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The Survival of Memory CD8 T Cells That Is Mediated by IL-15 Correlates with Sustained Protection Against Malaria

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Ag-specific memory T cell responses elicited by infections or vaccinations are inextricably linked to long-lasting protective immunity. Studies of protective immunity among residents of malaria endemic areas indicate that memory responses to Plasmodium Ags are not adequately developed or maintained, as people who survive episodes of childhood malaria are still vulnerable to either persistent or intermittent malaria infections. In contrast, multiple exposures to radiation-attenuated Plasmodium berghei sporozoites (Pb γ-spz) induce long-lasting protective immunity to experimental sporozoite challenge. We previously demonstrated that sterile protection induced by Pb γ-spz–immunized B6 mice are found predominantly in the liver and are sensitive to levels of liver-stage Ag depot and they express CD44hiCD62Llo markers indicative of effector/effector memory phenotype. The developmentally related central memory CD8 T (TCM) cells express elevated levels of CD122 (IL-15Rβ), which suggests that CD8 TCM cells depend on IL-15 for maintenance. Using IL-15–deficient mice, we demonstrate in this study that although protective immunity is inducible in these mice, protection is short-lived, mainly owing to the inability of CD8 TCM cells to survive in the IL-15–deficient milieu. We present a hypothesis consistent with a model whereby intrahepatic CD8 TCM cells, being maintained by IL-15–mediated survival and basal proliferation, are conscripted into the CD8 effector/effector memory T cell pool during subsequent infections. The Journal of Immunology, 2013, 190: 5128–5141.

One of the cardinal features of Ag-specific immune responses elicited by infections or vaccinations is the persistence of optimally effective memory T cells that are inextricably linked to long-lasting protective immunity (1). Adequately maintained memory T cell pools assure a fast, effective, and specific response against reoccurring infections. Both the induction and the maintenance of memory T cells have been the subject of many elegantly conducted studies. The results from these studies provide much needed information toward the development of effective vaccines against viral, bacterial, and protozoan infections, such as malaria.

Maintenance of memory T cells is a very complex process involving many signals that are not yet fully understood. In some instances, particularly for CD8 T cells, the initial MHC/peptide–TCR interaction provides a sufficiently strong signal that the presence of long-lasting memory T cells is independent of persisting Ag (2). In other instances, particularly for intracellular pathogens that display tropism for nonlymphoid organs such as the kidney, lungs, or liver, Ag depot is needed for the maintenance of memory CD8 T cells (3, 4). Signals provided to T cells by costimulatory molecules, for example, B7 or OX40, expressed on APCs do not appear to be essential for the maintenance of secondary memory responses (5, 6), although engagement of OX40 is needed for the induction of lasting protection to vaccinia virus (7). Among other extrinsic factors that have been shown to affect the development and persistence of memory T cells, cytokines, referred to as signal 3 providers, play a prominent role in supporting these processes (8). Nevertheless, even in these instances, the sorting of each cytokine regarding its specific effects upon the development, survival, and turnover of memory CD8 T cells is still being investigated. The γ-chain receptor-sharing cytokines IL-2, IL-7, IL-15, and, to some extent, IL-21 have been shown to have complementary and overlapping effects on CD8 T cell differentiation and function; however, each cytokine also exerts a unique effect. For example, in most studies concerning acute responses to viral infections, IL-7 and IL-15 influence different CD8 T cell subsets. IL-7 promotes the accumulation of KLRC1hiCD127hi cells, whereas IL-2 and IL-15 cause accumulation of KLRC1loCD127lo CD8 T cells (9). Additionally, IL-7 regulates the survival and viability of naive and memory CD8 T cells (10), whereas IL-15 promotes survival and homeostatic proliferation (11, 12) as well as composition and differentiation of memory CD8 T cells (13).

The results from most studies, particularly those dealing with viral infections, show reduced maintenance of memory CD8 T cells in IL-15–deficient mice (14, 15). Alternatively, studies detailing the role of IL-15 in protective immunity to the intracellular parasite Toxoplasma gondii show conflicting results; whereas studies from one laboratory illustrated that IL-15 aids in resistance to infection...
and memory CD8 T cell development (16), another study demonstrated that IL-15 is not required for NK and CD8 T cell expansion or for protracted protection (17).

In this study, we focused on the maintenance of protracted protection induced by radiation-attenuated Plasmodium berghei sporozoites (Pb γ-spz) and particularly on the role of CD8 T central memory (TCM) cells in this process. We demonstrated previously (3) that lasting protective immunity induced in this model is associated with the accumulation in the liver of CD8 T cells that can be divided into two major subsets: 1) an effector/effector memory (TE/EM) CD8 T cell phenotype (CD44hiCD45RBlo), which is the major IFN-γ producer and is liver-stage (LS) Ag-dependent (3); and 2) a CD8 TCM cell phenotype (CD44hi CD45RBhi CD62Llo), which is not affected by the level of the LS Ag depot. Unlike CD8 TE/EM cells, CD8 TCM cells display a high concentration of IL-15Rβ (CD122hi). We previously hypothesized (18) that CD8 TCM cells function as a memory reservoir of CD8 T cells that, under the influence of IL-15 and MHC class I/peptide complexes derived from LS Ag depot, give rise to IFN-γ-producing CD8 TE/EM cells during reinfections.

IL-15 exerts its effects primarily on memory CD8 T cells by promoting their homoeostatic proliferation and survival as well as differentiation into an effector population (14, 19); hence, during reinfection, IL-15 would promote a shift from KLRG-1loCD127hi short-lived effector cells (9, 20). Because Pb γ-spz-induced CD8 TE/EM and TCM cells express different densities of IL-15Rβ (CD122), we asked whether this differential receptor expression on each subset might predispose each memory CD8 T cell set to respond uniquely to IL-15. The unique responses to IL-15 by each subset would in turn suggest a different, yet related, role of each subset in protective immunity induced by Pb γ-spz.

To investigate the effects of IL-15 on the CD8 TE/EM and CD8 TCM cell subsets, we used IL-15−/− deficient mice and examined CD8 T cell responses following multiple immunizations with Pb γ-spz and 1° and 2° homologous challenge with infectious sporozoites. Because IL-15−/− deficient mice have greatly diminished numbers of CD8 T (especially CD44hi phenotype) and NK cells, and because the residual CD44hi CD8 T cells are nearly all IL-2Rβhi CD8 T cells are nearly all IL-2Rβhi (21), we asked whether effector and/or memory CD8 T cells are inducible in IL-15−/− deficient mice and whether these mice could manifest long-term protection against blood stage parasitemia upon challenge with infectious sporozoites.

Our results demonstrate that although IL-15−/− deficient mice immunized with Pb γ-spz were protected against 1° sporozoite challenge, they succumbed to parasitemia upon a 2° challenge 2 mo later. Apart from demonstrating an absolute need for IL-15 for durable protection, our results reveal a critical role of CD8 TCM cells in maintaining protection. These are novel observations in malaria preerythrocytic-stage infection and are extremely important toward the shaping of our understanding of Plasmodium-induced immunity and toward developing more effective antimalaria vaccines. For example, these observations are paramount in view of our current observations from malaria clinical trial studies in which we show that Ag-specific memory T cell phenotypes are inducible (22), and yet these memory T cells appear to be inadequately maintained to prevent malaria infection upon experimental rechallenge with Plasmodium falciparum.

Materials and Methods

**Mice**

Female C57BL/6 mice (6–8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Female IL-15−/− deficient (knockout [KO]) (6–8 wk old) mice were purchased from Taconic (Germantown, NY). All mice were housed in accordance with Walter Reed Army Institute of Research guidelines. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee and performed in a facility accredited by the Association for Assessment of Laboratory Animal Care International.

**P. berghei sporozoites, immunizations, and parasitemia**

P. berghei sporozoites (cloned ANKA strain) (23), maintained by cyclical transmission in mice and Anopheles stephensi, were isolated from the salivary glands of female mosquitoes as described previously (3). For the induction of protective immunity, P. berghei sporozoites were dissected from mosquitoes 18–22 d after blood meal and used either immediately or after attenuation with gamma radiation (15,000 rads) (111Cs) source, Mark 1 series; J.L. Sheppard and Associates, San Fernando, CA). Sham-dissected preparations obtained from the salivary glands of noninfected mosquitoes were treated identically to sporozoites and were used as controls. Mice were primed i.v. with 75,000 γ-spz followed by two weekly immunizations of 20,000 γ-spz. Naïve control and γ-spz-immunized mice were challenged i.v. with 10,000 sporozoites between 7 and 10 d after the last boost (1° challenge) or 2 mo (~60 d) following 1° challenge (2° challenge) (see Fig. 3A). Thin blood smears were prepared daily from individual mice starting on day 4 after the challenge and followed for up to 14 d, and parasitemia was determined microscopically from Giemsa-stained slides.

**Isolation of intrahepatic mononuclear cells**

Mice were euthanized by CO2 inhalation according to protocol guidelines. Intrahepatic mononuclear cells (IHMCs) were isolated from the liver as described previously (3). Briefly, livers were exposed and the inferior vena cava was cut for blood outflow. After perfusion with 10 ml PBS (24), livers were removed and pressed through a 70-μm nylon cell strainer (BD Biosciences, San Jose, CA). IHMCs were isolated from the liver cell suspension on a 5% Percoll gradient (GE Healthcare Biosciences, Piscataway, NJ) and RBCs were lysed with RBC lysis buffer (Sigma-Aldrich, St. Louis, MO). The IHMCs were washed and resuspended in complete medium consisting of RPMI 1640 containing 10% heat-inactivated FBS (Thermo Fisher Scientific, Waltham, MA), 10 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate, GlutaMAX, and nonessential amino acids (Invitrogen, Grand Island, NY) and 50 μM 2-ME (Sigma-Aldrich). Cell counts were performed by the trypan blue exclusion test.

**Lymphocyte separation**

For Pb γ-spz infection, splenic lymphocytes were sorted for CD8 T cell separation by using a magnetic bead isolation kit (Miltenyi Biotec, Auburn, CA) following the manufacturer’s instructions. Where indicated, CD8+ and CD8− T cell subsets were further separated into CD62L+ and CD62L− populations using CD62L+ positive selection kit (Miltenyi Biotec).

**[3H]Thymidine deoxyribose incorporation**

For [3H]thymidine deoxyribose ([3H]Tdr) uptake, magnetic bead isolated splenic CD8+ and CD8− T cells were cultured for 96 h in the presence of recombinant mouse IL-15 at 50 or 100 ng/ml (R&D Systems, Minneapolis, MN). The IHMCs were washed and resuspended in complete medium consisting of RPMI 1640 containing 10% heat-inactivated FBS (Thermo Fisher Scientific, Waltham, MA), 10 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate, GlutaMAX, and nonessential amino acids (Invitrogen, Grand Island, NY) and 50 μM 2-ME (Sigma-Aldrich). Cell counts were performed by the trypan blue exclusion test.

**Flow cytometry**

Surface staining of phenotypic markers was performed using anti-CD3 (17A2), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD44 (1M7), anti-CD45RB (16A), anti-CD426 (MEL-14), anti-CD122 (TM-1), and anti-CD127 (A7R34) (BD Biosciences) as indicated in the figure legends. FcR was blocked during surface staining using unlabeled anti-CD16/32 (2.4G2) (BD Biosciences). Caspase-3 was detected using clone 46A/ 1 caspase 3 (BD Biosciences). Dead cells were labeled using PE-labeled annexin V (BD Biosciences) and Live/Dead fixable blue dead cell stain for UV excitation (Invitrogen/Molecular Probes). Intracellular staining for Bcl-2 (BD Biosciences) was performed following surface staining using BD Cytofix/Cytoperm according to the manufacturer’s instructions. Flow cytometry data were collected using BD FACSCalibur or LSRII instruments and analyzed using CellQuest (BD Biosciences) or FlowJo (Tree Star) software. Cells (1 × 106) were stained in each sample, and >100,000 live lymphocyte events were collected. Where appropriate, cell counts were derived by multiplying the percentage of gated events by total cell counts obtained by a trypan blue exclusion test.

**CFSE labeling**

Total CD8+ T cells, isolated by negative magnetic bead selection, were stained with 10 nM CFSE (Invitrogen/Molecular Probes) in PBS for 30
min at 37°C, washed three times, and cultured for 7 d in the presence of recombinant mouse IL-15, IL-2 (R&D Systems), or plate-bound anti-CD3 mAb (145-2C11; BD Biosciences). Following culture, cells were stained with anti-CD4 and anti-CD45RB to delineate memory cell subsets.

**In vivo proliferation by BrdU incorporation**

In vivo proliferation of liver CD8 T cells, CD4 T cells, and NK1.1 T cells as well as NK cells was measured by a BrdU (Sigma-Aldrich) incorporation assay (25). Mice were fed 0.8 mg/ml BrdU in drinking water replaced daily for 7 d to permit analysis. IHMCs were isolated and stained using a FITC BrdU staining kit (BD Biosciences) according to the manufacturer’s instructions. Briefly, cells were stained for the indicated surface markers, after which cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and washed in Perm/Wash buffer (BD Biosciences). Cells were then incubated with Cytoperm Plus (BD Biosciences) and washed. BrdU was exposed by treatment with DNase and stained using anti-BrdU-FITC–labeled Ab. Total numbers of IHMCs were counted and were used to determine absolute number of CD8 TE/EM and CD8 TCM cell subsets.

**IFN-γ cytokine secretion assay**

IFN-γ determinations were performed using a secretion assay detection kit according to the manufacturer’s instructions (Milenyi Biotec) and as described previously (3). Briefly, IHMCs were washed twice with cold PBS/0.5% BSA (Sigma-Aldrich) and 2 nM EDTA (Sigma-Aldrich) and were resuspended at 106 cells/90 μl cold RPMI 1640 with 5% normal mouse serum plus 10 μl IFN-γ capture reagent and were incubated on ice for 5 min and then diluted up to 105 cells/ml with warm RPMI 1640 with 5% normal mouse serum, transferred into flat-bottom 12-well plates, and incubated for 45 min at 37°C. After two washes, cells were resuspended in 90 μl cold PBS (Invitrogen) and incubated with 10 μl IFN-γ detection reagent for 10 min at 4°C. After the final two washes in PBS, cells were resuspended in 100 μl freshly prepared propidium iodide at 50 μg/ml (Invitrogen) and analyzed by flow cytometry. Data were analyzed by CellQuest software (BD Biosciences).

**Preparation of Kupffer cells**

IHMCs were isolated from livers and incubated with anti-CD8 and anti-CD4–conjugated magnetic beads (Milenyi Biotec), washed, and then applied to a MS magnetic column. The flow-through cells were labeled with biotinylated anti-Mac3 Ab (Cedarlane, Burlington, ON, Canada) and anti-biotin beads (Milenyi Biotec) and applied to a second MS column. After removal of unlabeled cells, the bound Mac3+ Kupffer cells (KCs) were eluted. The identity of KCs was determined as described previously (26).

**RT-PCR**

RNA was isolated from the KCs lysed in TRizol (Invitrogen), and 5 μg RNA was reverse transcribed using SuperScript II (Invitrogen). Mouse genomic DNA was isolated using DNA STAT-60 (Iso-Test Diagnostics, Pearlland, TX) and diluted to 100 μg/μl, as previously described (26). Quantitative PCR reactions were established (Qiagen, Valencia, CA) with the following primers for IL-15 (forward 5′-CTCTACCTGGCAAACGACTC-3′, reverse 5′-CCGACATTGCTCATCATTCT-3′) and for GAPDH (forward 5′-TCCCTCACAATTTCCATCCC-3′, reverse 5′-CCTAGGCCCTCTGTTATT-3′; Invitrogen) using the incorporation of SYBR Green as the fluorescence reporter. The PCR products were performed using an ABI 7700 (PE Applied Biosystems) under the following conditions: 1 cycle at 95°C for 10 min, and 50 cycles of 95°C for 15 s and 55°C for 1 min. Standard curves were generated from a DNA standard and the amount of cDNA was determined for each sample. Quantitative IL-15 gene expression was done in triplicate and expressed as a ratio of IL-15 over GAPDH for each time point (26).

**Statistical analysis**

Data analysis was performed using GraphPad Prism 6.0 software using an unpaired two-tailed t test for unequal variances, a Mann–Whitney U test, or two-way ANOVA followed by a Bonferroni posttest where appropriate. A p value <0.05 was considered significant.

**Results**

**Liver CD8 TCM cells undergo selective in vitro proliferation and enhanced survival in the presence of IL-15**

It has been demonstrated that cytokines, by providing signals to T cells via the shared γ-chain receptor, maintain survival of naive and memory CD8 T cells (27). CD8 TCM cells (CD44highCD45RBhighCD62Llow) that accumulate in the liver of mice immunized with Pb γ-spz express considerably higher levels of CD122 (IL-15Rβ/IL-2Rβ) than the major IFN-γ–producing CD8 TEff cells (CD44highCD45RBhighCD62Llow) or CD8 T naive cells (CD44high) (3) (Supplemental Fig. 1), which suggests an enhanced sensitivity of CD8 TCM cells to either IL-15 or IL-2. We measured in vitro proliferation to soluble IL-15 (1H[TdR uptake] of enriched CD8+ and CD8– T cells isolated from livers of mice immunized thrice with Pb γ-spz. CD8+CD62Llow T cells proliferated in a dose-dependent manner (Fig. 1A), and all other cultures, including CD8+CD62Lhigh T cells and CD8–CD62Llow and CD8–CD62Lhigh T cells, exhibited ~5-fold lower proliferation, even at 100 ng/ml IL-15. We confirmed this observation by the dilution effect of CFSE-labeled total liver CD8 T cells isolated from Pb γ-spz–immunized mice. In the presence of soluble IL-15, CD8 TCM cells but not CD8 TEff cells underwent several rounds of division; IL-2 induced much less proliferative activity than did IL-15, but it had a discernible effect on both CD8 T cell subsets (Fig. 1B); anti-CD3 mAb, as expected, also induced proliferation of both CD8 TCM and CD8 TEff cells (Fig. 1B). A population of CD8 TCM cells that did not proliferate to anti-CD3 mAb likely belongs to terminally differentiated short-lived effector cells. Collectively, these observations establish that IL-15 selectively induced in vitro proliferative activity of liver CD8 TCM cells from mice exposed to protective doses of Pb γ-spz.

IL-15 also has been shown to enhance survival of memory CD8 T cells by upregulating antiapoptotic molecules such as Bcl-2 and Bcl-2 (28, 29). We evaluated the expression of Bcl-2 on liver CD8 T cells in our protection model. Among the IHMCs isolated 1 wk after the third immunization with Pb γ-spz and cultured with IL-15, CD8 TCM cells significantly upregulated the expression of Bcl-2 compared with control media and significantly exceeded the expression of Bcl-2 on CD8 TEff cells from the same group of mice (Fig. 1C). Whereas IL-15–stimulated cultures of CD8 TCM cells sustained Bcl-2 expression for 7 d, control cultures in medium alone, similar to cultures stimulated with anti-CD3 mAb (data not shown), showed downregulated expression of Bcl-2 after 3 d culture. Thus, as previously shown for spleen CD8 T cells (11), liver CD8 TCM cells also are the primary responders to IL-15 in regards to increased survival.

**IL-15-deficient mice respond to immunization with Pb γ-spz by expanding liver IFN-γ+ CD8 T cells**

T cell and B cell responses directed to the nonrepeat (30) and the repeat regions (31), respectively, of the circumsporozoite protein (CSP), the major protein Ag present on Plasmodium sporozoites, have been shown to be linked to protection induced by Plasmodium γ-spz (32, 33). Other Ags representing pre-erythrocytic stages, and specifically Ags expressed during LS development, have also been shown to be involved in protection (32, 34–36). We demonstrated previously that owing to drug-induced reduction of LS Ag depot, protection wanes concurrently with declining CD8 TCM cells (3), which, expectedly, is not the case with effector CD8 T cells specific for epitopes on CSP (37, 38). We, as well as others, have proposed that the longevity of protective immunity (3, 39) and the effectiveness of CD8 TCM cells (3) is linked to the levels of LS Ag depot derived from partially developed Plasmodium γ-spz. However, no links between CD8 TCM cells and Plasmodium γ-spz–induced protective immunity or immunity induced by natural exposure have thus far been investigated. CD8 TCM cells have been considered the key cells that maintain lasting protection (40), and in some infections protective immunity has been shown to be largely dependent on CD8 TCM cells (41).
Because liver CD8 T<sub>CM</sub> cells preferentially responded in vitro to IL-15 (Fig. 1), we used IL-15–deficient mice to test whether the absence of IL-15 might reveal a role of CD8 T<sub>CM</sub> cells in protective immunity in the Pb γ-spz model. IL-15 KO mice have reduced numbers of CD8 T cells, NKT cells, and NK cells (15); nonetheless, the Ag-specific repertoire of CD8 T cells remains intact (14, 16). We monitored liver CD3<sup>+</sup> CD8 T cells from Pb γ-spz–immunized wild-type (wt) and IL-15–deficient mice for the expression of an activation marker (CD44<sup>hi</sup>) indicative of Ag exposure and compared the numbers of CD44<sup>hi</sup> CD8 T cells between the two groups of mice. As shown previously for other organs (21), livers from naive wt mice had twice as many CD3<sup>+</sup> CD8 T cells as did IL-15–deficient mice. After the third immunization with Pb γ-spz, the number of total liver CD3<sup>+</sup> CD8 T cells doubled, with a concomitant increase of CD8 T<sub>EM/TCM</sub> cells in both groups of mice (Fig. 2A); however, the increase of CD8 T<sub>CM</sub> cells was considerably lower in the IL-15 KO mice compared with wt mice. Upon primary challenge (∼7 d after the last boost immunization with Pb γ-spz), the absolute number of CD3<sup>+</sup> CD8 T cells significantly increased in wt mice, whereas in the IL-15 KO mice the number of total CD3<sup>+</sup> CD8 T cells, although significantly higher relative to naive cells, did not change. The number of CD8 T<sub>EM/TCM</sub> cells rose in both groups and the ratio of T<sub>EM/TCM</sub> to T<sub>CM</sub> cells clearly favored the former subset in IL-15 KO mice (Fig. 2A). We also observed this skewing toward CD8 T<sub>EM/TCM</sub> cells in a separate experiment; 2 mo after the last Pb γ-spz boost.

**FIGURE 1.** CD8 T<sub>CM</sub> cells, having elevated expression of IL-15Rβ<sub>1</sub> (CD122), are the primary cells responding to IL-15. IHMCs were isolated from C57BL/6 mice (three per group) 1 mo after a tertiary immunization with Pb γ-spz. (A) CD62L<sup>+</sup> and CD62L<sup>−</sup> T cells were separated by magnetic bead isolation procedure; a second round of isolation using CD8 magnetic beads resulted in the following subpopulations: CD8<sup>+</sup>CD62L<sup>−</sup>, CD8<sup>+</sup>CD62L<sup>+</sup>, and CD8<sup>+</sup>CD62L<sup>+</sup> T cells as well as CD8<sup>+</sup>CD62L<sup>−</sup> and CD8<sup>+</sup>CD62L<sup>+</sup> T cells. Each T cell subset was cultured at 4 × 10<sup>5</sup> cells/0.2 ml culture medium for 96 h in the presence of the indicated concentrations of IL-15; during the last 16 h culture, 1 μCi [<sup>3</sup>H]Tdr was added to each well. Results are presented as the mean cpm ± SD of [<sup>3</sup>H]TdR uptake in triplicate wells and are representative of three separate experiments. 

(B) Liver CD8 T cells, isolated by negative magnetic beads selection, were stained with CFSE and incubated for 7 d in the presence of 100 ng/ml IL-15 or 100 ng/ml IL-2. Harvested cells were stained with mAbs against CD8, CD44, and CD84RB. Lymphocytes were gated on a forward/side scatter plot, and gates were applied to identify CD8<sup>+</sup> T cells that expressed either CD44<sup>hi</sup>, CD44<sup>lo</sup>, or CD44<sup>hi</sup>CD45RB<sup>lo</sup> T cells. These results represent one of two separate experiments. 

(C) Liver CD8 T cells were cultured in the presence of either 100 ng/ml IL-15 or medium alone. Cells were harvested on days 1, 3, and 7 and stained with mAbs against CD44 and CD44RB as described above, fixed, permeabilized, and stained with mAbs against Bcl-2. Mean fluorescence intensity (determined for each individual mouse and presented as the mean ± SD) of Bcl-2 expression of T<sub>CM</sub> or T<sub>EM/TCM</sub> cells is shown. Bcl-2 determinations on CD8 T<sub>CM</sub> and CD8 T<sub>EM/TCM</sub> cells prior to culture showed an MFI of 189 ± 45 for CD8 T<sub>CM</sub> cells and an MFI of 76 ± 35 for CD8 T<sub>EM/TCM</sub> cells. Results are representative of three separate experiments. *p < 0.05, **p < 0.01.

**FIGURE 2.** IL-15 KO CD8 T cells respond to Ag from Pb γ-spz. Wt and IL-15 KO mice (three to five mice per group) were immunized with 3 weekly doses of Pb γ-spz followed 1 wk later by infectious sporozoite challenge as described in Materials and Methods. (A) Livers from naive, immune, or immune/challenged (CH) mice were isolated 1 wk after last exposure to Ag and stained with anti-CD3, anti-CD8, anti-CD44, and anti-CD62L mAbs to determine the number of total CD8 T cells, CD8 T<sub>EM/TCM</sub> cells, and CD8 T<sub>CM</sub> cells. The results show the number of cells as the mean ± SD. (B) IHMCs were isolated from wt and IL-15 KO mice 1 wk following the third immunization with Pb γ-spz. IFN-γ was detected by cytokine secretion assay as described in Materials and Methods. CD8 T cells were enriched by magnetic bead separation and CD44<sup>hi</sup>CD44RB<sup>hi</sup> or CD44<sup>lo</sup>CD44RB<sup>lo</sup> T cells were identified by flow cytometry. The results are representative of several experiments performed under the same conditions and show the number of IFN-γ–secreting CD8 T<sub>CM</sub> cells and CD8 T<sub>EM/TCM</sub> cells per 10<sup>6</sup> IHMCs determined for each individual mouse and are presented as the mean ± SD. *p < 0.05, **p < 0.01.
immunization of wt and IL-15 KO mice, where the percentage of CD8 T_EM cells exceeded CD8 T_Cm by ~7-fold in IL-15 KO mice, but only ~1.4-fold in wt mice (Supplemental Fig. 2). The skewing toward the CD8 T_EM subset in IL-15 KO mice might have resulted from attrition of IL-15–dependent CD8 T_Cm cells or from a compensatory action of other γ-chain cytokines that enhances the survival of CD8 T_EM cells. As shown by others, differentiation of CD8 T cells is programmed following Ag exposure (41), and effector CD8 T cells do accumulate in γ-chain cytokine-deficient mice (42). Regardless of the mechanism responsible for the skewing toward CD8 T_EM cells, these results establish that IL-15 KO mice responded to pre-erythrocytic stage Ags expressed by the partly developed Pb γ-spz.

It has been established that IFN-γ-producing effector CD8 T cells are the main mediators of protection induced by attenuated Plasmodium sporozoites (43–45). Although it has been shown that IL-15 modulates the differentiation of CD8 T cells into IFN-γ-producing or perforin- and granzymes A– and B–expressing cells (13, 46, 47), we considered the possibility that the increased percentage, as well as cell number, of CD8 T_EM cells in IL-15 KO mice may also reflect an elevated IFN-γ production by CD8 T cells. Comparing IFN-γ CD8 T cells between wt and IL-15 KO mice immunized three times with Pb γ-spz revealed that IL-15 KO mice had significantly lower frequency of IFN-γ CD8 T cells than did wt mice: 3 × 10^3 versus 5 × 10^3 cells/10^6 HMCs, respectively (Fig. 2B). Nonetheless, IL-15 KO mice immunized with Pb γ-spz were able to mount inflammatory cytokine–producing CD40^hi CD8 T cells before 1˚ challenge, which further confirms that IL-15 KO mice were able to respond to Ags associated with Pb γ-spz.

**IL-15 is not required for induction but is needed for the maintenance of protective immunity induced by Pb γ-spz**

The observation that immunization with Pb γ-spz induced both IFN-γ CD8 T cells and the expansion of CD8 T_EM cells in IL-15–deficient mice prompted us to ask whether these responses were sufficient to confer protection against infectious sporozoite challenge. Groups of wt and IL-15 KO mice exposed to three weekly doses of Pb γ-spz were challenged with 10,000 infectious sporozoites between days 7 and 10 after the last boost immunization, along with naive infectivity control mice, and sterile protection was determined by blood smears starting on day 4 and continuing for 14 d after the challenge (Fig. 3A). Both wt as well as IL-15 KO mice were protected (14 d) and did not develop blood-stage parasitemia, whereas infectivity control mice became parasitic within 5–7 d after exposure to infectious P. berghei sporozoites (Fig. 3B). On the basis of these results, we conclude that IL-15 was not needed for the induction of protective immunity. Clearly, functional effector CD8 T cells arose to Pb γ-spz immunization in the absence of IL-15 and this response appeared to be sufficient to protect mice during 1˚ sporozoite challenge; severely reduced numbers of CD8 T_Cm cells did not affect this phase of protection induced with Pb γ-spz.

A sine qua non of a successful vaccine is Ag-specific durability of its protective effects. Exposure to Plasmodium γ-spz has been shown to provide sterile and long-lasting protection in both human and animal models, and, therefore, γ-spz vaccine is considered the gold standard of antimalaria vaccines (48). We and others have firmly established that sterile protection induced by Pb γ-spz in wt mice is long-lived (3, 33). We compared the durability of protection in wt and IL-15–deficient mice by rechallenging mice that were protected 2 mo previously (Fig. 3A). Whereas wt mice remained protected, most (85%) of previously protected IL-15 KO mice became parasitic between days 5 and 9 after rechallenge (Fig. 3B). All infectivity control mice became parasitic between days 4 and 7 after infection (data not shown). In contrast to the induction of protection, durability of protection was clearly IL-15–dependent. This single observation highlights the importance of IL-15 in the maintenance of protection induced by Pb γ-spz against experimental sporozoite challenge, and, by extension, it highlights the importance of CD8 T_Cm cells and suggests a link between IL-15 and CD8 T_Cm cells in this process.

We also considered that the failure to maintain long-term protection might be related to some deficits of NK or NKT cells in IL-15 KO mice, as IL-15 has a strong effect on the proliferation of NK and NKT cells (49). We measured absolute cell numbers and assessed proliferative responses (data not shown) of liver NK and NK1.1^+ T cells from wt and IL-15 KO mice. As expected, the cell numbers of both NK and NK1.1^+ T cells were reduced by 90% in IL-15 KO mice as compared with wt mice (Supplemental Fig. 3A). Because the decline of NK1.1^+ T cells and NK cells was already apparent at 1˚ challenge, when IL-15 KO mice were protected, these results confirm previously established observations that protection in B6 mice is mediated mainly by effector CD8 T cells (3, 45) rather than by NK or NK1.1^+ T cells. Analyses of liver CD4 T cells showed that neither the absolute cell number nor the number of proliferating cells (data not shown) was statistically different between the two groups of mice at 2˚ challenge, when IL-15 KO mice became parasitic (Supplemental Fig. 3B).
According to these observations, liver CD4 T cells have not been compromised by the lack of IL-15 and thus do not appear to have a role in the IL-15 impact on lasting protection induced by Pb γ-spz. Although it has been shown that IL-15 readily substitutes for CD4 T cell helper activity in CD8 T cell responses (50), our results do not suggest any reciprocal affects of CD4 T cells on CD8 T cell responses in IL-15 KO mice.

**IFN-γ–producing CD8 T cells are induced in IL-15 KO mice immunized with Pb γ-spz**

The disparate outcomes of 1˚ and 2˚ challenges were not completely surprising in view of our current understanding of the IL-15 effects on CD8 T cells. IL-15 affects mainly memory CD8 T cells by promoting differentiation of MPECs to terminal effector CD8 T cells (19), enhancing cellular turnover (14) and maintaining their survival (11). In consideration of these multiple effects, we hypothesized that the absence of IL-15 likely caused a global disturbance of memory CD8 T cell formation and/or function that ultimately resulted in the failure to maintain long-lasting protection induced by Pb γ-spz.

**IFN-γ+ CD8 T cells represent the hallmark of protective immunity induced in the Pb γ-spz model (45), and IFN-γ+ CD8 T cell responses, albeit significantly lower than in wt mice, were nonetheless present in IL-15 KO mice prior to primary challenge (Figs. 2B, 4). As has been originally proposed by Ahmed and Gray (1) and confirmed by others (51, 52), the breadth of the effector CD8 T cell population during the induction phase of the immune response determines the size of the memory pool. On the basis of the above observations (Fig. 2B), we considered the possibility that the failure of IL-15 KO mice to maintain lasting protection might be linked to reduced memory pool and hence fewer functioning CD8 T cells, for example, IFN-γ–producing, CD8 T_E cells upon rechallenge. If indeed memory CD8 T cells relied on IL-15 for the maintenance of memory cell pool size, or a transition from CD8 T_CM or CD8 T_E/EM to CD8 T_E cells, the numbers of IFN-γ+ CD8 T cells would be considerably lower in IL-15 KO mice at 2˚ challenge, when the mice were no longer protected.

Both IL-15 KO and wt mice were immunized thrice with Pb γ-spz, and we determined the numbers of IFN-γ+ CD8 T cells in both groups of mice at several critical time points during 1˚ and 2˚ challenges.
challenges. At 0 h (day of 1˚ challenge), IL-15-deficient mice responded robustly with nearly 3000 IFN-γ+ CD8 T cells/10^6 IHMCs versus 5000 IFN-γ+ CD8 T cells/10^6 IHMCs in wt mice. The IFN-γ responses doubled in each group 6 h after challenge; 24 h after challenge, the response peaked (18,000/10^6 IHMCs) in wt mice, whereas in IL-15-deficient mice the response remained nearly the same (7,000/10^6 IHMCs) (Fig. 4A). There was a small spike of reactivity, however insignificant relative to reactivity at 24 h in IL-15 KO mice at 72 h, at which time the response in wt mice decreased to a baseline level. We have observed (3) that IFN-γ+ CD8 T cell numbers fluctuate, particularly during the first week after the challenge. The key observation that emerged from these experiments was that similar to wt mice, IL-15 KO mice, despite significantly lower IFN-γ+ CD8 T cell responses prior to challenge, responded swiftly after the 1˚ challenge, which is essential to prevent blood-stage parasitemia (3). A significantly higher response relative to prechallenge was also maintained for 72 h. Because Abs also contribute to protection in this model, it should be noted that the anti-P. berghei CSP Ab responses were identical in both groups of mice (data not shown).

IFN-γ+ CD8 T cell responses declined in both groups of mice 2 mo after the 1˚ challenge (around day 60 or the day of 2˚ challenge). Six hours after the 2˚ challenge, the number of IFN-γ+ CD8 T cells rose significantly in both groups and responses remained at a plateau (Fig. 4B). However, the numbers of IFN-γ+ CD8 T cells in wt mice significantly exceeded those in IL-15 KO mice at 6 and 30 h. We chose not to monitor IFN-γ+ CD8 T cell responses beyond 30 h after 2˚ challenge primarily because the onset of parasitemia is known to interfere with responses induced by pre-erythrocytic-stage Ag. According to results from other studies, the absence of IL-15 has a negligible effect on the production of inflammatory cytokines by boosted memory CD8 T cells (17). In contrast, in the Pb γ-spz model, CD8 T cells were lower IFN-γ producers after challenge in IL-15 KO mice relative to wt mice. The reasons for this difference remain unclear, but because the initial number of the responding effector CD8 T cells determines the memory CD8 T cell pool size (1), the initial lower cell number of the IFN-γ+ CD8 T cells at prechallenge in IL-15 KO mice may have resulted in the lower breadth of responses that followed upon 2˚ challenge. It is also possible that Pb γ-spz-induced CD8 T CM cells are dependent on IL-15 for differentiation and/or transition to IFN-γ-producing terminal effector CD8 T cells.

A threshold of an effective IFN-γ+ CD8 T cell response needed for sterile protection has been suggested; however, such a threshold has not been clearly defined in terms of cell numbers or cytokine levels. The threshold also varies among different murine Plasmodium spp., the source and type of CD8 T cells, as well as the assay used for determinations (53). Additionally, variations have been observed from experiment to experiment. On the basis of our observations, it is clear that IL-15-deficient mice showed lower responses than wt mice (Fig. 4A, 4B). Nevertheless, the responses were not strikingly low to account for the failure of IL-15 KO mice to maintain protective immunity at rechallenge compared with primary challenge. Similar observations have been made in other systems showing that despite the absence of a single γ-chain cytokine, formation of robust effector CD8 T cells does occur (42).

If IL-15 indeed affects the expression of phenotypic markers and functional attributes of memory CD8 T cells (13, 42), such as the transition from CD127hiCD62LloCD8 T CM cells to a population of CD127loCD62LhiCD8 T E cells, such transition would be less evident in IL-15 KO mice than in wt mice exposed to Pb γ-spz. A compromised ability to transition to CD127loCD62LloCD8 T cells may explain the lower IFN-γ response observed in IL-15 KO mice, particularly during a rechallenge. We examined both CD44hiCD62LloCD8 T CM cells and CD44hiCD62LhiCD8 T E cells for the expression of CD127 at 24 h after 1˚ and 2˚ challenge. Liver CD8 T CM cells expressed nearly similar CD127 profiles in wt and IL-15 KO mice and they persisted for 2 mo as shown during the 2˚ challenge (Fig. 4C). Because IL-15 and IL-7 overlap in the induction of CD8 T cell response, in the absence of IL-15, IL-7 might have been responsible for the preservation of CD62LhiCD127loCD8 T cells in IL-15 KO mice. In contrast, 24 h after 2˚ challenge, we observed a transition of CD8 T E cells to CD127lo in wt mice, whereas in IL-15 KO mice, most CD62Llo CD8 T cells remained as CD127hi phenotype. Our observation that the loss of CD127, which indicates differentiation of MPECs into terminal effector cells (42), required IL-15 is in agreement with results from other studies that the availability of IL-15 allows long-lived memory to transition to short-lived IFN-γ-producing effector cells (13). Further studies are needed to determine whether CD127hi cells in IL-15 KO mice represent a true long-lived memory CD8 T cell, or whether they are sustained by IL-7, which may not be critical for the determination of memory formation as shown during acute viral infection (54).

Attrition of CD8 T CM cells is coincident with the failure to maintain protracted protection in IL-15 KO mice

Among the in vivo activities regulated by IL-15, basal proliferation of CD8 T cells is yet another of its signatures. We hypothesized that proliferation of CD8 T CM cells might be significantly reduced in Pb γ-spz–immunized IL-15–deficient mice, because as we have shown here (Fig. 1) and as was shown by others (55), these cells depend on IL-15 more than CD8 T EM cells by the virtue of having elevated expression of CD122 (Supplemental Fig. 1C). A decreased cellular turnover, which has been reported previously for 2˚ memory CD8 T cells (13), would clearly lead to a severely contracted reservoir of CD8 T CM cells. Partly activated CD8 T CM cells are needed for a quick differentiation into effectors to sustain functional properties of CD8 T cells that provide a robust response during reinfection.

To examine whether indeed CD8 T CM cells in IL-15 KO mice have reduced proliferative activity in relationship to wt mice, we measured BrdU incorporation by CD8 T cells in wt and IL-15 KO mice for 7 d prior to either 1˚ or 2˚ challenge. Results from initial BrdU uptake experiments that were conducted in mice immunized with Pb γ-spz were ruled out the possibility that proliferation of CD8 T cells stems from contaminants present in the hand-dissected sporozoite preparations (Supplemental Fig. 4). The gating strategies for liver CD3+ CD8 T cells and the respective CD8 T CM (CD44hiCD45RBhi) and CD8 T EM (CD44hiCD45RBlo) phenotypes are depicted for wt and IL-15 KO mice in Fig. 5A and 5B, respectively. Additionally, we show gating strategies for BrdU+ total liver CD3+ CD8 T cells and the respective CD8 T CM and CD8 T EM cell subsets (Fig. 5A, 5B), where the numbers in each panel reflect the percentage of the designated BrdU+ CD8 T cells from a representative single mouse from each group prior to 2˚ challenge.

Despite the fact that the numbers of total BrdU+ CD8 T cells as well as CD8 T cell memory subsets in IL-15 KO mice represented approximately half of the respective BrdU+ CD8 T cells in wt mice, the percentage of BrdU+ CD8 T cells were remarkably similar between the two groups of mice at prechallenge (Fig. 5C). These results suggest that an equivalent fraction of the respective CD8 T cell subset from each group proliferated similarly after immunizations with Pb γ-spz. Prior to 2˚ challenge (prechallenge), considerably fewer BrdU+ CD8 T cells, regardless
of the phenotype, were detected in both wt and IL-15–deficient mice (Fig. 5C) and this is in agreement with observations made in bacterial and viral systems (13). The percentages of BrdU+ CD8 T cells also declined in both groups of mice, but the percentage of BrdU+ CD8 T cells was higher in IL-15 KO mice than in wt mice. This was particularly true for CD8 TCM cells, as ∼52% of these cells were proliferating, presumably driven by cytokines other than IL-15 or possibly by the LS Ag depot. This is worth noting because we recovered very few CD8 T cells from IL-15–deficient mice prior to 2˚ challenge (Fig. 5D). It is possible that the proliferating CD8 TCM cells were undergoing an accelerated attrition in the absence of the survival promoting IL-15 (11, 28), which ultimately led to a reduced number of cells in the memory pool, hence the inability to confer protection upon rechallenge. Although results from other studies suggest that 2˚ memory responses are less sensitive to endogenous IL-15 (56), it appears that in the Pb γ-spz system, cellular turnover of secondary memory CD8 T cells is dependent, in part, on the provision of IL-15.

According to absolute cell counts of the liver CD3+ CD8 T cells and the two CD8 T cell memory subsets, a significant cellular attrition did occur in both groups prior to 2˚ challenge (Fig. 5D). A >10-fold reduction in IL-15 KO mice was quite striking in comparison with cellular loss in wt mice. To assess the relative loss of CD8 T cells in both the wt and IL-15 KO mice during the interval between the challenges, we determined the percentages of the total liver CD8 T cells, CD8 T EM cells, and CD8 TCM cells remaining at time of 2˚ challenge. The percentage of the remaining cells was calculated as follows: at 1˚ challenge, the number of total CD8 T cells and each of the CD8 T cell subset was considered as 100% and the number of each of the respective CD8 T cells counted at 2˚ challenge was considered as a percentage of the cells at 1˚ challenge, which is indicated by the filled bars for wt mice and by the open bars for IL-15 KO mice. *p < 0.05.
to ∼7% of CD8 T<sub>CM</sub> cells that remained in wt mice. Similar observations were made with regard to level of memory CD8 T cells that remained in wt mice with other infections (54, 57).

Decreased expression of antiapoptotic molecules on CD8 T<sub>CM</sub> cells in IL-15 KO mice is responsible for decreased survival and lack of protection

Because the most severe attrition occurred within the CD8 T<sub>CM</sub> cell subset in IL-15 KO mice prior to 2<sup>°</sup> challenge (2 mo after the 1<sup>°</sup> challenge), we asked whether this cell loss could be explained by a reduced expression of antiapoptotic proteins (e.g., Bcl-2). Prior to 2<sup>°</sup> challenge, CD8 T<sub>CM</sub> cells from wt mice expressed the highest level of Bcl-2; CD8 T<sub>EM</sub> cells also expressed a high level of Bcl-2 in comparison with naive CD8 T cells and isotype controls (Fig. 6A). Strikingly different results were observed from identically immunized and challenged IL-15–deficient mice, as Bcl-2 was downregulated not only on CD8 T<sub>CM</sub> cells but also on CD8 T<sub>EM</sub> cells (Fig. 6A, lower panel). Determinations of caspase-3 levels confirmed that the reduced levels of antiapoptotic Bcl-2 on IL-15 KO D<sub>C</sub> T cell subsets related to the extensive apoptosis (Fig. 6B). In comparison with caspase-3 levels on CD8 T<sub>CM</sub> cells in wt mice, significantly elevated levels (%) of caspase-3<sup>+</sup> CD8 T<sub>CM</sub> cells were found in IL-15–deficient mice at 2 mo after immunization/challenge (Fig. 6B). Increased caspase-3<sup>+</sup> CD8 T<sub>CM</sub> cells were already found in naive IL-15 KO mice, as the absence of IL-15 could not support survival of these cells.

According to results shown in Fig. 5C, the percentage of BrdU<sup>+</sup> CD8 T cells, including CD8 T<sub>CM</sub> and CD8 T<sub>EM</sub> cells, was comparable in wt and IL-15 KO mice prior to 1<sup>°</sup> challenge. Nonetheless, CD8 T<sub>CM</sub> cells underwent greater attrition in IL-15 KO mice compared with wt mice, whereas CD8 T<sub>EM</sub> cells were comparable between the two strains of mice. In the next set of experiments we specifically evaluated proliferating BrdU<sup>+</sup> CD8 T<sub>CM</sub> cells for both the expression of Bcl-2 and the level of apoptosis after Pb γ-spz immunization (prior to 1<sup>°</sup> challenge) and 1 mo and 6 mo after 1<sup>°</sup> challenge. BrdU<sup>+</sup> CD8 T<sub>CM</sub> cells from IL-15 KO mice expressed significantly decreased levels (2- to 3-fold) of antiapoptotic Bcl-2 at all indicated time points relative to wt mice (Fig. 7A). Similarly, IL-15 KO BrdU<sup>+</sup> CD8 T<sub>CM</sub> cell populations also contained a significantly greater percentage of annexin V<sup>+</sup> staining cells than did BrdU<sup>+</sup> CD8 T<sub>CM</sub> cells, indicating an increase in cell death within the proliferative subset at 1 mo and 6 mo after 1<sup>°</sup> challenge (Fig. 7B). Although BrdU<sup>+</sup> CD8 T<sub>CM</sub> cells in wt mice also showed higher annexin V<sup>+</sup> cells than BrdU<sup>+</sup> CD8 T<sub>CM</sub> cells, the percentage was significantly lower in IL-15 KO mice (Fig. 7B).

**IL-15 is produced in a sustained manner in KCs from mice immunized and challenged with Pb γ-spz**

Because liver CD8 T cells are a prominent cellular component in Pb γ-spz–induced protection, a question arose of whether the liver could be a source of IL-15 for the maintenance of memory CD8 T cells. IL-15 is produced by hepatocytes (58, 59) as well as by other cells in the liver, including KCs (60). The precise involvement of KCs in response to pre-erythrocytic stage Ags remains somewhat controversial (61, 62), and the role of KCs in the maintenance of liver memory CD8 T cells has not been investigated. We hypothesized that if immunization with Pb γ-spz induces IL-15 in KCs, these abundant liver macrophages could be involved in the maintenance of protected protection. We determined the levels of IL-15 transcript in KCs isolated at different time points during the immunization and challenge regimen with
P. berghei sporozoites (Fig. 8). Relative to KCs from naive mice, IL-15 transcripts increased in KCs during immunization with Pb γ-spz. IL-15 transcripts increased in KCs 24 h after challenge of both naive and Pb γ-spz–immunized mice. However, although the IL-15 transcripts decreased to baseline in KCs from naive-infected mice, they remained elevated in KCs for 288 h (12 d) after challenge of Pb γ-spz–immunized mice (Fig. 8). We have observed similar results at the IL-15 protein levels in liver CD11c+ dendritic cells (DCs) (S. Dalai, manuscript in preparation). Although these results are not conclusive with regard to the role of KCs in maintaining memory liver CD8 T cells, nor do they suggest the only source of IL-15 for memory CD8 T cells, they strongly suggest that apart from liver CD11c+ DCs, KCs provide IL-15 needed for protracted protection induced by Pb γ-spz.

Discussion
In this study we demonstrate that the presence of liver memory CD8 T cells correlates with maintaining protracted protective immunity induced by immunization with Pb γ-spz. Because CD8 TCM cells depend on IL-15 for proliferation (11, 27), effector function (42), and survival (28, 63), we tested the contribution of CD8 TCM cells to protection in IL-15 KO mice. Despite reduced numbers of CD8 T cells, IL-15 KO mice respond to a large spectrum of Ags and infectious agents (13, 14, 16, 17, 64). In this study we expanded this list by adding responses to Pb pre-erythrocytic or LS Ag. The activation of Ag-specific effector CD8 T cells was further confirmed by sterile protection of IL-15 KO mice at 1° challenge. Of note, and as shown previously in wt mice (3, 45), protection in IL-15 KO mice was mainly CD8 T cell–dependent; neither NK nor...
NK1.1+ T cells affected the outcome of protection at 1˚ challenge. Liver CD4 T cells appeared not to play a role in the IL-15 impact on duration of protection. The key observation that emerged from this study, however, was that unlike wt mice, most IL-15 KO mice succumbed to blood-stage parasitemia at 2˚ challenge because they were unable to develop and/or maintain secondary memory CD8 T cell responses. The ability of IL-15 KO mice to manifest only short-term protection highlights the differences in the requirements for the induction and maintenance of sterile immunity specific for LS Ags in the Pb γ-spz model; maintenance, but not induction, of protection was IL-15-dependent.

**Memory CD8 T cells play a crucial role in lasting protection**

The failure to maintain sterile protection in IL-15 KO mice against 2˚ sporozoite challenge was clearly linked to the dependence of memory CD8 T cells on IL-15, and immunologic deficits caused by the lack of IL-15 became evident during 2˚ challenge of previously protected mice. In comparison with wt mice, IL-15 KO mice had lower numbers of IFN-γ-producing CD8 T cells, reduced basal proliferation, as well as fewer cells transiting from a central/long-term memory phenotype to an effector phenotype. The essentiality of IL-15, however, was apparent mainly in the inability of CD8 T CM cells to survive in IL-15 KO mice. Survival is critical for the maintenance of adequate CD8 T cell numbers to combat parasites at rechallenge. In malaria, these numbers have been shown to be exceedingly higher than in other infections (65). Consequently, long-term protection was not sustainable in the absence of IL-15.

We propose that CD8 T CM cells represent a key cellular element required for long-term protection against the pre-erythrocytic stage of infection and perhaps malaria infection in general. The dependence on CD8 T CM cells rather than CD8 T E cells or CD8 T EM cells for lasting protection has been demonstrated in other systems, including acute viral and bacterial infections (40, 41), as well as another protozoan parasite, Trypanosoma cruzi (66). IL-15 is also critical for sustaining protective responses to vaccinia (67) and Toxoplasma gondii (16), as memory CD8 T cells are impaired and they gradually decline in numbers owing to a decrease in replication in the absence of IL-15. Although IL-15 has been shown to support early control and resolution of blood-stage parasites (68), to our knowledge our study is the first demonstration of an important role of IL-15 in maintaining CD8 T CM cells required for protracted protection in the Plasmodium γ-spz model, which is considered to be the gold standard of antimalaria vaccines.

**Lasting protection requires a reservoir of CD8 T CM cells ready to combat the parasite**

We previously hypothesized (18) that in the Pb γ-spz model of protection, CD8 T CM cells form a memory reservoir, which assures a quick differentiation of memory into effector cells upon reinfections. The memory reservoir is likely composed of CD8 T cells that avoid becoming effectors upon activation with LS Ags during repeated immunizations with Pb γ-spz and CD8 T E cells that survive the contraction phase after sporozoite challenge and then transition to CD8 T CM cell pool, where they are capable of replicating. CD8 T CM cells did indeed increase in both groups, but the increase in IL-15 KO mice was to a lesser extent during immunizations with Pb γ-spz. After the primary challenge, liver CD8 T cells declined in both wt and IL-15 KO mice, but again the loss was particularly evident among CD8 T CM cells in IL-15 KO mice. Similar to results from other systems (57), ~7% of CD8 T CM cells survived in wt mice, whereas only ~1% of CD8 T CM cells were found in the memory compartment in IL-15 KO mice prior to 2˚ challenge. This suggests that a near absence of the surviving CD8 T cells limited the number of cells entering into the CD8 T CM reservoir. Because IL-15 enhances survival of CD8 T cells during the contraction phase (9, 29), thus assuring the formation of a durable CD8 T CM compartment, our results suggest that the unavailability of IL-15 (in IL-15 KO mice) during the contraction phase resulted in a severe loss of cells otherwise destined to form the memory reservoir.

**IL-15 is needed for the conversion of memory to effector function during secondary challenge**

The significantly reduced memory pool explains in part the inadequate differentiation of memory CD8 T cells into IFN-γ-producing CD8 T E cells at rechallenge. Although soon (6 h) after rechallenge, IFN-γ-producing cells increased in wt and IL-15 KO mice, the responses remained at a plateau in IL-15 KO mice during subsequent time points, whereas in wt mice the responses continued to climb. These observations are in agreement with the findings from separate experiments showing that the transition of CD127 hi CD8 T CM cells to CD127 lo CD8 T E cells diminished at 2˚ challenge in IL-15 KO mice relative to wt mice. Taken together, these results support the involvement of IL-15 in conditioning differentiation of CD8 T cells into an effector population, and they are consistent with the observation that IL-15 promotes the conversion of CD127 hi to CD127 lo populations (20) and affect the composition and function of CD8 T cells at the 2˚ memory stage (13).

Despite a two-fold higher percentage of BrdU+ CD8 T CM in IL-15 KO mice compared to wt at 2˚ challenge, rather low cell numbers remained. Although this difference could be indicative of a difference in the rate of CD8 T cell division between these two groups of mice, it most likely resulted from increased apoptosis of CD8 T cells in IL-15 KO mice. CD8 T CM cells displayed lower levels of Bcl-2 and increased caspase-3 and annexin V at 2˚ challenge of IL-15 KO mice relative to wt mice. The preferential survival-enhancing effects of IL-15 on CD8 T CM cells were con-

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**FIGURE 8.** KCs are a source of IL-15 in the liver. IHMCs were isolated from livers of naive, Pb γ-spz–immunized, and immunized/challenge mice at the indicated time points. Mac3+ KCs were isolated as described in Materials and Methods and were lysed in TRIzol for RNA isolation and subsequently reverse transcribed for RT-PCR assay. Assays were done on a 96-well plate format and the detection of the PCR products was performed as described in the Materials and Methods. Quantitative IL-15 gene expression is shown as a ratio of IL-15 to GAPDH for each time point, and results are expressed as the mean ± SD of triplicate wells. Similar results were observed in experiments performed three times. *p < 0.05, **p < 0.01.
firmed by results from in vitro studies. Thus, despite the apparently proportional cellular turnover in wt and IL-15 KO mice, many more dividing cells underwent apoptosis in the latter group. Although other cytokines, for example, IL-7, have been shown to exert a dominant effect over IL-15 in enhancing memory cell formation among recently activated CD8 T cells (57), it appears that memory CD8 TCM cells that arise to P. berghei LS Ags are extremely IL-15–sensitive particularly during the formation and/or maintenance of 2’ memory cells. Our observation is in contrast to reports from other systems where 2’ memory is less dependent on IL-15 than are 1’ memory CD8 T cells (56); however, our observations are in agreement that IL-15 still affected the composition of the memory CD8 T cell pool (13). Neither of the γ-chain cytokines that are presumed to have been active in IL-15 KO mice was sufficient to compensate for IL-15 prior to 2’ challenge.

IL-15 is indispensable for the induction of protection but not for its maintenance

In contrast to the immunologic impairment caused by the absence of IL-15 at 2’ challenge, IL-15 seemed rather dispensable during the initial induction, differentiation, and expansion of CD8 T cells in response to immunization with Pb γ-spz, including the 1’ challenge. These observations are similar to those made in other systems where the absence of γ-chain cytokines does not affect the processes associated with the initial CD8 T cell expansion and differentiation (42). Instead, the accumulation of CD8 T E cells in lymphoid organs proceeds quite normally, as did the accumulation of CD8 T E/EM cells in the livers of 15 KO mice immunized with Pb γ-spz. It is entirely possible, therefore, that in the absence of IL-15, other cytokines with functional activities similar to those of IL-15 substituted for the effectiveness of IL-15 and promoted both cellular proliferation and effector function during primary challenge.

The two cytokines that likely supported activation of CD8 T cells during the priming and boosting immunizations with Pb γ-spz were IL-7, known to act on naïve CD8 T cells, (10) and IL-21, produced by CD4 T cells (69) that help CD8 T cells. The supporting action of these cytokines in IL-15 KO mice may be considered as evident by the increase of CD8 T E/EM cells. It is clear that the Pb LS Ag–induced initial expansion and acquisition of an effector function was independent of the signals provided by IL-15. We presume that the swiftness of the response observed at 6 h after the challenge combined with the overall magnitude of the response must have been sufficient to eliminate the pre-erythrocytic parasites and to prevent blood-stage parasitism.

Although the absence of IL-15 did not interfere with or prevent the induction of protective immunity delivered by CD8 T cells at 1’ challenge in Pb γ-spz, the absence of other γ-chain cytokines does show an impairment of CD8 T cell survival early after infection (10). The differences in the need for a particular γ-chain cytokine over another, (e.g., IL-7 versus IL-15) may stem from the differences associated with the type of infectious agents, mode of delivery of infection, as well as homing or localization of the infectious agent to a particular organ or tissue, where each γ-chain cytokine may have a unique pattern or even a level of expression.

Liver APCs support the induction and maintenance of CD8 T cells

According to recently published studies, nonlymphoid organs contain resident memory CD8 T cells that confer local lasting protection of infected extralymphoidal organs (70). It remains unknown whether memory CD8 T cells specific for P. berghei LS Ags were induced in liver or whether early during the effector phase CD8 T cells migrated from lymphoid organs, as has been shown for P. yoelii CSP-specific CD8 T cells (43), and lodged in the liver where they formed a memory CD8 T cell pool, which is partly supported by the local availability of IL-15. Recently, it was also demonstrated that CD8 T cell survival during influenza infection is promoted in the lung by transpresentation of IL-15 by pulmonary DCxs (71). On the basis of our published results, liver, but not splenic, CD11c+CD8α+ DCs from Pb γ-spz–immunized/challenged mice activate CD8 T cells to express CD44+ in a MHC class I–dependent manner; CD11c+ DCs also upregulate IL-15 mRNA (72) and expressed detectable IL-15 protein (S. Dalai, unpublished observations). KCs, the major liver macrophages, also upregulate MHC class I molecules, secrete proinflammatory cytokines (26), and, as shown in this study, maintained upregulated IL-15 mRNA transcripts, but only after Pb γ-spz immunization and challenge. The issue of LS Ag processing and presentation as well as the various signals needed for the survival of memory CD8 T cells remain to be sorted out in future studies. According to our collective results, however, we suggest that in the Pb γ-spz model of protective immunity, liver KCs and DCs function as APCs of LS Ags and as IL-15 producers that target only liver CD8 T CM cells because CD8 T E/EM cells were not responsive to IL-15. Similar observations were made in in vitro studies where only CD8 T CM cells require transpresentation of IL-15 in the context of a concurrent signaling via TCR for optimal recall response, as responses by CD8 T E cells are not augmented by IL-15 (55). It could be envisaged, therefore, that the TCR signal is delivered by MHC/peptide complex presented by KCs or liver DCs that hold the potential repository of LS Ag depot, which is also needed for the maintenance of lasting protection in the Pb γ-spz model (3, 39).

In summary, the general cellular disregulations caused by the absence of IL-15 contributed to the severely reduced CD8 T CM cell reservoir, which is critical for the maintenance of protective immunity induced with Pb γ-spz against homologous reinfections. IL-15 was crucial for the maintenance, but not for the induction, of protection, as IL-15 KO mice showed sterile protection at 1’ challenge. The need for IL-15 in the maintenance of lasting protection to protozoan infection has been controversial; however, most studies clearly demonstrate that memory CD8 T cells are exclusively IL-15–dependent (11, 13, 14, 27, 42, 56, 73). IL-15 extends the lifespan of CD8 T CM cells and, hence, protective immunity. Our observations are in agreement with those that support models of IL-15 as the critical signal for the expansion, survival, and thus long-term maintenance of the CD8 T CM cell pool.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Material

Supplemental Figure 1. Intrahepatic CD8 T cells from Pbγ-spz immunized mice segregate into Te/Em and Tcm subsets. (A) Gating strategy for liver CD8 T cells based on the expression of CD44 and CD62L (B) In a separate experiment, total CD44hi gated cells were divided on the basis of CD62L expression and were analyzed for expression of (C) CD122 (IL-15Rβ/IL-2Rβ) and (D) IFN-γ by the cytokine secretion assay as described in Materials and Methods.

Supplemental Figure 2. Immunization with Pbγ-spz favors induction of Te/Em subset in IL-15KO mice. IHMC were isolated 2 months following the last boost immunization with Pbγ-spz from either wt or IL-15KO mice and analyzed for expression of CD3, CD8, CD44 and CD45RB.

Supplemental Figure 3. Liver NK and NKT cells as well as CD4 T cells do not play a role in protective immunity against Pb sporozoite challenge of IL-15KO mice. Wt (n=3) and IL-15KO (n=4) mice were immunized with Pbγ-spz and then challenged as described in the legend for Fig. 3. (A) IHMC were isolated one week following immunization and stained for CD3 and NK1.1. (B) IHMC were isolated 2 months post challenge and stained for CD3 and CD4. Total numbers of each population were calculated for individual mice and are represented as mean ± SD for each group, ** p<0.01.

Supplemental Figure 4. Proliferation of IHMC CD8 T cells is induced by Pbγ-spz. Proliferation of intrahepatic CD8 Tnaive cells (CD44lo), CD8 Tcm, or CD8 Te/Em after i.v. immunization with sham dissected salivary gland material or 75K Pbγ-spz. Mice were fed BrdU in drinking water for 7 days following immunization and IHMC were isolated and stained for
surface expression of CD3, CD8, CD44 and CD45RB followed by intranuclear staining of BrdU as described in the legend for Fig. 5.