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Protracted Protection to Plasmodium berghei Malaria Is Linked to Functionally and Phenotypically Heterogeneous Liver Memory CD8+ T Cells

Dmitri Berenzon,* Robert J. Schwenk,* Lisa Letellier,* Mimi Guebre-Xabier,2* Jackie Williams,† and Urszula Krzych3*

We previously demonstrated that protection induced by radiation-attenuated (γ) Plasmodium berghei sporozoites is linked to MHC class I-restricted CD8+ T cells specific for exoerythrocytic-stage Ags, and that activated intrahepatic memory CD8+ T cells are associated with protracted protection. In this study, we further investigated intrahepatic memory CD8+ T cells to elucidate mechanisms required for their maintenance. Using phenotypic markers indicative of activation (CD44, CD45RB), migration (CD62L), and IFN-γ production, we identified two subsets of intrahepatic memory CD8+ T cells: the CD44highCD45RBlowCD62LlowCD122low phenotype, representing the dominant effector memory set, and the CD44highCD45RBhighCD62Llow/highCD122high phenotype, representing the central memory set. Only the effector memory CD8+ T cells responded swiftly to sporozoite challenge by producing sustained IFN-γ; the central memory T cells responded with delay, and the IFN-γ reactivity was short-lived. In addition, the subsets of liver memory CD8+ T cells segregated according to the expression of CD122 (IL-15R) in that only the central memory CD8+ T cells were CD122high, whereas the effector memory CD8+ T cells declined as protection waned in mice treated with primaquine, a drug that interferes with the formation of liver-stage Ags. We propose that protracted protection induced by P. berghei radiation-attenuated sporozoites depends in part on a network of interactive liver memory CD8+ T cell subsets, each representing a different phase of activation or differentiation, and the balance of which is profoundly affected by the repository of liver-stage Ag and IL-15. The Journal of Immunology, 2003, 171: 2024–2034.

The formation of optimally effective memory T cells is one of the cardinal features of Ag-specific immune responses elicited by infections or vaccinations, and it is inextricably linked to long-lasting protective immunity. Memory T cells are thought to arise either directly from naive populations or from Ag-expanded effector cells that are spared apoptosis. However, the mechanisms involved in the long-term maintenance of memory T cells are less well defined. Recent evidence suggests that maintenance of memory CD8+ T cells in peripheral immune organs relies on mechanisms that are unique for each of the two T cell sets. For example, although maintenance of memory CD8+ T cells is independent of antigenic stimulation (1), the need for signaling through the TCR on memory CD4+ T cells is less clear (2). The requirement for cytokines also differs between CD4+ and CD8+ T cells; only memory CD8+ T cells require IL-15 (3), and the presence of IL-4 during T cell activation has also been shown to favor long-term survival of memory CD8+ T cells (4).

Apart from memory T cells that reside in the spleen or lymph nodes, we and others have shown that memory CD4+ and CD8+ T cells persist in nonlymphoid organs such as the liver (5), lungs (6), and kidneys (7) following protozoan or viral infections. Unlike memory T cells in peripheral lymphoid organs, the memory T cells found in the nonlymphoid organs appear to express cell surface markers characteristic of activated T cells (8). We have shown (9) that radiation-attenuated Plasmodium berghei sporozoite (γ-spz)-immune intrahepatic mononuclear cells (IHMC), which adoptively transfer lasting protective immunity to naive recipients, contain CD4+ and CD8+ T cells that express a CD44high and CD45RBlow phenotype. The kinetics of the expression of these surface markers differs significantly between the two T cell subsets. In contrast to CD44highCD45RBlow T cells that appear transiently following each immunization with γ-spz, the emergence of CD8+CD45RBlow coincides with the induction of protection, which in C57BL/6 mice requires three weekly immunizations with γ-spz. Significantly, the CD8+CD44highCD45RBlow T cell phenotype is still found in livers, but not spleens, of γ-spz-immune mice 4 mo after challenge with infectious spz. Therefore, it appears that the long-term protection induced by P. berghei γ-spz is linked, at least in part, to the persistence of activated memory CD8+ T cells in the liver (9). The activated status of the memory cells that localize to the liver most likely ensures a rapid reactivity to Plasmodium infection, hence a very efficient effector function against the malaria parasite.

The liver is the key organ in malaria infection. It is the first organ targeted by plasmodia sporozoites (spz), because hepatocytes are an obligatory venue for schizogony, a process of plasmodia differentiation associated with amplification and molecular...
changes that result in an acquisition of novel Ags and increased Ag load. Intrahepatic lymphocytes and Kupffer cells (10–12) also render the liver a critical organ for the host’s defense. Protection in naturally exposed persons has been directly linked with liver-stage Ag (LSA)-specific immune responses (13). For example, LSA-specific T cells have been shown to be associated with both reduced incidence of severe malaria (14) as well as resistance to Plasmodium falciparum (15) in naturally exposed persons. Hence, the liver is considered not only pivotal for the survival of the parasite but also as a crucial organ for the induction of anti-Plasmodium protective immune responses.

In contrast to natural infection characterized by a slow acquisition of nonsterile protective immunity that wanes when exposure to the parasite ceases, immunization of humans (16) and laboratory rodents (17) with multiple doses of γ-spz leads to a sterile and lasting protection. It is believed that radiation partially retards the development of the parasite (18, 19), so that the underdeveloped or blocked liver schizonts cannot infect RBCs remain in the liver, a step that is considered critical for the induction of a local Ag-specific protective immunity (20). Findings by Scheller and Azad (21) support the notion that, indeed, LSAs are required for prolonged protection. By extension, we propose that the persistence of memory T cells in the liver, which appears essential for the long-lived protection induced by γ-spz, requires the presence of LSA.

The location of memory T cell pools is particularly crucial for the prophylactic potential of memory T cell responses during re-encounter with a pathogen. Hence, the importance of organ- or tissue-specific memory T cells cannot be overstated, because it is one of the key elements for consideration in the planning and development of vaccines to prevent or abate infectious diseases.

In this study, we asked how P. berghei γ-spz-induced memory CD8 T cells are maintained in the liver. We approached our analyses by further characterization of liver memory T cells on the basis of their phenotype and functional activity. In this study, we show that the liver memory CD8 T cell pool consists of at least two subsets, each performing a unique function and displaying somewhat different maintenance requirements. On the basis of these observations, we propose that liver memory CD8 T cells form a network of interactive T cell sets, each representing a different phase of activation or differentiation, the balance of which is profoundly affected by the local availability of specific Ags as well as the cytokine milieu.

Materials and Methods

Animals

Female C57BL/6 (H-2b) mice, purchased from The Jackson Laboratory (Bar Harbor, ME), were used at 6–8 wk of age. IL-15 knockout (KO) female mice (C57BL/6 background), purchased from Taconic Farms (Germantown, NY), were used at 5–6 wk of age. Animals were housed and handled according to institutional guidelines. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council publication, 1996 edition). All procedures were reviewed and approved by the Institute’s Animal Care and Use Committee, and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Preparation of spz

P. berghei (ANKA strain) spz were maintained by cyclical transmission in mice and Anopheles stephensi mosquitoes. spz were dissected from the salivary glands of mosquitoes 21 days after an infective blood meal, as described previously (5). For immunization, spz were attenuated by exposure to 15,000 rad of radiation using a cesium-137 source (Mark I series; JL Shepherd & Associates, San Fernando, CA), counted, and adjusted to a given concentration in RPMI 1640 (Life Technologies, Grand Island, NY) with 10% normal mouse serum (NMS). For challenge, spz were used immediately after dissection to ensure maximal infectivity. Sham-dissected salivary gland preparations obtained from noninfected mosquitoes were attenuated and treated according to the same procedure as described for spz.

Immunization

Mice were primed i.v. with 75,000 γ-spz followed by two boost immunizations of 20,000 γ-spz given 1 wk apart. Control mice were given three i.v. injections 1 wk apart of medium alone. One week after the last immunization, mice were challenged i.v. with 10,000 infectious spz. When warranted, mice were rechallenged 6 or 10 mo after the first challenge. For controls, mice were immunized three times with γ-irradiated preparations of sham-dissected salivary gland debris from noninfected mosquitoes. The number of salivary glands for dissection was estimated on the basis of an average number of spz yields per 10 mosquitoes.

Determination of parasitemia

Thin blood smears were prepared from individual mice starting on days 2–4 postchallenge, and parasitemia was determined microscopically using Giemsa stain, as described previously (5). Up to 40 oil-immersion fields were screened for the presence of parasitized erythrocytes. Mice were considered protected if parasites were not detected by day 14 after challenge. Nontreated naïve mice that were challenged with P. berghei spz usually became parasitemic by day 7 and died by day 20 after challenge.

Primaquine treatment

Mice received 60 mg/kg body weight of primaquine phosphate (a gift from W. Ellis, Walter Reed Army Institute of Research) in double-distilled H2O. In the first two experiments, primaquine was administered s.c. at 5, 10, 20, and 23 h after each of the three immunizations with γ-spz. In the third experiment, two primaquine doses were administered 5 h apart after each γ-spz immunization. γ-spz-immune mice, γ-spz-immune and primaquine-treated mice, as well as naïve mice were challenged with 10,000 infectious spz 1 wk after the last boost immunization and were rechallenged 6 mo later.

HMMC

Mice were anesthetized, and the exposed livers were perfused via the hepatic portal vein with 10 ml of 0.1% solution of collagenase IV (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 as described previously (5). The livers were resected and incubated for 30 min at 37°C, after which the livers were gently pressed through a Falcon cell strainer (BD Labware, Franklin Lakes, NJ) and resuspended in HBSS (Life Technologies). The bulky debris was removed, and the cell suspension was centrifuged at 1200 rpm for 10 min. The pellet was suspended in 13% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden), and centrifuged at 2000 rpm for 20 min. Isolated liver mononuclear cells were washed twice in HBSS. Whereas the cell numbers from naïve mice yielded on average 2 × 106 lymphocytes/liver, the numbers of lymphocytes from γ-spz immune mice ranged between 3 and 5 × 106 cells/liver.

Cytofluorometry

Cells were washed twice and resuspended in 0.2% NMS PBS at 106 cells/100 μl. To block non specific binding of mAb, cells were preincubated with the Fc-block (anti-FcγRI/II) CD16/CD32 mAb; BD PharMingen, San Diego, CA) at 1 μg/106 cells at 4°C for 30 min. After one wash, cells were incubated with 0.5 μg of the relevant mAb per 106 cells at 4°C for 30 min, followed by two washes with cold PBS. Four-color flow-cytometric analysis was performed on a FACSCalibur (BD Biosciences, Mountain View, CA). The following mAb were used: FITC-anti-CD45RB (553099), FITC-anti-CD122 (553361), PE-anti-CD44 (553134), PerCP-anti-CD4 (553052), PerCP-anti-CD8a (553036), and allophycocyanin-anti-CD62L (553152) (BD Pharmingen).

Positive selection of CD4+ and CD8+ T cells

The procedure for positive selection was done according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). Briefly, isolated HMMC were washed twice with PBS-0.5% BSA buffer, and the cell pellet (107 cells) was resuspended in 90 μl of buffer to which 10 μl of MACS CD8a or CD4 MicroBeads 130-049-401 and 130-049-201, respectively, was added, and the reaction mixture was incubated for 15 min at 6°C. Cells were washed by adding 20× labeling volume of PBS-0.5% BSA and 2 mM EDTA, centrifuged at 300 × g for 10 min. The cell pellet was resuspended in 500 μl of buffer, and the cell suspension was applied to a positive-selection MS column. After the negative cells passed through, the column
was rinsed three times with 500 µl of buffer. The column was removed from the separator, and 1 ml of buffer was added onto the column to flush out the positive fraction.

**IFN-γ secretion assay**

The IFN-γ determinations were performed using the secretion assay detection kit according to the manufacturer’s instructions (Miltenyi Biotec). Briefly, IHMC were resuspended at 10⁶ cells/tube and washed twice with cold PBS-0.5% BSA and 2 mM EDTA. Cells in 90 µl of cold RPMI 1640 with 5% NMS plus 10 µl of IFN-γ capture reagent were incubated on ice for 5 min and then diluted up to 10⁴/ml with warm RPMI 1640 with 5% NMS, transferred into flat-bottom 12-well plate, and incubated for 45 min at 37°C. After two washes, cells were resuspended in 90 µl of cold PBS and incubated with 10 µl of IFN-γ detection reagent for 10 min at 4°C. After the final two washes in PBS, cells were resuspended in 100 µl of freshly prepared 50 µg/ml propidium iodide and analyzed by flow cytometry. Data was analyzed by CellQuest software (BD Biosciences).

**Statistical analysis**

Data are shown as the mean ± SD of cell numbers from at least three separate experiments. Significance between data points was compared by ANOVA and Tukey’s postest. Values of p < 0.01 were considered significant.

**Results**

**Immunization with γ-spz induces phenotypically distinct subpopulations of liver CD8⁺ T cells**

We have shown that repeated exposure to *P. berghei* γ-spz induces lasting protective immunity that is associated with activated intrahepatic memory CD8⁺ CD44brightCD45RBlow T cells (9). Other studies have shown that distinct subsets of memory CD8⁺ T cells could be revealed by the expression of migration ligands as well as by functional activity. For example, effector memory T cells express CD62Llow and CCR5 and migrate to nonlymphoid tissue where they engage in an effector function without additional activation; central memory T cells express CD62Lhigh and CCR7 and home to T cell areas of secondary lymphoid organs, possibly to be reactivated by future encounter with the Ag (22, 23). In the present study, we extended the characterization of the intrahepatic memory CD8⁺ T cells to include both CD62L expression and IFN-γ production.

In the first set of experiments, we analyzed intrahepatic CD8⁺ T cells to identify the subsets on the basis of their phenotypic markers following immunization with γ-spz. Approximately 53% of liver CD8⁺ T cells expressed CD44brightCD45RBlow (Fig. 1B) and nearly the entire (96%) subset was CD62Llow (Fig. 1D). In naive mice, the CD44brightCD45RBlow phenotype ranged between 10 and 27% of CD8⁺ T cells (data from a representative experiment is shown in Fig. 1A). Among this subset, ~80% were CD62Llow (Fig. 1C). Following tertiary immunization with γ-spz, the CD8⁺ CD44brightCD45RBlow T cells declined 2-fold (compare Fig. 1, A and B), but a new fraction expressing CD62Llow emerged, and then the CD62Llow/bright phenotypes were represented in nearly equal proportions (Fig. 1, E and F).

Thus, compartmentalization of intrahepatic CD8⁺ CD44bright T cells into phenotypically distinct subsets was already evident in naive mice. Following Immunization with γ-spz, the CD44bright CD45RBlow/CD62Llow T cells became the dominant subset, and a new, intermediate subset emerged that expressed a mixture of CD62Lhigh/bright. Presently, we do not know whether these subsets represent stages of CD8⁺ T cell differentiation, expansion, or immigration from the peripheral immune organs to the liver. However, these observations imply that protective immunity induced with γ-spz depends on a network of T cells with distinct migratory pattern, maturational diversity, and possibly distinct functions. For example, the major CD8⁺ CD44bright/CD45RBlow/CD62Llow T cell subset might represent the effector memory pool, and the CD8⁺ CD44bright/CD45RBlow/CD62Llow/bright phenotype might represent a central memory population, whereas the CD8⁺ CD44bright/CD45RBlow/CD62Llow/bright might indeed belong to an intermediate subset ready to be conscripted into the effector population during reinfection.

Regarding liver CD4⁺ T cells, we confirmed our previous observations that a large percentage, ranging from 28 to 55% of naive CD4⁺ T cells, already expressed the memory phenotype (5). Following the third immunization with γ-spz, the memory subset

![FIGURE 1](http://www.jimmunol.org/Downloadedfrom/http://www.jimmunol.org)
transiently increased ∼1.5-fold with nearly equally distributed CD62L\textsubscript{low} and CD62L\textsubscript{high} phenotype (data not shown).

It has been demonstrated that immunization with LPS influences the expression of phenotype and expansion of memory CD8\textsuperscript{+} T cells (24). Therefore, as a control for nonspecific expression of memory phenotype CD8\textsuperscript{+} T cells, we examined memory markers among the intrahepatic CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells isolated 7 days after tertiary immunization with γ-irradiated salivary gland debris dissected from noninfected mosquitoes. In comparison with intrahepatic CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells from naive mice, LPS induced only negligible shifts in the expression of the memory markers; hence no changes were induced in the phenotypically distinct liver T cell subsets (data not shown).

**Effect memory CD8\textsuperscript{+} CD44\textsuperscript{high}CD45RB\textsuperscript{low}CD62L\textsuperscript{low} T cells are maintained in the liver following challenge with infectious spz**

In the next set of experiments, we asked whether the arrangement of activated memory CD8\textsuperscript{+} T cell subsets persisted in livers of γ-spz-immune mice following spz challenge. For comparison, we analyzed liver CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells from naive, γ-spz-immune (after tertiary immunization), and γ-spz-immune/challenged mice. At preimmunity (naive), the CD8\textsuperscript{+} CD44\textsuperscript{high}CD45RB\textsuperscript{low}CD62L\textsuperscript{low} T cells ranged between 50 and 100 \times 10\textsuperscript{3} cells/liver; however, this subset rose to nearly 500 \times 10\textsuperscript{3} T cells following tertiary immunization with γ-spz (Fig. 2A). Following spz challenge, the subset increased 4-fold, reaching 2000 \times 10\textsuperscript{3} cells/liver, thus representing between 45 and 75\% of total liver CD8\textsuperscript{+} T cells (Fig. 2, A and E); most importantly, however, effector memory T cells persisted for up to 28 days, and the level was ∼10-fold higher that at preimmunity. By contrast, the most pronounced increase (2-fold) of the CD4\textsuperscript{+} CD44\textsuperscript{high}CD45RB\textsuperscript{low}CD62L\textsuperscript{low} T cells occurred on day 14 after the challenge (750 \times 10\textsuperscript{3} cells/liver), but the subset declined to the preimmune level at day 28 (300 \times 10\textsuperscript{3} cells/liver) (Fig. 2A).

Upon closer examination of the distribution of CD8\textsuperscript{+} T cells into CD44\textsuperscript{high}CD45RB\textsuperscript{low} (effector memory) and CD44\textsuperscript{high}CD45RB\textsuperscript{high} (central memory) T cell subsets under conditions of immunization and challenge (Fig. 2, B–F), it became clear that the central memory (CD44\textsuperscript{high}CD45RB\textsuperscript{high}) CD8\textsuperscript{+} T cells expanded following priming with γ-spz (from 39 to 57\%), whereas the effector T cells required three doses of γ-spz for the expansion to occur (from 27 to 50\%); and they continued to expand (up to 75\%) following the challenge. Moreover, the effector memory CD8\textsuperscript{+} T cells remained elevated (67\%) even 30 days after the challenge, whereas the central memory T cells decreased 2-fold in comparison with preimmunity. The corresponding CD4\textsuperscript{+} T cell phenotype remained unchanged (data not shown). Although these observations do not provide direct evidence that Ag exposure induces central memory T cells to differentiate into effector memory T cells, they nonetheless suggest the possibility of a propinquous relationship between these two memory CD8\textsuperscript{+} T cells subsets, which we are currently investigating.

**Only effector memory CD8\textsuperscript{+} (CD44\textsuperscript{high}CD45RB\textsuperscript{low}) T cells responded swiftly by producing IFN-γ**

It has been demonstrated previously that functional differences can be discerned among separate subsets of memory CD8\textsuperscript{+} T cells (23, 25, 26). Accordingly, we hypothesized that only the effector memory T cells would produce mediators, such as IFN-γ, that have been associated with protection against malaria (27, 28). To avoid detection of IFN-γ responses from cells that might have been induced nonspecifically during immunization with γ-spz (24), we examined both the CD44\textsuperscript{high}CD45RB\textsuperscript{low} and CD44\textsuperscript{high}CD45RB\textsuperscript{high}CD8\textsuperscript{+} T cells for IFN-γ production 1 mo after the last boost immunization.
with γ-spz (0 h) and at the indicated time points (hours and days) after the challenge. We performed an ex vivo cytokine capture assay using enriched (>95%) liver CD8+ T cells and CD4+ T cells, and the responses are expressed as the number of IFN-γ-producing cells/10^6 IHMC. For CD8+CD44highCD45RBlow T cells, the response rose from 2000 1 mo after the last immunization with γ-spz to 8000 at 1 h following spz challenge, and the elevated level of reactivity persisted for 12 h after the challenge (Fig. 3A). Subsequent analyses revealed a peak reactivity (30,000) 7 days after the challenge, and a decline to ~15,000 on days 14 and 28 (Fig. 3B).

The reactivity of the central memory (CD44highCD45RBhigh) T cells differed sharply from that of the effector memory CD8+ T cells. Before challenge (0 h), we measured only 500 IFN-γ-producing cells. The response increased slightly following spz challenge with a peak reactivity of ~5000 at 6 h after the challenge. However, at 12 h, the response declined to preimmune level, at which it remained for the duration of the analyses (28 days). Because the distinction in the functional activity between the two subsets of CD8+CD44high T cells was marked not only by their celerity but also by the magnitude as well as the duration of the response, these observations provide evidence that only the effector memory CD8+CD44highCD45RBlow T cells persisted to be rapidly recalled into an effector mode even 1 mo after the last immunization with γ-spz.

CD8+CD44highCD45RBlow T cells isolated from livers of naive mice showed negligible responses that ranged between 100 and 200 IFN-γ-producing cells. Immediately following the infection, the CD8+CD44highCD45RBlow T cells remained indolent IFN-γ producers, but at 12 h, the response rose to ~6000 IFN-γ-producing cells. In contrast to the declining responses observed among γ-spz-immune and -challenged mice, the IFN-γ responses of the naive mice declined very little and remained at a level of 150 IFN-γ-producing cells.

**FIGURE 3.** Secretion of IFN-γ by liver memory CD8+ and CD4+ T cells. IHMC were isolated at the indicated times from the following groups of mice (three mice per group): naive, γ-spz immune, and γ-spz immune and challenged. CD8+ and CD4+ T cells were enriched by magnetic separation, and CD45RB and CD44 T cells subsets were identified, as described in Fig. 1, and analyzed for the production of IFN-γ by cytokine secretion assay (see Materials and Methods). A and B, Results from a representative of two experiments are presented as the number of IFN-γ-secreting cells/10^6 liver IHMC at the indicated time points in hours (A) and in days (B) following challenge by effector memory (CD44highCD45RBhigh) CD8+ T cells from γ-spz-immune mice (solid line, □), central memory (CD44highCD45RBlow) T cells from γ-spz-immune mice (solid line, ▪), and effector memory CD8+ T cells from naive mice (dotted line, ○). C and D, IFN-γ secretion by CD4+ T cells at the indicated time points following challenge: in hours (C) and in days (D) by effector memory T cells from γ-spz-immune mice (solid line, ○) and effector memory T cells from naive mice (dotted line, ○).
infected control mice rose to 10,000 IFN-γ-producing cells during the following 2 wk. This uncontrolled inflammatory response is presumed to have been associated with parasitemia and immunopathology of malaria infection.

In contrast to CD8⁺ T cells, both naive and γ-spz-immune CD4⁺CD44highCD45RBlow cells secreted IFN-γ at nearly comparable levels during the early time points (hours) after infection. However, although the responses abated over time in protected mice, they rose in infected control mice to a remarkable level (40,000 IFN-γ-producing cells/10⁶IHMC). Hence, it appears that, although the protective component of the IFN-γ response stems from CD8⁺ T cells, IFN-γ-producing CD4⁺ T cells are associated with the pathology caused by parasitemia.

**Effector memory T cells producing IFN-γ persisted in the liver for ~1 year in γ-spz-immune mice**

In the next experiment, we asked whether the effector memory T cells producing IFN-γ persisted in the livers beyond 1 mo after the challenge. A group of mice immunized three times with γ-spz was challenged and then rested for ~10 mo. Upon rechallenge, we observed 100% protection (data not shown). Moreover, examination of the liver CD8⁺ T cells revealed persistence of the effector memory phenotype (54%) (Fig. 4B). Analyses of liver lymphocytes from age-matched naive control mice showed that effector memory phenotype CD8⁺CD44highCD45RBlow T cells accumulated in the livers of older mice, and their numbers appeared indistinguishable (40%) from the effector memory phenotype CD8⁺ T cells found in γ-spz-immune/challenged mice (Fig. 4A). However, IFN-γ responses differed sharply between the effector memory CD8⁺ T cells from naive and γ-spz-protected mice. In naive (Fig. 4C) mice, only 7% of memory phenotype CD8⁺ T cells produced IFN-γ, whereas in protected mice, ~20% of CD8⁺CD44highCD45RBlow T cells were IFN-γ producers (Fig. 4D) at 10 mo after the challenge.

Analyses of effector and central memory CD8⁺ T cell subsets for the production of IFN-γ revealed similar results to those observed earlier, after the challenge: only the effector memory CD8⁺ T cells responded rapidly, and within 6 h after rechallenge, the number of IFN-γ-producing cells rose from 2500 to 8000/10⁶IHMC. The central memory CD8⁺ T cells responded with a delay, and the magnitude of response was considerably lower (Fig. 4E). Age-matched infectivity control mice showed a hugely delayed reactivity, which at the initial time points was very low. Interestingly, however, we observed that, during a challenge 10 mo earlier, the effector memory T cells had a swifter IFN-γ reactivity, which occurred 1 h after the challenge.

Ag depot in the liver is necessary for maintenance of CD8⁺CD44highCD45RBlowCD62Llow T cells

According to one of two rival hypotheses, periodic exposure to Ag is necessary for the maintenance of long-term memory T cells (29, 30). An alternative view proposes that the memory pool consists of long-lived T cells capable of surviving without Ag stimulation (1, 31). In a study by Scheller and Azad (21), long-term protection against *P. berghei* malaria induced by immunization with γ-spz could be abrogated by primaquine, if the drug is administered during priming and boosting with γ-spz. Primaquine disrupts the developing liver schizonts and thus prevents the accumulation of plasmodia-derived Ags in the liver. Therefore, we asked whether, coincidental with primaquine-mediated loss of protection, we could detect a reduction of either the central or effector memory liver CD8⁺ T cells. Importantly, selective Ag sensitivity of one of the two subsets of liver memory CD8⁺ T cells would support our view that each set requires a distinct mechanism for its persistence.

We performed three separate experiments with two groups of 5–10 mice/group. Each group was immunized with three weekly doses of γ-spz, and one of the two groups was treated with primaquine during immunization. Upon spz challenge, both groups of mice remained fully protected. As expected, naive control mice became parasitemic (data not shown). Upon a rechallenge 5 mo
later, the γ-spz-immune mice remained fully protected; however, 40–60% of the primaquine-treated mice became parasitemic, as did 100% of the control mice (data not shown).

In the first two experiments, we analyzed only the persistence of liver CD8⁺CD45RBlow T cells. In comparison to γ-spz-immune mice that preserved the effector memory phenotype of CD8⁺ T cells, primaquine-treated mice that became parasitemic clearly had a decreased level of the analogous memory T cells as indicated by the shift in the expression of CD45RB (Fig. 5A). Mice that maintained protective immunity despite primaquine treatment behaved like the untreated, γ-spz-immune controls and showed no changes in the memory CD8⁺ T cells (Fig. 5B).

In the third experiment, we extended the analyses of the CD8⁺ T cells to include CD44highCD45RBlow and CD44highCD45RBhigh phenotypes to determine whether the shift in the memory subsets of CD8⁺ T cells was random or whether it selectively affected a particular subset of the memory population. In comparison to effector memory CD8⁺ T cells found in nontreated mice at rechallenge, a ~5-fold reduction was observed among the liver effector memory CD8⁺ CD44highCD45RBlow T cells in primaquine-treated mice that became parasitemic upon rechallenge (compare 120 vs 25 × 10³ cells) (Fig. 6A). This was not the case for cells from treated mice that remained protected, because that reduction was much smaller in comparison with nontreated rechallenged mice. More significantly, the central memory CD8⁺ CD44highCD45RBhigh T cells remained unchanged in both groups (Fig. 6B). Identical cytofluorometric analyses of CD4⁺ lymphocytes revealed no differences in populations among all groups of mice (data not shown). Although additional experiments demonstrating a reduction of P. berghei Ags in the liver as well as Ag specificity of the responding CD8⁺ T cells will be needed for the confirmation of these results, it is clear that, coincidental with the loss of protective immunity, there was a significant reduction in the effector memory but not the central memory subpopulations of liver CD8⁺ T cells.

Only central memory CD8⁺ T cells express CD122high (IL-15R)

Recently, it has been shown that IL-15 provides a signal necessary for the survival of long-term memory CD8⁺ T cells, whereas IL-2 stimulates both the initial expansion and subsequent contraction of T lymphocytes (32–35). This has been demonstrated in viral and bacterial model systems, in which a slow cellular division in response to IL-15 maintains memory CD8⁺ T cells (36). Determination of CD122, the β-chain shared by IL-2R and IL-15R, expressed on various subtypes of liver lymphocytes from γ-spz-immune mice (data not shown) confirmed previous report(s) (37) that CD4⁺ T cells were CD122⁻neg. We have observed (N. Steers, manuscript in preparation) that immunization with γ-spz caused up-regulation of IL-15 mRNA in Kupffer cells, but not in cells isolated from spleens of γ-spz-immune mice. Therefore, we asked whether the presence of IL-15 in the liver might be involved in the maintenance of local CD8⁺ T cells. We approached this investigation by determining whether the expression of CD122 might be up-regulated on CD8⁺ CD44highCD45RBlow (effector memory) as well as CD8⁺ CD44highCD45RBhigh (central memory) T cells as a consequence of γ-spz immunization and challenge. Although both subsets expressed CD122, to our surprise, the density of CD122 expression on the central memory phenotype was much higher, and thus, this subset was designated as CD44highCD45RBhigh CD122high (Fig. 7). Twice as many central memory T cells expressed CD122high compared with the effector memory T cells. This ratio of CD8⁺ CD44highCD45RBhighCD122high to CD8⁺ CD44highCD45RBlowCD122low T cells was maintained for 14 days following spz challenge of γ-spz-immune mice (data not shown). Although presently we have no direct evidence that IL-15 is involved in the maintenance of liver CD8⁺ CD44high T cells, the difference in the expression of IL-15R indicates that IL-15 could indeed exert distinct regulatory processes on the two sets of liver CD8⁺ CD44high T cells. Further confirmatory evidence is needed.

**FIGURE 5.** Primaquine causes a decline of memory CD8⁺ CD45RBlow T cells. Following priming and each boost immunization with γ-spz, a group of five mice received primaquine (60 mg/kg) (see Materials and Methods). The mice were challenged 7 days after last immunization with γ-spz and then rechallenged 6 mo later. A group of five mice that were immunized with γ-spz, challenged, and rechallenged was used as a control. Parasitemia was determined by thin blood smears. IHMC were isolated from livers 9 (A) and 18 (B) days after rechallenge. Liver CD8⁺ CD45RBlow T cells were identified by two-color FACS as described in Fig. 1. Results in A show CD8⁺ CD45RBlow T cells from γ-spz-immune control mice that were protected at challenge and rechallenge (filled histogram) and γ-spz-immune, primaquine-treated mice that were protected upon rechallenge (bold gray line). The results are representative of two separately performed experiments (n = 20).
to validate the effects of IL-15 on the maturation/maintenance of liver memory T cells; nonetheless, these results demonstrated that CD122\textsuperscript{low/high} expression is a distinguishing feature of intrahepatic memory CD8\textsuperscript{T} T cell subsets. Data from a recently published study (3) also strongly imply that only CD8\textsuperscript{T} CD44\textsuperscript{high}/CD122\textsuperscript{high} T cells are dependent on IL-15.

Even if IL-15 selectively affects the maintenance of the central memory CD8\textsuperscript{T} T cells in the liver, by extension it will also affect the effector memory sets, providing that the latter stems from the differentiation of the former memory CD8\textsuperscript{T} T cells in response to immunization with \gspz. We compared the expression of CD122 on central and effector liver memory CD8\textsuperscript{T} T cells in naive mice (Fig. 8). On the basis of our observations, it appears that, indeed, even in naive mice, the liver central memory CD8\textsuperscript{T} T cell subset expressed CD122\textsuperscript{high}. A comparison of the two CD8\textsuperscript{T} cell sets derived from the livers of wild-type and IL-15 KO mice showed that the numbers of both the central and the effector CD8\textsuperscript{T} T cells were markedly reduced in the IL-15 KO mice (Table 1); however, only the central memory CD8\textsuperscript{T} T cells in the IL-15 KO mice showed a reduced expression of CD122 (Fig. 8B).

**Discussion**

MHC class I-restricted CD8\textsuperscript{T} T cells that produce IFN-\(\gamma\) are considered a key effector mechanism in protection induced by \textit{P. berghei} (28, 38) and \textit{Plasmodium yoelii} (27, 4) \gspz against experimental infection. Protection induced by this process is long-lived and associated with activated intrahepatic memory CD8\textsuperscript{T} T cells (5). On the basis of the IFN-\(\gamma\) response and cell surface markers indicative of either activation (CD44, CD45RB) or migration (CD62L), we demonstrate that intrahepatic memory CD8\textsuperscript{T} T cells generated by immunization with \textit{P. berghei} \gspz consisted of at least two distinct subsets: the dominant, IFN-\(\gamma\)-producing CD44\textsuperscript{high}/CD45RB\textsuperscript{low}/CD62L\textsuperscript{low} phenotype, hence effector memory set; and the indolent IFN-\(\gamma\)-producing CD44\textsuperscript{high}/CD45RB\textsuperscript{high}/CD62L\textsuperscript{low/high} phenotype, hence central memory set. We propose that these functionally and phenotypically unique subsets of liver memory CD8\textsuperscript{T} T cells form an interactive network involving different phases of cell activation and differentiation, the balance of
which is regulated by the local availability of specific LSA and IL-15. Whereas the effector memory CD8+ T cells were primarily sensitive to the availability of LSA, the central memory CD8+ T cells were dependent on the provision of IL-15.

The copresence of distinct subsets within the intrahepatic memory CD8+ T cell pool in mice protected against malaria is consistent with an earlier view that virally induced memory CD8+ T cells are organized into subsets based on distinct functional activities and the maturation/activation status (25, 26). Similar to the rapid lysis mediated by influenza- and Sendai-specific effector memory CD8+ T cells (39), intrahepatic γ-spz-immune effector memory CD8+ T cells produced a copious amount of IFN-γ within 1–6 h after spz infection. Over time, after the challenge, the pool of effector memory T cells contracted, and the IFN-γ response became reduced, but the population of IFN-γ-producing memory cells persisted, exceeding by ~3-fold those found in naive mice. IFN-γ-producing effector memory CD8+ T cells were also observed in the livers of long-term γ-spz-immune mice (10 mo) that maintained protracted protection against a rechallenge. This contrasted significantly with the effector memory phenotype CD8+ T cells that accumulated in the livers of age-matched naive controls, because these cells produced comparatively low levels of IFN-γ. Hence, the long-lived intrahepatic effector memory CD8+ T cells that were recalled to rapidly produce IFN-γ, play a decisive role in protection induced with P. berghei γ-spz. It could be argued that liver CD8+ T cells from naive mice exposed to infectious spz were also high IFN-γ producers, yet these mice failed to be protected. The marked delay in the IFN-γ response most likely contributed in part to the onset of parasitemia as an absence of an inflammatory response might have permitted uncontrolled proliferation as well as development of the parasites to occur.

By contrast to the effector (CD44highCD45RBlowCD62Llow) memory CD8+ T cells, the IFN-γ reactivity of the central (CD44highCD45RBhighCD62Llow/high) memory population exhibited a lag period, and the responses were considerably lower and relatively short-lived. Therefore, these cells did not appear to be directly involved in the elimination of the parasite. Instead, by acquiring the CD122high phenotype, the central memory CD8+ T cells most likely engaged in a proliferation, which qualified them to function as a reservoir to maintain the size of memory CD8+ T cell pools. Indeed, recent evidence from studies of Ag-specific memory CD8+ T cells present in the lung airways of mice infected with Sendai virus demonstrate the copresence of two memory CD8+ T cell subsets, one of which is maintained in the lung by homeostatic proliferation (40). The maintenance of memory pools is one of the prerequisites of a memory T cell response, because attrition, particularly of the effector CD8+ T cells, is inevitable during any infection (41).

Memory CD8+CD44high T cells segregated into two distinct populations on the basis of the IFN-γ response and CD122 expression: the central memory CD44highCD45RBhigh T cells were CD122high whereas effector memory CD44highCD45RBlow T cells did not differ from naive and effector cells in that they were CD122low. Judge et al. (3) have recently made similar observations that CD8+CD44highCD122high T cells are highly dependent on IL-15 for proliferation and survival. Moreover, proportionally, there were at least twice as many central as effector memory CD8+ T cells expressing CD122. It is not unreasonable to presume that the effector memory T cells, being efficient IFN-γ producers, were refractory to the proliferative process, hence their CD122low phenotype. Therefore, the observation that γ-spz immune mice contained proportionally more CD122high central memory CD8+ T cells, which were highly dependent on IL-15, as shown in the IL-15 KO mice, fully supports the view that the role of this subset is distinct from that of the effector memory T cells.

Conscription of central memory CD8+ T cells into the effector memory pool could be a continuous, albeit slow, process occurring in the liver as a result of an increased Ag load following repeated immunizations with γ-spz. The process could also occur during infection, when large numbers of effector memory CD8+ T cells would be most needed to combat the parasite. In either case, the cells in the central memory CD8+CD44highCD45RBlowCD122high T cell pool would expand under the influence of IL-15 up-regulated by γ-spz immunization. Upon encounter with specific Ags from the liver repository, the central memory CD8+ T cells would be driven to differentiate into the CD44highCD45RBlowCD122low phenotype that is easily triggered by infectious spz to produce IFN-γ. The effector memory T cells might, in addition, proliferate to Ag, a notion that is consistent with the recent findings showing Ag-specific proliferation of terminally differentiated memory CD8+CD45RA+CD27− T cells (42). Regardless of which of the two mechanisms operates in the liver to preserve memory CD8+ T cells, each relies on Ag for the maintenance of intrahepatic effector

Table I. Both effector and central memory phenotype liver CD8+ T cells are reduced in IL-15 KO mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Effector Memory (%)</th>
<th>Central Memory (%)</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>27</td>
<td>35</td>
</tr>
<tr>
<td>IL-15 KO</td>
<td>5</td>
<td>7</td>
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*IHMC were isolated from livers of naive wild-type and IL-15 KO mice and stained with anti-CD8, anti-CD44, and anti-CD45RB mAbs. Subsets of CD8+ T cells were identified as described in Fig. 1. The results shown are from one of two representative experiments. The numbers indicate percentages of liver CD8+ T cells.
memory CD8⁺ T cells in mice protected by immunization with γ-spz. There are several, often opposing, views regarding the need for Ag for the persistence of memory T cells. We suggest that lasting protective immunity in the γ-spz model depends upon a depot of Ag in the liver, but this requirement for Ag could be delineated only through the analysis of very specific circuits of interacting T cell subsets engaged in protracted protection. This process typically requires many different phases of T cell memory development, each with unique cellular and molecular requirements. For example, loss of protection in mice treated with primaquine, a drug that disrupts intrahepatic development of the parasite and hence blocks the maintenance of protective immunity (21), was accompanied by a 5-fold decrease in the number of intrahepatic effector memory CD8⁺ T cells. Significantly, the central memory subset was not affected. We assume that disruption of the intrahepatic-stage parasite development prevented the formation of a local Ag depot, which impeded the conscription or differentiation of central into effector memory CD8⁺ T cells. Although the majority of results from other studies convincingly argue against the need for Ag to maintain long-lived memory CD8⁺ T cells, we suggest that Ag requirements might be quite different in instances in which a parasite exhibits tropism to an immune-privileged organ, such as the liver in plasmodia infection. It is possible that pathogens such as plasmodia spz establish an Ag repository in the liver parenchyma or in Kupffer cells. The chronic Ag depot may modify the local, normally immunosuppressive environment (9) to create conditions favorable for the maintenance of the activated memory T cells. In some instances, the liver Ag repository may be sufficient to set apart the local activated liver memory T cells from those found in the spleen or lymph nodes.

Our hypothesis provides a partial explanation for the need of intermittent boost immunizations with γ-spz to maintain protective immunity induced in humans by immunization with P. falciparum γ-spz (16, 43). Moreover, it is supported by recent evidence from our laboratory that memory CD4⁺ T cells that characterize long-lasting protection induced in humans by P. falciparum γ-spz (44) are also organized into an interactive network of subsets, each displaying a unique phase of maturation/differentiation, as demonstrated by phenotypic and functional features.

More recent evidence based on chemokines and migration markers (22, 23) also support the organization of memory T cells into a central (CD62L⁺CCR7) phenotype that localizes primarily to lymph nodes and an effector set, which, upon acquiring the CD62L⁻ or CCR7 phenotype, migrates from immune organs toward the site of inflammation in a nonlymphoid tissue. The intrahepatic memory CD8⁺ T cells surprisingly appear to contain both subsets. Although it is likely that both sets emigrated from the spleen and lymph nodes to the site of inflammation in the liver, it is also possible that both activation and maintenance of plasmodia Ag-specific CD8⁺ T cells occurred locally in the liver. The local availability of highly active effector memory T cells in the liver clearly presents an advantageous situation for the host, because these cells are poised to mount a rapid defense against the parasite.

In summary, the success of protection induced by γ-spz depends on the long-lived intrahepatic memory CD8⁺ T cells, which reside in distinct subpopulations as central and effector memory T cells. Although the effector memory T cells are maintained by the Ag-driven conscription of the central memory T cells, the latter, representing a very broad spectrum of Ag-specific T cells, is maintained by IL-15. This arrangement, based on a conscription process from the effector to the effector memory CD8⁺ T cell phenotype, ensures the availability of Ag-specific T cells, should they be required to combat an infection. The dependence on specific Ag essentially controls the balance between the two phenotypes, and the differential expression of IL-15R prevents the effector memory CD8⁺ T cells from becoming activated in the event of sporadic coinfections. However, it is the activated status of the intrahepatic memory CD8⁺ T cells that really distinguishes them from the memory CD8⁺ T cells in the spleen and lymph node, because it represents the sentinel of a local, organ-specific infection.

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References


