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Development of Memory CD8⁺ T Cells and Their Recall Responses during Blood-Stage Infection with Plasmodium berghei ANKA

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Conditions required for establishing protective immune memory vary depending on the infecting microbe. Although the memory immune response against malaria infection is generally thought to be relatively slow to develop and can be lost rapidly, experimental evidence is insufficient. In this report, we investigated the generation, maintenance, and recall responses of Ag-specific memory CD8⁺ T cells using Plasmodium berghei ANKA expressing OVA (PbA-OVA) as a model system. Mice were transferred with OVA-specific CD8⁺ T (OT-I) cells and infected with PbA-OVA or control Listeria monocytogenes expressing OVA (LM-OVA). Central memory type OT-I cells were maintained for >2 mo postinfection and recovery from PbA-OVA. Memory OT-I cells produced IFN-γ as well as TNF-α upon activation and were protective against challenge with a tumor expressing OVA, indicating that functional memory CD8⁺ T cells can be generated and maintained postinfection with P. berghei ANKA. Cotransfer of memory OT-I cells with naive OT-I cells to mice followed by infection with PbA-OVA or LM-OVA revealed that clonal expansion of memory OT-I cells was limited during PbA-OVA infection compared with expansion of naive OT-I cells, whereas it was more rapid during LM-OVA infection. The expression of inhibitory receptors programmed cell death-1 and LAG-3 was higher in memory-derived OT-I cells than naive-derived OT-I cells during infection with PbA-OVA. These results suggest that memory CD8⁺ T cells can be established postinfection with P. berghei ANKA, but their recall responses during reinfection are more profoundly inhibited than responses of naive CD8⁺ T cells. The Journal of Immunology, 2012, 189: 4396–4404.

Malaria, which is mediated by infection with Plasmodium species, is one of the most serious infectious diseases in the world. During blood-stage infection with malaria parasites, specific protective immune responses of both the humoral and cellular arms develop, which are both critical for the establishment of protective immunity. Within the cellular immune responses, both CD4⁺ and CD8⁺ T cells are specifically activated during the blood stage of malaria (1, 2). CD4⁺ T cells help B cells to develop into Ab-producing cells and activate dendritic cells (DC) as well as macrophages promoting protective immune responses. Although malaria-infected RBCs do not express MHC, DC present malaria Ag by cross-presentation and activate a large population of malaria-specific CD8⁺ T cells (3, 4). Although CD8⁺ T cells may play some protective role under specific conditions, the protective role of CD8⁺ T cells during blood-stage infection is unclear. CD8⁺ T cells appear to have a pathogenic role in the generation of cerebral malaria (6) and are reported to modulate immune responses by destroying marginal metallophilic macrophages in the spleen (7). Therefore, activation of malaria-specific CD8⁺ T cells plays a pivotal role in the course of malaria pathology.

Studies of the natural history of malaria patients in endemic areas suggest that immunity to malaria infection is relatively slow to develop and incomplete (8). Anecdotal evidence suggests rapid loss of malarial immunity when people leave endemic areas followed by risk of developing symptomatic malaria upon later revisits to the endemic area (8). However, experimental evidence for the generation and maintenance of malaria immunological memory is relatively scarce (9). Several mechanisms have been proposed to explain the poor generation and maintenance of memory immune responses in malaria. First, blood-stage malaria infection modulates function and maturation of DCs and interferes with their Ag-presentation capacity (10, 11). Decreased DC function during blood-stage malaria infection results in poor priming of CD8⁺ T cells and consequently interferes with CD8⁺ T cell-mediated protection during the liver stage of the disease. Second, adoptive transfer of cultured malaria-specific CD4⁺ T cells resulted in deletion of malaria-specific T cells during infection with P. berghei, suggesting that apoptosis of malaria-specific CD4⁺ T cells is accelerated during infection (12, 13). However, this massive depletion could not explain the presence of malaria-specific CD4⁺ T cells in infected mice, and it was unclear whether this depletion was malaria-specific or whether it could occur in other situations when T cells clonally expand in vivo (9).

Accumulating evidence indicates that naive CD8⁺ T cells dramatically expand after priming, acquire effector function, and then...
undergo a contraction phase after clearance of the Ag-bearing microbes, leaving a certain proportion of the burst size as long-lived memory cells (14). Studies on T cell memory are, however, hampered because populations of specific memory T cells in vivo are almost undetectable. One way to circumvent this difficulty is by use of T cells from TCR-transgenic mice, thus enabling monitoring of the response of the defined specific T cell population. We developed a system to monitor CD8+ T cells specific for a model malaria Ag, OVA, using a combination of OVA-specific TCR-transgenic CD8+ T cells, OT-I, and Plasmodium berghei ANKA expressing rOVA (3). In this study, we examine whether memory CD8+ T cells can be generated postinfection with malaria parasites and whether the response of memory CD8+ T cells is modulated during malaria infection using this model system. Throughout this study, we compared the responses of OT-I T cells postinfection with P. berghei ANKA expressing OVA (Pha-OVA) with Listeria monocytogenes expressing OVA (LM-OVA) (15), thus enabling a comparison of CD8+ T cell responses of the same specificity expressed by different pathogens. Through these studies, we examined the memory CD8+ T cell responses unique to infection with Plasmodium species. The results suggest that memory CD8+ T cells can be generated postinfection with Plasmodium species, but their recall responses during infection with Pha are hampered when compared with their recall responses during infection with L. monocytogenes.

Materials and Methods

Mice

OT-I transgenic mice expressing the TCR specific for OVA257–264/Kb (16) were provided by Dr. H. Kosaka (Osaka University), B6.SJL-Ptprc congenic (B6.SJL) mice (CD45.1+) by Dr. Y. Takahama (Tokushima University), and Rag2 knockout (KO) mice (17) by Dr. Y. Yoshikai (Kyushu University). C57BL/6 (B6) mice were purchased from SLC. OT-I and B6.SJL-Ptprc congenic mice were bred, and offspring were intercrossed to obtain CD45.1+ OT-I mice. Rag2 KO mice and CD45.1+ OT-I mice were intercrossed to obtain CD45.1+ Rag2 KO OT-I mice. These mice were maintained in the Laboratory Animal Center for Animal Research at Nagasaki University and were used at the age of 8–14 wk. The animal experiments reported in this study were approved by the Institutional Animal Care and Use Committee of Nagasaki University and conducted according to the guidelines for Animal Experimentation, Nagasaki University.

Microorganisms

Pha-OVA (previously described as OVA-Pha), which constitutively expresses OVA driven by hsp70 promoter, was maintained by serial pasegpassage in BALB/c mice as described previously (3). Mice were infected with Pha-OVA by i.p. injection of parasitized RBCs (5 × 10⁶ infected RBC). LM-OVA (15) was kindly provided by Dr. Y. Yoshikai (Kyushu University) and Dr. H. Shen (University of Pennsylvania). Mice were infected i.p. with 3 × 10⁶ LM-OVA (0.1 LD50).

Generation of memory cells

Memory CD8+ T cells were generated both in vivo and in vitro. For in vivo memory generation, B6 mice were adoptively transferred with CD8+ T cells (1 × 10⁶) from CD45.1 Rag2 KO OT-I mice and infected with Pha-OVA or LM-OVA (18). Postinfection with Pha-OVA, mice were treated with chloroquine and sulfadiazine (Sigma-Aldrich) for 2 wk between days 6 (parasitemia 2.1–12.1%) and 20 as described previously with slight modification (19). Chloroquine (10 mg/kg body weight/d) was injected i.p. for 4 consecutive d in each week, and sulfadiazine was provided in the form of daily drinking water containing sulfadiazine (30 mg/l). For in vitro memory generation, splenocytes (5 × 10⁵) from Rag2 KO OT-I mice were stimulated in RPMI 1640 medium supplemented with nonessential amino acids, glutamine, sodium pyruvate, antibiotics, 10% FCS, and 2-ME (5 × 10⁻⁵ M) in the presence of OVA257–264 (1 µg/ml) in 24-well plates overnight. After washing three times, cells (1 × 10⁶/well) were cultured in 24-well plates in the presence of recombinant mouse IL-7 (5 ng/ml; PeproTech) for >2 wk before being used in adoptive transfer experiments (20).

Adaptive transfer of memory and naive OT-I cells

B6 mice were adoptively transferred with 1:1 mixture of memory and naive OT-I cells, and B6 host, memory OT-I, and naive OT-I cells were distinguished by CD45.1/2 markers. For cotransfer with unsorted memory OT-I cells, naive OT-I cells were isolated from the spleen of Rag2 KO OT-I mice using anti-CD8 IMag (BD Biosciences). To purify central and effector memory CD8+ T cells, memory OT-I cells generated in vitro were stained for CD8, CD26, and CD44 and sorted into central (CD62L+CD44−) and effector memory (CD62L−CD44+) populations using FACS Aria II (BD Biosciences). Memory OT-I cells generated in vivo were purified by cell sorting after staining for CD45.1 and CD8. For cotransfer of sorted memory OT-I cells, naive OT-I cells were purified from the spleen of Rag2 KO OT-I mice by sorting after staining for CD8. To examine DNA synthesis, mice that received OT-I cells and were infected with Pha-OVA or LM-OVA were injected i.p. with BrdU (2 mg). After 20 h, splenocytes were intracellularly stained with anti-BrdU Ab according to the manufacturer’s instructions (BD Biosciences).

Flow cytometry

The staining reagents used in this study include PECy7–anti-CD45.1, PE–or allophycocyanin–anti-CD8, allophycocyanin–eFluor780- or allophycocyanin–anti-CD45.2, PE–anti-CD26, PE–anti-CD44, FITC–anti-CD4, FITC–anti-CD127, allophycocyanin–anti-killer cell lectin-like receptor G1 (KLRG1), allophycocyanin–anti-programmed cell death-1 (PD-1), PE–anti-CD160, PE–anti-CD244 mAbs (eBioscience), and allophycocyanin–anti-LAG-3 mAbs (BioLegend). The number of OT-I cells in each organ was calculated by multiplying the number of total cells with the proportion of OT-I cells as determined by flow cytometry. Staining with Annexin V was performed in Annexin V binding buffer (Sigma-Aldrich) in the presence of 20 µg/ml 7-aminoactinomycin D (7-AAD) was added.

For intracellular staining, CD8+ T cells (1 × 10⁶) were stimulated with DC (3 × 10⁵) pulsed with OVA257–264 peptide (1 µg/ml) in 24-well plates for 3 to 4 h at 37°C in the presence of monensin. Cells were incubated with Fc block (anti-Fc receptor mAb), and cell-surface molecules were stained with appropriate mAbs, fixed, and permeabilized using Cytofix/Cytoperm buffer in accordance with the manufacturer’s instructions (Sigma-Aldrich). To exclude dead cells from the analysis 7-aminoactinomycin D (7-AAD) was added.

For intracellular staining, CD8+ T cells (1 × 10⁶) were stimulated with DC (3 × 10⁵) pulsed with OVA257–264 peptide (1 µg/ml) in 24-well plates for 3 to 4 h at 37°C in the presence of monensin. Cells were incubated with Fc block (anti-Fc receptor mAb), and cell-surface molecules were stained with appropriate mAbs, fixed, and permeabilized using Cytofix/Cytoperm buffer in accordance with the manufacturer’s recommendations (BD Biosciences). Cells were stained with PE–anti–IFN-γ or Alexa Fluor 488–anti–TNF-α mAbs (eBioscience) and analyzed using FACSCanto II (BD Biosciences).

FIGURE 1. Generation of memory CD8+ T cells postinfection with PhaA and L. monocytogenes. B6 mice (CD45.2+) were adoptively transferred with Rag2 KO OT-I CD8+ T cells (CD45.1+ 1 × 10⁶) (day −1) and uninfected or infected with Pha-OVA or LM-OVA on day 0. Mice infected with Pha-OVA were treated with chloroquine (CQ) and sulfadiazine (SDF) for 2 wk between days 6 and 20 (A). The parasitemia level was 6.8 ± 2.7% on day 6 and became undetectable 10 d after the infection. PBMCs were collected from each mouse and stained for CD45.1, CD45.2, and CD8. The proportions of OT-I cells in total CD8+ T cells were determined in each mouse (B). Mice that maintained OT-I cells above the level of detection limit (0.08%) are shown with a black line, and those below detection limit are shown with a gray line.
Tumor rejection assay

The tumor rejection assay was performed using EL-4 thymoma and its OVA-transfected E.G7 as previously described with slight modification (21). Mice received $2 \times 10^6$ cells each of EL-4 and E.G7 on the left and right back, respectively. Tumor diameter was monitored every 1 to 2 d, and the average tumor diameter was determined as: average tumor diameter = [longest tumor diameter + shortest tumor diameter]/2.

FTY720 treatment

B6 mice received FTY720 (20 mg/mouse; Mitsubishi Tanabe Pharma) i.p. 2 h prior to the adoptive transfer of OT-I cells. The next day, the mice were infected with PbA-OVA or LM-OVA. During the infection, mice received a daily injection of FTY720.

Statistical analysis

Comparison of three groups (uninfected, PbA-OVA, and LM-OVA) was first made by one-way ANOVA at the significance level of 0.05, and, if significant, Tukey’s multiple comparison test was used to compare uninfected and PbA-OVA–infected mice or uninfected and LM-OVA–infected mice. Comparison of two independent groups was performed using the Mann–Whitney U test. The paired t test was used to compare naive and memory OT-I cells transferred into each mouse.

Results

Persistence of Ag-specific CD8+ T cells postinfection with PbA-OVA

To investigate whether memory CD8+ T cells can be generated postinfection with PbA, female B6 mice were adoptively transferred with CD8+ T cells from female OT-I mice, infected with PbA-OVA or LM-OVA, and the fate of OT-I cells compared. Infected mice with PbA-OVA or LM-OVA enabled a comparison of CD8+ T cell responses of the same specificity expressed by different pathogens.

Because PbA infection is lethal, mice were treated with the antimalarial drugs chloroquine and sulfadiaidine for 2 wk starting 6 d postinfection (Fig. 1A). This treatment completely cured the mice, and no parasitemia was detected for >9 wk after treatment (data not shown). Drug treatment did not directly affect the response of OT-I cells, because no significant differences in the immune responses of treated and untreated mice to LM-OVA were observed (data not shown). To analyze changes in the number of OT-I cells in each mouse, the proportion of OT-I cells in CD8+ T cells was monitored in PBL. Seven days postinfection, the proportion of OT-I cells in CD8+ T cells reached the peak level of 2.3 ± 1.1 and 49.3 ± 11.9% in mice infected with PbA-OVA and LM-OVA, respectively (Fig. 1B). The proportions of OT-I cells declined afterward, but were detectable in PBL for >8 wk in 12 out of 32 mice (37.5%) postinfection with PbA-OVA and in 16 out of 26 mice (61.5%) postinfection with LM-OVA. The presence of OT-I cells in PBL strongly correlated with their maintenance in lymphoid organs (data not shown). However, we did not observe a clear correlation between the length of OT-I maintenance and the peak levels of OT-I cell ratios (Fig. 1B). Levels of parasitemia (day 6) in mice that maintained OT-I cells for >8 wk and in those mice that did not maintain OT-I cells for >8 wk were 7.7 ± 3.3 and 6.2 ± 2.2%, respectively. This suggests that long-term maintenance of memory OT-I cells does not directly correlate with parasitic load and the initial burst of T cell clonal expansion.

Functional memory CD8+ T cells can be maintained in mice after malaria infection

Next, the phenotype of transferred OT-I cells was examined (Fig. 2). The majority of OT-I CD8+ T cells purified from Rag 2 KO
OT-I mice were the CD62L<sup>lo</sup>CD44<sup>hi</sup> naive phenotype (Fig. 2A). Seven days postinfection with PbA-OVA or LM-OVA, the majority of OT-I cells became CD62L<sup>lo</sup>CD44<sup>hi</sup> effector phenotype. The proportion of short-lived effector cells (KLRG1<sup>hi</sup>CD127<sup>lo</sup>) (22) was lower in mice infected with PbA-OVA than those infected with LM-OVA (Fig. 2B). Two months postinfection, the proportion of central memory (CD62L<sup>hi</sup>CD44<sup>hi</sup>) OT-I cells in mice infected with PbA-OVA was slightly higher than those infected with LM-OVA. There was no significant difference in the proportion of long-lived memory (KLRG1<sup>lo</sup>CD127<sup>hi</sup>) cells between mice infected with PbA-OVA and LM-OVA. Next, we evaluated the ability of OT-I cells to produce IFN-γ and TNF-α by intracellular staining (Fig. 2, bottom panels). Naive OT-I cells did not produce IFN-γ and TNF-α. Two months postinfection, there was no significant difference in the proportions of cytokine-producing OT-I cells between mice infected with PbA-OVA and LM-OVA (Fig. 2C). Taken together, these results indicated that memory CD8<sup>+</sup> T cells can develop postinfection with PbA-OVA.

Finally, we evaluated the function of memory OT-I cells in vivo by tumor rejection assay. Two months postinfection with PbA-OVA or LM-OVA, mice that maintained memory OT-I cells in PBL were transplanted with OVA-expressing E.G7 tumor cells on the right flank and control EL-4 tumor cells on the left flank (Fig. 3). E.G7 cells did not grow in mice infected either PbA-OVA or LM-OVA, whereas they grew in uninfected mice. Control EL-4 cells grew in all three groups of mice (Fig. 3A). OT-I cells in PBL were monitored after tumor challenge. The proportion of OT-I cells increased in mice that rejected E.G7 (Fig. 3B). These results indicate that OT-I cells recognized the OVA epitope expressed in E.G7 and mounted specific recall effector responses. Taken together, functional memory cells were generated and maintained for >2 mo postinfection with PbA-OVA, and the established memory CD8<sup>+</sup> T cells were phenotypically and functionally indistinguishable from those generated postinfection with LM-OVA.

Expansion of memory CD8<sup>+</sup> T cells was reduced during malaria infection

Next, the response of memory OT-I cells was examined during malaria infection. Naive OT-I cells were prepared from Rag2 KO OT-I mice (CD45.1<sup>+</sup>/2<sup>+</sup>). Memory OT-I cells were generated in vitro by culturing Ag-stimulated CD8<sup>+</sup> T cells from Rag2 KO OT-I mice (CD45.1<sup>+</sup>/2<sup>+</sup>) in the presence of IL-7 for >14 d as previously described (20). B6 mice were adoptively transferred with a 1:1 mixture of naive (CD45.1<sup>+</sup>/2<sup>+</sup>) and memory (CD45.1<sup>+</sup>/2<sup>+</sup>) OT-I cells and infected with PbA-OVA or LM-OVA, a microbe for which memory CD8<sup>+</sup> T cell responses have been extensively investigated (Fig. 4A) (23, 24). This approach allowed us to monitor
the response of both naive and memory OT-I cells postinfection in the same mouse. The proportion of naive-derived OT-I cells increased for 8 d in the spleen of PbA-OVA infected mice, whereas the proportion of memory-derived OT-I cells peaked on day 6. The change in numbers of naive or memory OT-I cells postinfection was calculated and shown as fold increase relative to uninfected mice (Fig. 4B). Significantly increased expansion of naive OT-I cells occurred in spleen, lymph node, and brain postinfection with PbA-OVA compared with uninfected mice. However, the expansion of memory OT-I cells postinfection with PbA-OVA was less robust than that of naive OT-I cells in spleen, lymph nodes, and brain. The reduced expansion of memory-derived OT-I cells was unlikely to be due to their redistribution to other organs because we did not observe a significant increase in memory-derived OT-I cells in bone marrow, brain, liver, and peripheral blood (Fig. 5, saline). In mice infected with LM-OVA, however, the proportions of both naive- and memory-derived OT-I cells reached peak levels 4 d postinfection (Fig. 4A). During this period, memory OT-I cells expanded more rapidly than naive OT-I cells expanded in the spleen of LM-OVA–infected mice (Fig. 4B). The expansion of memory OT-I cells was also higher than naive OT-I cells in bone marrow, brain, liver, and peripheral blood in LM-OVA–infected mice (Fig. 5, saline). These results suggest that clonal expansion of memory CD8+ T cells is limited in mice infected with malaria parasites.

Because memory OT-I cells generated in vitro include both central and effector memory cells, we examined expansion of each memory subpopulation postinfection. The CD62LhiCD44hi effector memory and CD62LhiCD44hi central memory OT-I cells were purified by sorting, each mixed with naive OT-I cells at 1:1 ratio, and adoptively transferred into B6 mice. The expansion of both central and effector memory cells postinfection with PbA-OVA was lower than that of naive OT-I cells (Fig. 6A). We also examined the response of memory OT-I cells generated in vivo. In vivo-generated memory OT-I cells were purified from OT-I inoculated B6 mice after 104 d of LM-OVA infection and then transferred into B6 mice as a 1:1 mixture with naive OT-I cells. The mice were then infected with PbA-OVA or LM-OVA. The in vivo-generated memory CD8+ T cells also expanded less than naive cells during infection with PbA-OVA (Fig. 6B). We also examined the ability of these OT-I cells to produce IFN-γ in response to Ag. The proportion of OT-I cells that produce IFN-γ was not significantly different between naive-derived and memory-derived OT-I cells during infection with PbA-OVA and LM-OVA (Fig. 6C).

**Distribution of memory CD8+ T cells was not disturbed during malaria infection**

One possibility to account for the reduced expansion of memory OT-I cells in lymphoid organs during PbA-OVA infection is that they migrated to other sites in the body. To address this possibility, we used the immunosuppressive drug FTY720, which acts as an agonist of the sphingosine 1-phosphate receptor and inhibits lymphocyte egress from lymphoid organs (25, 26). Mice were transferred with a 1:1 mixture of naive and memory OT-I cells, infected with PbA-OVA or LM-OVA, and the expansion of naive- and memory-derived OT-I cells was examined 6 or 4 d postinfection, respectively (Fig. 5). This time frame corresponded with maximal OT-I cell expansion for each infection. During the course of infection, mice received FTY720 daily. The levels of parasitemia in PBS- and FTY720-treated groups were 6.3 ± 2.0 and 7.0 ± 1.7%, respectively, indicating that FTY720 treatment had no direct effect on parasitemia levels. The proportion of CD8+ T cells in PBL of untreated and FTY720-treated mice was 6.6 ± 3.9 and 2.6 ± 1.2% in PbA-OVA–infected mice and 23.0 ± 5.8 and 15.3 ± 9.7% in LM-OVA–infected mice, respectively, indicating that FTY720 was effective in retaining the primed CD8+ T cells within the lymphoid organs. In each organ, memory OT-I cells generally expanded less than naive OT-I cells both in the absence and presence of FTY720 during infection with PbA-OVA. One exception was inguinal lymph nodes in which expansion of memory OT-I cells was less robust in the absence of FTY720, but was not significantly different in its presence. The result suggested that lymph nodes were not the primary sites of OT-I expansion during infection with PbA-OVA. When the mice were infected with LM-OVA, memory OT-I cells generally expanded to a greater extent than naive cells in all organs except LN. This tendency was not changed in the presence of FTY720 in all organs except spleen. These results suggested that the reduction in the increase of memory OT-I cells in PbA-OVA–infected mice, when compared with that of naive OT-I cells, was not due to altered distribution of these cells. Taken together,

![FIGURE 5. Effect of FTY720 on the number of naive/memory OT-I cells during infection. B6 mice were transferred with 1 × 10^6 cells each of a 1:1 mixture of in vitro memory (CD45.1+2+, ○) and naive OT-I cells (CD45.1+2- , ●) and uninfected or infected with PbA-OVA or LM-OVA the following day. Mice were injected i.p. with FTY720 (20 μg/mouse) or saline daily starting 1 d before OT-I transfer until sacrifice. Six days postinfection with PbA-OVA (parasitemia, FTY720-treated group 6.3 ± 2.0%, saline-treated group 7.0 ± 1.7%) or four days postinfection with LM-OVA, cells were collected and stained for CD8, CD45.1, and CD45.2. The number of naive- and memory-derived OT-I cells was determined by multiplying the total cell number with the proportion of each cell type. The values of naive- and memory-derived OT-I cells in the same mouse are connected with a line. Paired t test, *p < 0.05, **p < 0.01.](http://www.jimmunol.org/DownloadedFrom)
we concluded that PbA-OVA infection impairs the Ag-driven expansion of memory CD8+ T cells in mice.

Mechanisms underlying the impaired expansion of memory CD8+ T cells during malaria infection

CD8+ T cells express inhibitory receptors including PD-1, LAG-3, CD160, and CD244 in mice infected with P. yoelii, and their blockade improves antiparasite response (27). Therefore, we examined the expression of these inhibitory receptors on CD8+ T cells on naive- and memory-derived OT-I cells during infection with PbA-OVA and LM-OVA (Fig. 7). OT-I cells in mice infected with PbA-OVA expressed PD-1, LAG-3, and CD160 at levels higher than those in LM-OVA–infected mice. Importantly, within mice infected with PbA-OVA, memory-derived OT-I cells expressed PD-1 and LAG-3 at levels significantly higher than naive-derived OT-I cells. In mice infected with LM-OVA, however, memory-derived OT-I cells expressed PD-1 and CD160 at a level similar to naive-derived OT-I cells and LAG-3 at a slightly higher level.

To examine mechanisms underlying the reduced expansion of memory OT-I cells during infection with PbA-OVA, B6 mice were transferred with naive and memory OT-I cells, and their DNA synthesis was monitored by incorporation of BrdU during infection with PbA-OVA or LM-OVA (Fig. 8A). Although memory OT-I cells incorporated more BrdU than naive OT-I cells during infection with LM-OVA, memory and naive OT-I cells incorporated BrdU similarly during infection with PbA-OVA. This suggests that proliferation of memory OT-I cells was limited in mice infected with PbA-OVA, although the difference was modest. Apoptosis of OT-I cells was evaluated during their active expansion 6 and 4 d postinfection with PbA-OVA and LM-OVA, respectively. B6 mice were transferred with naive and memory OT-I cells, infected with PbA-OVA or LM-OVA, and spleen cells were stained with Annexin V and 7-AAD. The proportion of Annexin V+7-AAD+ early apoptotic cells in memory OT-I cells was higher than in naive OT-I cells in both PbA-OVA– and LM-OVA–infected mice (Fig. 8B), suggesting that the reduced expansion of memory OT-I cells during malaria infection was not due to differences in apoptosis compared with LM-OVA infection.

Discussion

In this study, the generation of CD8+ memory T cells after clearance of malaria infection was evaluated, and the CD8+ memory T cell recall responses of OT-I T cells during infection with recombinant PbA-OVA or LM-OVA was compared, thus enabling a comparison of CD8+ T cell responses of the same specificity expressed by different pathogens. This study showed that memory CD8+ T cells can be generated and maintained after clearance of malaria infection. Phenotypic and functional studies indicated that OT-I cells that persisted postinfection were true central memory T cells (CD62L+CD44+) that produced IFN-γ and TNF-α. In addition, they elicited specific protective immune responses against tumor challenge. Second, adoptively transferring memory and naive OT-I cells into the same mice revealed that the capacity of clonal expansion of memory CD8+ T cells during infection with PbA is limited when compared with naive CD8+ T cells or compared with expansion of memory CD8+ T cells during infection with LM-OVA.

To determine maintenance of OT-I cells postinfection, we inoculated a small number of OT-I cells (1 × 10^3/mouse) and infected with PbA-OVA or LM-OVA. The proportion of OT-I cells reached peak levels 7 d postinfection and gradually decreased.
thereafter. OT-I cells were maintained for >2 mo postinfection in 37.5% of mice infected with PbA-OVA and in 61.5% of LM-OVA–infected mice. Differences in OT-I cell maintenance between mice infected with PbA-OVA and LM-OVA may reflect the lower peak response of OT-I cells postinfection with PbA-OVA compared with infection with LM-OVA. We did not observe a direct correlation between peak levels of OT-I cell ratios and the length of OT-I cell maintenance within a group of mice infected with PbA-OVA or LM-OVA. It was reported that levels of MHC/epitope density on DC have a more profound effect on the generation of memory T cells than on the expansion of effector T cells (28). Differences in maintenance of memory OT-I cells in individual mice may be explained by differences in levels of epitope density on DC (28). During infection, the effective microbial Ag dose may have varied in each mouse, affecting DC antigenic density and thus influencing generation and maintenance of memory OT-I cells in individual mice. An alternative possibility is that the niche of memory cells was limited under the conditions employed, and competition for the niche between OT-I and endogenous memory populations affected the maintenance of OT-I cells in a stochastic manner, resulting in variation in the maintenance period of OT-I cells in individual mice.

Recall responses of memory OT-I cells during infection with PbA-OVA and LM-OVA were evaluated by their ability to clonally expand in vivo after Ag encounter. Memory OT-I cells expanded more than naive OT-I cells in the majority of organs, except lymph nodes postinfection with LM-OVA. In contrast, the expansion of memory OT-I cells postinfection with PbA-OVA was lower than that of naive OT-I cells in all organs examined. This trend was observed in both effector and central memory-type CD8+ T cells.

**FIGURE 7.** B6 mice were adoptively transferred with a 1:1 mixture of naive and memory OT-I cells generated in vitro and uninfected or infected with PbA-OVA or LM-OVA. Six days postinfection with PbA-OVA (parasitemia 9.58 ± 0.54%) or four days postinfection with LM-OVA, spleen cells were stained for CD8, CD45.1, CD45.2, and inhibitory receptors (PD-1, LAG-3, CD160, CD244). The representative result (A) and the summary of the expression (B) of inhibitory receptors on memory- (○) and naive-derived (●) OT-I cells are shown. Paired t test, *p < 0.05, **p < 0.01.
generated in vitro and central memory CD8 T cells generated in vivo. It is unlikely that this reduction in memory OT-I cell expansion was due to altered distribution of OT-I cells, such as sequestration of OT-I cells in a particular organ during malaria infection, because even in the presence of FTY720, which inhibits egress of T cells from lymphoid organs (25, 26), the expansion of memory OT-I cells was lower than naive OT-I cells in the majority of organs examined. Two possibilities can be considered to account for the reduced expansion of memory OT-I cells during infection with Plasmodium: enhanced apoptosis and reduction in proliferation. This study suggested that apoptosis of memory OT-I cells was not increased during Plasmodium infection compared with LM-OVA infection. In contrast, proliferation of memory OT-I cells was slightly suppressed during Plasmodium infection compared with LM-OVA infection, suggesting that reduced proliferation, rather than cell death, limits the expansion of memory OT-I cells in Plasmodium-infected mice. Recently, Martin et al. (29) reported an experimental system similar to ours and suggested that naive CD8 T cells undergo greater expansion than memory CD8 T cells after antigenic encounter. They transferred naive and memory OT-I cells into the same mice, challenged with LM-OVA, and showed that the expansion of naive OT-I cells was greater than memory OT-I cells on a per-cell basis. In our experimental model, expansion of memory OT-I cells was always higher than naive OT-I cells postinfection with LM-OVA. Although the two systems are quite similar, there are some important differences. We used memory OT-I cells generated in vitro or splenic in vivo memory cells, whereas Martin et al. (29) used memory OT-I cells from spleens of mice infected with vaccinia virus expressing OVA peptide. We obtained naive cells from spleens of Rag2 KO OT-I mice, whereas they used OT-I cells from PBL. In addition, we transferred 1 x 10^6 OT-I cells, whereas they used 1 x 10^5 cells. We showed that OT-I cell numbers reached peak levels 4 d postinfection, when we observed a greater increase of memory OT-I cells than naive cells. In the model of Martin et al. (29), the numbers of naive- and memory-derived OT-I cells were similar 5 d after LM-OVA infection, but these cells continued to increase until 7 d postinfection, when they observed a greater increase of naive OT-I cells. Therefore, we believe that these two experimental results are not contradictory, but are likely to reflect the differences in the type of memory cells used and/or differences in the kinetics of OT-I cell expansion during the course of LM-OVA infection.

The fate of T cells after antigenic stimulation can be influenced by the TCR and costimulatory signals delivered by APCs during Ag recognition and by microenvironmental factors including cytokines (30). We reported that the expansion of memory OT-I cells was poor when compared with naive OT-I cells during infection with Plasmodium. Several possibilities can be considered to account for the differential responses. First, naive and memory CD8 T cells express different patterns of homing receptors and may encounter different types of DC. The major APCs that activate CD8 T cells are DC8a+ DC in the spleen (4). CD317 (plasmacytoid DC Ag1)+ DC can support the survival of malaria parasites (31) and regulatory DC that are induced during malaria infection may be involved in the activation of naive and memory CD8 T cells (32). Second, the inflammatory environment induced during malaria infection may affect the expansion of CD8 T cells. DC produce reduced levels of IL-12 and increased levels of IL-10 in response to TLR ligands during malaria infection (11, 33). CD4 T cell immune responses are also modulated during infection with malaria parasites, and their production of IL-2 is severely diminished (34–36). IL-2 and IL-12 have an important role in regulating differentiation of memory CD8 T cells (22, 24, 30). Thus, the cytokine milieu may be unfavorable for the clonal expansion of memory CD8 T cells during infection with Plasmodium species. Finally, the intrinsic differences in naive and memory CD8 T cells might play a role. Both naive- and memory-derived OT-I cells expressed inhibitory receptors PD-1, LAG-3, and CD160 during infection with Plasmodium. Importantly, the levels of PD-1 and LAG-3 expression were higher in memory-derived OT-I cells when compared with naive-derived OT-I cells, consistent with their reduced proliferative responses. These results suggest that memory CD8 T cells are more likely to become exhausted during Plasmodium infection than naive CD8 T cells (27, 37). This defect is not inherent to Plasmodium-specific CD8 memory T cells and might be linked to the poor recall responses of memory CD8 T cells to various pathogens during malaria infection. The precise mechanisms underlying the defect remain to be understood. It would be interesting to examine the correlation between environmental factors and T cell-intrinsic factors leading to the regulation of CD8 T cell responses during infection with malaria parasites.
Our study showed that memory CD8+ T cells can be established postinfection of malaria parasites, but their secondary recall responses are limited during reinfection. Accumulating evidence suggests pathogenic roles for CD8+ T cells during the blood stage of malaria infection. It is therefore possible that memory CD8+ T cells specific for blood-stage malaria Ags are unfavorable for the host. In this regard, understanding the mechanisms underlying the generation, maintenance, and recall responses of malaria-specific CD8+ T cells is important to better understand their pathogenesis. In addition, these studies provide models for understanding the general mechanism of T cell memory during malaria infection. Memory CD4+ T cells play critical roles in the protective immune response against malaria blood-stage infection. CD4+ and CD8+ T cell responses are modulated during blood-stage infection with malaria parasites (2, 27). In our experimental system, the clonal expansion of OVA-specific OT-II CD4+ T cells was poor postinfection with PBA-OVA and LM-OVA, consistent with the previous study, and we were unable to examine the response of OVA-specific CD4+ T cells during infection (Supplemental Fig. 1) (38).

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Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure 1

The response of OT-II CD4+ T cells during infection with PbA-OVA and LM-OVA.

(A) B6 mice (CD45.2+) were adoptively transferred with CFSE-labeled 1x10^6 cells each of a 1:1 mixture of naive OT-I cells (CD45.1+/2+) and CD4+ cells from OT-II mice (CD45.1+/2-). Six days after infection with PbA-OVA (parasitemia 4.6%) or 4 days after infection with LM-OVA, spleen cells were stained for CD8, CD45.1 and CD45.2. OT-I and OT-II cells were distinguished by the expression of CD45.1/2.

(B) B6 mice were adoptively transferred with 1x10^6 cells each of a 1:1 mixture of memory OT-II cells (CD45.1+) generated in vitro and CD4+ T cells from DsRed/OT-II mice (DsRed+CD45.1+). Seven days after infection with PbA-OVA (n=4, parasitemia 13.9 ± 6.3%) or 5 days after infection with LM-OVA, cells isolated from spleen, inguinal lymph nodes (iLN) and blood were stained for CD8 and CD45.1.

The fold increase in naive (●) or memory (○) OT-II cells in mice infected with PbA-OVA or LM-OVA in relationship to the mean number of OT-II cells in uninfected mice was calculated for each organ. The values for naive- and memory-derived OT-II cells in the same mice are connected with lines.