Cutting Edge: Attrition of Plasmodium-Specific Memory CD8 T Cells Results in Decreased Protection That Is Rescued by Booster Immunization

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Sterile sporozoites require high numbers of memory CD8 T cells. However, infections with unrelated pathogens, as may occur in areas endemic to malaria, can dramatically decrease pre-existing memory CD8 T cells. It remains unknown whether unrelated infections will compromise numbers of Plasmodium-specific memory CD8 T cells and thus limit the duration of antimalarial immunity generated by subunit vaccination. We show that P. berghei circumsporozoite-specific memory CD8 T cells underwent significant attrition in numbers in mice subjected to unrelated infections. Attrition was associated with preferential loss of effector memory CD8 T cells and reduced immunity to P. berghei sporozoite challenge. However, and of relevance to deployment of Plasmodium vaccines in areas endemic to malaria, attrition of memory CD8 T cells was reversed by booster immunization, which restored protection. These data suggest that regular booster immunizations may be required to sustain protective vaccine-induced Plasmodium-specific memory CD8 T cells in the face of attrition caused by unrelated infections. The Journal of Immunology, 2011, 186: 3836–3840.

Malaria-causing Plasmodium species create an enormous global health burden, resulting in ~250 million infections and 900,000 deaths in 2008 (1). The mammalian life cycle of Plasmodium begins when infected mosquitoes deposit sporozoites into the dermal tissue, which then travel to the liver (2), differentiate in infected hepatocytes, and release merozoites that initiate RBC infection and malarial disease (3, 4). Both radiation-attenuated sporozoites (RAS) (5) and subunit vaccines (6) provide some protection of humans against sporozoite challenge; however, achieving long-term sterilizing immunity remains difficult. Importantly, protection mediated by these vaccines is CD8 T cell dependent in rodent and primate models of malaria (6, 7), although this has not been definitively proven in humans. However, CD4 T cells, Abs, and NK cells also contribute to protective immunity in rodents following RAS vaccination (7–13).

Memory CD8 T cells can remain stable in numbers for essentially the life of laboratory mice (14). However, memory CD8 T cells undergo attrition in frequency among total CD8 T cells and in some cases total numbers when mice are infected with unrelated pathogens or exposed to inflammation (15–22). One study showed that Ag-specific CD8 T cell numbers can increase dramatically upon repeated infection of mice, which resulted in a modest (~33%) but statistically significant decrease in pre-existing memory CD8 T cell numbers (19). This finding suggested that it may be possible to generate large CD8 T cell responses to protect against highly successful pathogens, without negatively impacting pre-existing memory CD8 T cell protection against other pathogens. However, a recent study showed that attrition of infection-induced memory CD8 T cells compromised immunity to a very high dose, but not low dose, challenge with Listeria monocytogenes (21). Furthermore, in scenarios in which large numbers of memory CD8 T cells are required for protection, as is the case for resistance to Plasmodium infection (23, 24), attrition of vaccine-induced memory CD8 T cells may compromise protection. In this article we address the impact of subsequent infections on the maintenance of pre-existing Plasmodium-specific memory CD8 T cell numbers, subset composition, protective capacity, and ability to respond to booster immunizations.

Materials and Methods

Mice, immunizations, and infections

BALB/c mice (National Cancer Institute, Frederick, MD) were housed at the University of Iowa. Mice were primed with 0.4–1 × 10⁶ dendritic cells (DCs) coated with circumsporozoite protein (CS)252–260 (DC-CS) i.v. and boosted 7 d later with 1 × 10⁶ dendritic cells (DCs) expressing CS252–260 (L. monocytogenes-CS) i.v., as described (23). Naive and (DC-CS + L. monocytogenes-CS) mice were infected with 2 × 10⁶ PFU lymphocytic choriomeningitis virus (LCMV) Armstrong i.p., 5 × 10⁶ actA-deficient L. monocytogenes (DP-L1942) (LM) i.v., and 1 × 10⁶ PFU vaccinia virus Western Reserve (VacV) i.p., and 2 × 10⁶ PFU mouse hepatitis virus-1 (MHV-1) i.p. Memory CS252-specific CD8 T cells were boosted with 2 ×...
FIGURE 1. CS252-specific CD8 T cells undergo attrition in multiple tissues. A, Experimental design. B, Frequency of CS252-specific T cells among all CD8 T cells as detected by intracellular cytokine staining (ICS) for IFN-γ. C, Total number of CS252-specific CD8 T cells per spleen. D, Percentage of PBLs that are CS252-specific CD8 T cells. E, Total number of CS252-specific CD8 T cells per liver. B–E, Cumulative data (mean ± SEM) from six mice from two experiments. Data were analyzed by unpaired two-tailed t test.

Quantification and phenotypic analysis of CS252-specific CD8 T cells

Spleens were disrupted into single-cell suspensions. Livers were perfused with cold HBSS through the hepatic portal vein and made into single-cell suspensions. Liver mononuclear cells were collected by centrifugation in 35% Percoll/HBSS. Spleen and liver mononuclear cells were treated with Trypsin–ammonium chloride to lyse RBCs. PBLs were obtained by treating blood with Tris–ammonium chloride.

Spleen CS252-specific CD8 T cells were identified by intracellular cytokine stain for IFN-γ after a 5-h incubation in brefeldin A (Biolgend, San Diego, CA), in the presence or absence of 200 nM CS252–260 peptide. P815 APCs were added to the stimulation to identify CS252-specific CD8 T cells in PBLs and livers. After stimulation, cells were fixed, permeabilized, and stained for intracellular IFN-γ, TNF, and IL-2 as recommended by the manufacturer (BD Biosciences). Cell surface expression of CD62L was detected by incubating cells with 0.1 mM TAPI-2 (Peptides International, Louisville, KY) prior to and during stimulation with CS252–260 peptide.

Abs

The following Abs from eBioscience (San Diego, CA) were used: CD4–allophycocyanin (RM4-5), CD8–FITC/PE/PE-Cy7 (53–6.7), CD11a–FITC (M17/4), CD27–PE (LC.7A9), CD127–allophycocyanin (A7R34), KLRG1–allophycocyanin (2F1), Golden Syrian Hamster IgG–allophycocyanin, IFN-γ–allophycocyanin/PE-Cy7 (XMGL1.2), TNF–allophycocyanin (MP6-XT22), IL-2–PE (JES6-5H4), rat IgG2a–PE/allophycocyanin (eBR2a). The following Ab from BD Pharmingen was used: CD62L–PE (MEL-14).

Plasmodium berghei ANKA clone 234 sporozoites were isolated from the salivary glands of infected Anopheles stephensi mosquitoes. Mice were challenged with 1000 P. berghei sporozoites i.v. Parasitized RBCs were identified by Giemsa stain 10 d postchallenge. Protection is defined as the absence of blood-stage parasites. At least 10 fields were examined for each mouse designated as protected.

Statistical analysis

Data were analyzed using Prism4 software.

Results and Discussion

Memory CS252-specific CD8 T cells decrease in frequency and total numbers following infection with unrelated pathogens

To address the impact of unrelated infections on maintenance and protection by epitope-specific vaccine-induced memory CD8 T cells, we employed a quantitative model of sterilizing immunity to Plasmodium sporozoite infection (23). BALB/c mice were immunized with DC-CS and boosted 1 wk later with attenuated L. monocytogenes-CS. This vaccination approach provides essentially life-long protection to BALB/c mice in a CD8 T cell-dependent manner against repeated challenge with P. berghei sporozoites (23, 26). Following the L. monocytogenes-CS boost, mice were left alone or infected at 3-wk intervals with LCMV, L. monocytogenes that do not express the CS252–260 peptide, VacV, and finally MHV-1 (Fig. 1A). Controls were age-matched naive mice, and mice that were infected only with LCMV, L. monocytogenes, VacV, and MHV-1 to control for any nonspecific resistance against sporozoite challenge. T cell analyses and Plasmodium challenge infections were undertaken ∼6 wk after the last unrelated infection (Fig. 1A). DC-CS + L. monocytogenes-CS immunization and infection with multiple pathogens had no impact on the frequency of CD4 T cells among the PBLs, compared with naive mice (Supplemental Fig. 1A, 1B). However, the frequency of CD8 T cells in the PBLs was higher in mice infected with multiple pathogens than in naive mice (Supplemental Fig. 1A, 1C). The increase in circulating CD8 T cells tracked with increased frequency of Ag-experienced CD8 T cells (Supplemental Fig. 1A, 1D, 1E), which were detected by downregulation of CD8α and upregulation of CD11a (27). Consistent with a previous report (19), these results show that as the number of infections in-
FIGURE 3. Attrition of CS252-specific CD8 T cells results in decreased protection against P. berghei sporozoite challenge. Mice were treated as shown in Fig. 1A. Percentage of PBLs that are CS252-specific CD8 T cells as detected by ICS for IFN-γ. Data (mean ± SD) are from 10 mice per group. Data were analyzed by unpaired two-tailed t test. Mice were challenged with 1000 P. berghei sporozoites. Numbers to right of bar graph represent the percent of mice protected against the development of blood-stage parasitemia (number protected/number challenged). Data are representative of two experiments. Protection results were analyzed by Fisher’s exact test.

Increased, so did the frequency of total CD8 T cells and Ag-experienced CD8 T cells among the PBLs.

In contrast to total CD8 T cell numbers, repeated infections correlate with decreased frequencies of infection-induced pre-existing memory CD8 T cells (19). To address this issue for epitope-specific vaccine-induced CD8 T cell responses, we analyzed memory CS252-specific CD8 T cells in the spleen, blood, and liver of control immune mice and those subjected to unrelated infection. Consistent with previous reports that demonstrate varying degrees of attrition (15–22), we observed a substantial decrease in the frequency of CD8 T cells that were CS252 specific in multiple tissues of DC-CS + L. monocytogenes-CS-immunized mice subsequently infected with multiple pathogens (Supplemental Fig. 1B, 1F). Importantly, we also observed decreases in the total number of memory CS252-specific CD8 T cells in the spleen and liver and frequency of total PBLs in DC-CS + L. monocytogenes-CS-immunized mice subjected to unrelated infections (Fig. 1C–E). These data demonstrate that pre-existing epitope-specific vaccine-induced memory CD8 T cells undergo substantial attrition in total cell numbers in multiple tissues after encounter with unrelated pathogens.

Multiple infections change the subset composition of memory CD8 T cells

Previous work has suggested that effector memory CD8 T cells (Tem, CD62L⁺, CD27low, IL-2⁻), in contrast to central memory CD8 T cells (Tcm, CD62L⁺, CD27hi, IL-2⁺), are critical for immunity to liver-stage Plasmodium infection (24). However, it is unclear how attrition impacts the composition of pre-existing memory CD8 T cell populations. L. monocytogenes-specific memory CD8 T cells that had or had not undergone attrition after modified vaccinia virus Ankara infection exhibited no differences in CD62L, CD127, IL-2, or TNF production (21). To address this issue for epitope-specific vaccine-induced memory CD8 T cells, we analyzed the phenotype and polyfunctionality of IFN-γ-producing memory CS252-specific CD8 T cells that had or had not undergone infection-induced attrition. Attrition had no impact on the frequency of memory CS252-specific CD8 T cells that were CD127⁺ or TNF⁺ (Fig. 2A, Supplemental Fig. 1G–I). Conversely, attrition increased the percentage of memory CS252-specific CD8 T cell populations that were CD27hi, CD62L⁺, and IL-2⁺ [canonical Tcm markers (28)] (Fig. 2A, Supplemental Fig. 1G–I). In addition, attrition decreased the percentage of memory CS252-specific CD8 T cells that were KLRG1⁺ (Fig. 2A, Supplemental Fig. 1G–I). These results show that attrition can decrease the fraction of pre-existing memory CD8 T cells that are Tem.

Although reproducible, the attrition-induced decrease in the percentage of CS252-specific Tem was relatively modest. However, coupling the changes in percentage of Tem with the attrition-induced decrease in total CS252-specific memory CD8 T cells revealed a preferential loss of the Tem subset. Specifically, we observed that Tem (CD62L⁺) CS252-specific CD8 T cells decreased in number approximately twice as much as Tcm (CD62L⁺) CS252-specific CD8 T cells in the spleen (4.3-fold versus 2.3-fold reduction) and liver (5.6-fold versus 2.8-fold reduction) (Supplemental Fig. 1B and Fig. 2B, respectively). This change in subset composition is likely to have functional consequences because Tem and Tcm CD8 T cells provide different levels of protection for specific pathogens. For example, Tem CD8 T cells may be more effective at providing protection against Plasmodium sporozoite challenge than are Tcm CD8 T cells (24, 29). In contrast, Tcm CD8 T cells provide increased protection against infection with LCMV clone 13 and vaccinia virus (28). Thus, the combined impact of infection-induced attrition in memory CD8 T cell numbers and memory subsets on protective immunity may be pathogen dependent, with serious consequences for infections like Plasmodium, for which protection may be tightly linked to high numbers of Tem CD8 T cells. Furthermore, future studies should determine if the
characteristics of the CD8 T cells (i.e., ratios of Tem and Tcm) induced using specific vaccine delivery vectors and prime-boost regimens could also influence the impact of attrition on vaccine-induced memory CD8 T cell numbers and protective immunity.

**Attrition of memory CS252-specific CD8 T cells results in decreased protection against Plasmodium sporozoites that can be rescued by booster immunization**

We next determined if multiple unrelated infections decrease CS252-specific CD8 T cell-mediated protection against sporozoite challenge. To address the degree of attrition in the same mice used for challenge studies, we quantified the fraction of PBLs that were memory CS252-specific CD8 T cells. Consistent with our previous analysis (Fig. 1D), there was a decrease in the percentage of PBLs that were memory CS252-specific CD8 T cells in DC-CS + L. monocytogenes-CS–immunized mice infected with multiple pathogens, compared with DC-CS + L. monocytogenes-CS–immunized mice (Fig. 3). We then challenged those same mice and age-matched naïve mice with a stringent dose of 1000 P. berghei sporozoites whereby 100% of the naïve mice developed blood-stage parasitemia (Fig. 3). Consistent with our previous results (23), a high proportion (70%) of DC-CS + L. monocytogenes-CS–immunized mice were protected (Fig. 3). However, only 10% of DC-CS + L. monocytogenes-CS–immunized mice infected with multiple pathogens were protected. Finally, 100% of the LCMV + L. monocytogenes + VacV + MHV-1–infected mice that did not contain CS-specific memory CD8 T cells also developed blood-stage parasitemia (Fig. 3), showing that the unrelated infections do not induce nonspecific resistance to P. berghei sporozoite infection. Thus, infection-induced attrition and/or phenotypic changes of pre-existing Plasmodium-specific memory CD8 T cells profoundly decrease protection against Plasmodium sporozoite challenge.

A well-defined characteristic of memory CD8 T cells is their ability to expand in numbers upon Ag re-encounter and provide increased protection (30). However, it is not known whether attrition impacts these attributes of memory CD8 T cells. To determine if booster immunization could rescue CD8 T cell numbers and protection, we immunized a cohort of mice with DC-CS + L. monocytogenes-CS and subsequently infected a subset of animals with LCMV and MHV-1 to induce attrition (Fig. 4A). We chose to use only these two pathogens because single infections with LCMV and MHV-1 induced the greatest attrition of CS252-specific memory CD8 T cells (data not shown). CS252-specific CD8 T cells were reduced in the blood, spleen, and liver (Fig. 4B, 4C; Supplemental Fig. 2A) in mice subjected to unrelated infections. In addition to decreases in cell numbers, CS252-specific CD8 T cells in mice infected with LCMV and MHV-1 also exhibited changes in the expression of CD27, CD62L, and KLRG1 and production of IL-2 (Supplemental Fig. 2B), consistent with previous results (Fig. 2). Finally, DC-CS + L. monocytogenes-CS–immunized mice that had undergone infection-induced attrition were less resistant to P. berghei sporozoite challenge (Fig. 4D). Booster immunization with L. monocytogenes-CS, however, induced substantial expansion of memory CS252-specific CD8 T cells that had undergone attrition (Fig. 4B, 4C, Supplemental Fig. 2A). In addition, phenotypic and functional analyses revealed that the resulting memory CS252-specific CD8 T cells in the boosted mice exhibited a Tem phenotype (CD62L−, CD27lo, IL-2−) (Supplemental Fig. 2B). Strikingly, booster immunizations completely rescued protection against P. berghei sporozoite challenge (Fig. 4D). These data demonstrate that a population of memory CS252-specific CD8 T cells that has undergone attrition retains its capacity to be boosted in numbers, which in turn rescues protection against sporozoite challenge. It is also possible that immunization to generate extremely large memory CS252-specific CD8 T cells, perhaps by additional booster immunizations, would ameliorate the impact of attrition due to unrelated infection and preserve long-term protection from Plasmodium challenge. Vaccination with RAS has long been viewed as the “gold” standard in malaria vaccine development. RAS vaccination elicits a multifactorial immune response, including CD8 T cells, CD4 T cells, Abs, and NK cells that contribute to protective immunity (7–13). Of note, it has been shown in both rodent and nonhuman primates that protection is CD8 T cell dependent (6, 7). It will be of great interest to determine if RAS-induced protective immunity also decreases following infection with multiple pathogens. However, these experiments are complicated by the fact that, owing to the paucity of defined epitopes, RAS-induced CD8 T cells can be tracked using only a surrogate activation marker approach (24), which will not be informative once mice are subsequently infected with different pathogens that will induce T cell populations specific for the unrelated pathogens but exhibiting the same activation markers as the Plasmodium-specific T cells (27).

Given that multiple pathogens are endemic to the same regions of the world as plasmodia, it is likely that infection-induced attrition of vaccine-generated Plasmodium-specific memory CD8 T cells will be an important issue to overcome for long-term protection in areas endemic to malaria. We demonstrate that Plasmodium-specific memory CD8 T cells that have undergone infection-induced attrition retain their capacity to respond to booster immunizations, which rescues protection against challenge with Plasmodium sporozoites. Therefore, booster immunizations provided at determined intervals will likely be required to maintain the large number of memory CD8 T cells required for protection against Plasmodium sporozoites.

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**Disclosures**

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**References**


