CD8⁺ T cells obtained from spleen were costained with anti-CD62L. Line graph shows CD62L expression of tetramer⁺/CD8⁺ T cells. (B) To assess the antiparasitic activity of CD62L^{low} and CD62L^{high} memory CD8⁺ T cells, these subpopulations were purified by cell sorting using flow cytometry. Equal numbers (1.5×10^6) of CD62L^{lo} or CD62L^{hi} tetramer-specific CD8⁺ T cells were transferred to naive mice. 2 d later, recipient mice were challenged with 3×10^4 viable sporozoites. 34 h after parasite challenge, the livers of infected mice were excised to measure the parasite load using real-time PCR (20). The histograms represent the number of plasmodial rRNA copies. The data are representative of two independent experiments, using four mice per group.

ever, as the response develops there is a drastic reduction of memory IL-4R KO CD8⁺ T cells in nonlymphoid organs, and the establishment of memory populations in these tissues is affected severely. This loss is particularly striking because memory cells seem to develop normally in lymphoid tissues.

Experiments in which IL-4 was neutralized *in vivo* after immunization clearly demonstrated that the effect of this cytokine is exerted early during the first week after immunization, at the time when the activated cells migrate to nonlymphoid organs. These findings indicate that IL-4/IL-4R interactions that occur early after T cell activation are critical to establish long living memory CD8⁺ T cell populations in nonlymphoid organs at a critical point when homeostatic mechanisms are inducing a massive apoptosis on activated cells that results in a severe contraction of the CD8⁺ T cell re-

sponse. Once CD8⁺ T cells enter a resting phase and begin to differentiate into memory cells, IL-4 seems to be dispensable. Together, these results demonstrate, for the first time, that IL-4 can modulate the differentiation of memory CD8⁺ T cells directly through the IL-4R that is expressed on these cells.

The results of the protection studies indicating that IL-4R KO CD8⁺ T cells lose their antiparasitic activity after failing to maintain memory cells in nonlymphoid organs suggest that this memory population—characterized by their capacity to reside in nonlymphoid tissue—represents the first line of defense against parasite infection in immune hosts. Adoptive transfer experiments using purified memory spleen cells revealed additional features of this memory population. Transfer of memory IL-4R KO cells obtained from spleen 25 d after immunization, a time point at which they are IL-4 independent and express CD62L^{hi}, indicated that these cells can home to the liver and become capable of inhibiting parasite development as efficiently as normal memory cells. The results on the homing behavior of these cells are not unexpected because it is known that memory CD8⁺ T cells, regardless of their tissue origin or CD62 expression, home with similar efficiency to lymphoid and nonlymphoid organs after adoptive transfer (22, 23). It is unclear whether this migration behavior represents a physiologic trafficking pattern accelerated by the adoptive transfer, or if this is an abnormal effect that is induced by the *ex vivo* purification procedures and intravenous injection. Nevertheless, the fact that memory IL-4R KO cells can protect after adoptive transfer indicates that they are fully capable of inhibiting parasite development if they reside in the proper tissue compartments.

These findings appear to differ, at least in part, from those obtained in studies using vaccinia and lymphocytic choriomeningitis virus. In these viral systems, adoptively transferred “central memory” CD8⁺ T cells—defined by the expression of CD62L^{hi}—represent the main protective cell subset (7, 24). In the malaria system, we found that adoptively transferred normal CD62L^{lo} and CD62L^{hi} memory cells display a comparable antiparasitic activity. Most importantly, the lack of protective activity by lymphoid resident CD62L^{hi} IL-4R KO memory cells of unmanipulated mice suggests that this subset may not have a protective role that is as prominent as that observed in viral infections. This apparent discrepancy between the viral and parasite systems most likely reflects the biologic differences that exist between these microbial infections. Particularly important is the fact that the parasite stage that is susceptible to CD8⁺ T cells develops only in the liver and last for only 40–42 h, whereas viral infections affect several organs, including lymphoid tissues, and remain detectable for several days. In this situation, during viral infections, memory CD8⁺ T cells that reside in lymphoid organs may have more opportunities to be reactivated and become full effector cells, and mask the early protective activity of memory cells from nonlymphoid organs.

The selective effect of IL-4 in the development of memory CD8⁺ T cells in nonlymphoid organs is intriguing because it seems to differ from that of IL-7 and IL-15, both of which seem to affect the entire memory population at different time points (i.e., IL-7 helps to maintain memory populations after they have developed, whereas IL-15 seems to be important during T cell activation and after memory populations have been developed; references 8, 11). The molecular mechanism that underlies the effect of these cytokines of the γ -chain receptor family on CD8⁺ T cells remains poorly understood. Although the importance of IL-15/IL-15R interactions at different stages of the CD8⁺ T cell responses is well documented, little is known about the molecular basis of this cytokine effect. As for IL-7, recent studies indicate that this cytokine promotes the survival of memory CD8⁺ T cells by increasing the expression of antiapoptotic molecules, such as Bcl-X_L and Bcl-2.

The mechanism by which the IL-4R modulates the development of memory CD8⁺ T cells in nonlymphoid organs remains to be elucidated. It is conceivable that the decrease of IL-4R KO memory cells in nonlymphoid organs may result from a reduced supply of memory cells from spleen, a selective migration of activated cells to lymphoid organs, or a decreased survival of memory cells in nonlymphoid organs. Our results show that the magnitude of normal and IL-4R KO CD8⁺ T cell responses at day 4 are identical in every organ. Moreover, at this time point the number of activated cells in each organ reaches their highest levels; this indicates that there is no lack of T cell migration from spleen or lymph nodes toward nonlymphoid tissues. After day 4 there is a pronounced reduction in the number of IL-4R KO CD8⁺ T cells in nonlymphoid organs. This may be explained by an accelerated emigration of activated cells toward lymphoid tissues facilitated by a high expression of CD62L, or it could be due to an increased death rate of activated cells in nonlymphoid organs. The idea that IL-4 enhances the survival of activated CD8⁺ T cells is supported by previous studies which described an antiapoptotic effect of IL-4 on CD4⁺ T cells (25), and by our current findings that activated IL-4R KO CD8⁺ T cells express lower levels of the antiapoptotic molecule, Bcl-X_L when compared with activated normal cells. Further research is necessary to fully resolve this issue.

The existence of various cytokine-mediated mechanisms that affect the development of memory CD8⁺ T cell responses raises questions regarding the possible interactions that these cytokines may establish between themselves during this process. Perhaps these cytokines act in concert—with a certain degree of specialization—to perform distinct tasks at different developmental phases of the T cell response. Alternatively, it is conceivable that these cytokine/cytokine receptor interactions might represent redundant functions that ensure a proper development and establishment of memory cells. Perhaps more important, it has yet to be determined whether the same set of cytokines participates in the

development of all antimicrobial CD8⁺ T cell responses. A “one size fits all” model of CD8⁺ T cell development may be too simplistic; the degree of participation of each cytokine may vary depending on the virulence of the infectious pathogen, the tissue compartments that are affected, or the pathology caused by the infection. A better characterization of the mechanisms by which these cytokines sustain and regulate the development of memory CD8⁺ T cells should open new avenues for research that are aimed at manipulating this differentiation process with a view to enhance the efficacy of T cell responses induced by vaccination.

MATERIALS AND METHODS

Mice and parasites. BALB/c mice were purchased from Taconic. The generation of the *P. yoelii*-specific transgenic mice expressing a TCR specific for the SYVPSAEQI epitope using C57BL/6 oocytes was described elsewhere (2). Original founders were crossed to BALB/c mice, and the F20 backcross generation was used for the experiments. TCR transgenic/IL-4R KO mice were obtained after crossing BALB/c IL-4R KO mice (16) (provided by N. Noben-Trauth, George Washington University Medical Center, Washington DC) with the *P. yoelii*-specific TCR transgenic mice. Experiments with mice were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

Antibodies, flow cytometry, and ELISPOT. All antibodies were purchased from BD Biosciences, except antibody to mouse Bcl-X_L and Bax (Santa Cruz Biotechnology, Inc). Staining for Granzyme B was performed using FITC-labeled anti-granzyme B obtained from Caltag (19). To stain for CCR7, cells were incubated with CCL19 ligand-IgG at 1 μg/ml (provided by J.G. Cyster, University of California, San Francisco, CA), followed by goat anti-human Fc biotin and streptavidin-FITC. SYVPSAEQI-specific H2Kd tetramer was prepared as described (2). FACS analysis of cells was done using a FACSCalibur flow cytometer (200,000–500,000 gated events per sample). CELLQuest software (Becton Dickinson) was used to analyze the data. The ELISPOT assay to detect IFN-γ and IL-2-secreting cells was performed as described in detail (14, 26). Statistical analysis was done by using analysis of variance, and a significance level of P < 0.05 was used.

Adoptive transfer of transgenic cells, immunizations, and evaluation of protective immunity. Spleen cells from transgenic mice containing 10⁶ tetramer⁺/CD8⁺ cells were used for adoptive transfers, unless otherwise specified. Before transfer, the total number of transgenic cells present in spleen was determined by FACS analysis after staining cells with anti-CD8 and SYVPSAEQI-tetramers. Immunization with *P. yoelii* (17X NL strain) sporozoites was done by i.v. injection of γ-irradiated parasites. To deplete endogenous CD8⁺ T cell populations, BALB/c mice received daily injections of 0.1 mg anti-CD8 antibody YTS 169 for 3 d, 4 d before transfer of TCR transgenic CD8⁺ T cells. Endogenous CD4⁺ T cells were depleted by treating mice with anti-CD4 antibodies (GK1.5) administered by daily injections of 0.15 mg for 3 d before parasite challenge. The evaluation of parasite development in liver of mice challenged with parasites was performed by real time PCR, as described (20).

In vivo neutralization of IL-4. At the indicated days, mice received daily i.p. injections of 0.1 mg of purified rat mAb 11B11 specific for mouse IL-4, obtained from NCI Biological Resources Branch. Control mice received no treatment or similar amounts of purified rat IgG (Pierce Chemical Co.).

Isolation of memory T cell subsets from spleen. Memory CD8⁺ T cells were obtained from spleen of mice that received normal TCR transgenic CD8⁺ T cells and were immunized with parasites, as described (2, 14). 20 d after immunization, spleen cells were enriched for CD8⁺ T cells by negative selection of CD4⁺ T cells and B cells through magnetic sorting

using anti-CD4-FITC (L3T4), B220-FITC, and anti-FITC beads, following the manufacturer's instructions (Miltenyi Biotec). The total number of tetramer⁺/CD8⁺ T cells was determined by FACS analysis. The purification of the memory CD8⁺ T cell subsets, CD62L^{hi} or CD62L^{lo}, was obtained by FACS cell sorting using tetramer-PE, anti CD8-APC, and anti-CD62L-FITC.

Isolation of lymphocytes from nonlymphoid tissues. Before tissue removal, mice were perfused with phosphate-buffered saline and heparin (75 U/ml). Tissues were disrupted mechanically, suspended in HBSS with 2% FCS and 10 mM HEPES, and filtered through a 70-μm mesh. This cell suspension was centrifuged, the pellet was resuspended in a solution of Percoll (Amersham Biosciences) as described (10), centrifuged at 600 g, and washed extensively before use.

CFSE labeling and proliferation of memory cells. BALB/c mice receiving 10⁶ transgenic CD8⁺ T cells were immunized with 50,000 γ-irradiated sporozoites; 25 d later, lymphocytes were isolated from their lymph nodes. After labeling in vitro with CFSE (Molecular Probes), the cells were transferred i.v. into naive BALB/c mice that were immunized with 50,000 sporozoites. 3 d after immunization, the dilution profile of the CFSE label in spleen cells from recipient mice was evaluated by FACS, gating in tetramer⁺/CD8⁺ lymphocytes.

Online supplemental material. Fig. S1 shows the kinetics of the CD8⁺ T cell responses and the total numbers of tetramer⁺/CD8⁺ T cells in the spleen. Fig. S2 shows the expression of Bcl-X_L in activated tetramer⁺/CD8⁺ T cells. Fig. S3 shows the phenotypes of activated and naive CD8⁺ T cells obtained from spleen. Fig. S4 presents the activation profile of normal and IL-4R KO memory CD8⁺ T cells obtained from spleen. Fig. S5 shows the kinetics of the CD8⁺ T cell responses in the lymph nodes. Fig. S6 presents the quantification of the CD8⁺ T cell responses in the lungs and kidneys. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20042463/DC1>.

Flow cytometry was performed in the Becton Dickinson Immune Function Laboratory at the Johns Hopkins Bloomberg School of Public Health.

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REFERENCES

- Wong, P., and E.G.P. Am. 2003. CD8 T cell responses to infectious pathogens. *Annu. Rev. Immunol.* 21:29–70.
- Sano, G., J.C. Hafalla, A. Morrot, R. Abe, J.J. Lafaille, and F. Zavala. 2001. Swift development of protective effector functions in naive CD8(+) T cells against malaria liver stages. *J. Exp. Med.* 194:173–180.
- Mueller, S.N., C.M. Jones, C.M. Smith, W.R. Heath, and F.R. Carbone. 2002. Rapid cytotoxic T lymphocyte activation occurs in the draining lymph nodes after cutaneous herpes simplex virus infection as a result of early antigen presentation and not the presence of virus. *J. Exp. Med.* 195:651–656.
- Kaech, S.M., S. Hemby, E. Kersh, and R. Ahmed. 2002. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell.* 111:837–851.
- Lanzavecchia, A., and F. Sallusto. 2002. Progressive differentiation and selection of the fittest in the immune response. *Nat. Rev. Immunol.* 2:982–987.
- Fahmy, T.M., J.G. Bieler, M. Edidin, and J.P. Schneck. 2001. Increased TCR avidity after T cell activation: a mechanism for sensing low-density antigen. *Immunity.* 14:135–143.
- Wherry, E.J., and R. Ahmed. 2004. Memory CD8 T-cell differentiation during viral infection. *J. Virol.* 78:5535–5545.
- Kaech, S.M., J.T. Tan, E.J. Wherry, B.T. Konieczny, C.D. Such, and

- R. Ahmed. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* 4:1191–1198.
9. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* 401:708–712.
 10. Masopust, D., V. Vezys, A.L. Marzo, and L. Lefrancois. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. *Science.* 291:2413–2417.
 11. Schluns, K.S., and L. Lefrancois. 2003. Cytokine control of memory T-cell development and survival. *Nat. Rev. Immunol.* 3:269–279.
 12. Huang, L.R., F.L. Chen, Y.T. Chen, Y.M. Lin, and J.T. Kung. 2000. Potent induction of long-term CD8⁺ T cell memory by short-term IL-4 exposure during T cell receptor stimulation. *Proc. Natl. Acad. Sci. USA.* 97:3406–3411.
 13. Schuler, T., Z. Qin, S. Ibe, N. Noben-Trauth, and T. Blankenstein. 1999. T helper cell type 1-associated and cytotoxic T lymphocyte-mediated tumor immunity is impaired in interleukin 4-deficient mice. *J. Exp. Med.* 189:803–810.
 14. Carvalho, L.H., G. Sano, J.C. Haffala, A. Morrot, M.A. Curotto de Lafaille, and F. Zavala. 2002. IL-4-secreting CD4⁺ T cells are crucial to the development of CD8⁺ T-cell responses against malaria liver stages. *Nat. Med.* 8:166–170.
 15. Stager, S., J. Alexander, A.C. Kirby, M. Botto, N.V. Rooijem, D.F. Smith, F. Brombacher, and P.M. Kaye. 2003. Natural antibodies and complement are endogenous adjuvants for vaccine-induced CD8(+) T-cell responses. *Nat. Med.* 9:1287–1292.
 16. Noben-Trauth, N., L.D. Shultz, F. Brombacher, J.F. Urban Jr., H. Gu, and W.E. Paul. 1997. An interleukin 4 (IL-4)-independent pathway for CD4⁺ T cell IL-4 production is revealed in IL-4 receptor-deficient mice. *Proc. Natl. Acad. Sci. USA.* 94:10838–10843.
 17. van Engeland, M., L.J. Nieland, F.C. Ramaekers, B. Schutte, and C.P. Reutelingsperger. 1998. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry.* 31:1–9.
 18. Wang, X.Z., S.E. Stepp, M.A. Brehm, H.D. Chen, L.K. Selin, and R.M. Welsh. 2003. Virus-specific CD8 T cells in peripheral tissues are more resistant to apoptosis than those in lymphoid organs. *Immunity.* 18:631–642.
 19. Wolint, P., M.R. Betts, R.A. Koup, and A. Oxenius. 2004. Immediate cytotoxicity but not degranulation distinguishes effector and memory subsets of CD8⁺ T cells. *J. Exp. Med.* 199:925–936.
 20. Bruna-Romero, O., J.C. Haffalla, G. Gonzalez-Aseguinolaza, G. Sano, M. Tsuji, and F. Zavala. 2001. Detection of malaria liver-stages in mice infected through the bite of a single Anopheles mosquito using a highly sensitive real-time PCR. *Int. J. Parasitol.* 31:1499–1502.
 21. Masopust, D., V. Vezys, E.J. Usherwood, L.S. Cauley, S. Olson, A.L. Marzo, R.L. Ward, D.L. Woodland, and L. Lefrancois. 2004. Activated primary and memory CD8 T cells migrate to nonlymphoid tissues regardless of site of activation or tissue of origin. *J. Immunol.* 172:4876–4881.
 22. Klonowski, K.D., K.J. Williams, A.L. Marzo, D.A. Blair, E.G. Lingenheld, and L. Lefrancois. 2004. Dynamics of blood-borne CD8 memory T cell migration in vivo. *Immunity.* 20:551–562.
 23. Wherry, E.J., V. Teichgraber, T.C. Becker, D. Masopust, S.M. Kaech, R. Antia, U.H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* 4:225–234.
 24. Unsoeld, H., S. Krautwald, D. Voehringer, U. Kunzendorf, and H. Pircher. 2002. Cutting edge: CCR7⁺ and CCR7⁻ memory T cells do not differ in immediate effector cell function. *J. Immunol.* 169:638–641.
 25. Vella, A., T.K. Teague, J. Ihle, J. Kappler, and P. Marrack. 1997. Interleukin 4 (IL-4) or IL-7 prevents the death of resting T cells: stat6 is probably not required for the effect of IL-4. *J. Exp. Med.* 186:325–330.
 26. Fujihashi, K., J.R. McGhee, K.W. Beagley, D.T. McPherson, S.A. McPherson, C.M. Huang, and H. Kiyono. 1993. Cytokine-specific ELISPOT assay. Single cell analysis of IL-2, IL-4 and IL-6 producing cells. *J. Immunol. Methods.* 160:181–189.