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 protection against infection with Plasmodium sporozoites requires 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) promise 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 0.4−1×10^6 dendritic cells (DCs) coated with circumsporozoite protein (CS)252–260 (DC-CS) i.v. and boosted 7 d later with 1×10^6 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 2×10^6 PFU lymphocytic choriomeningitis virus (LCMV) Armstrong i.p., 5×10^6 actA-deficient L. monocytogenes (DP-L1942) (LM) i.v., 1×10^6 PFU vaccinia virus Western Reserve (VacV) i.p., and 2×10^5 PFU mouse hepatitis virus-1 (MHV-1) i.p. Memory CS252-specific CD8 T cells were boosted with 2×...
Study of immune responses to sporozoite challenge of P. berghei in mice infected with multiple unrelated pathogens. BALB/c mice were given DC-CS + LM-CS + VacV + MHV-1 inoculation to induce memory CS252-specific CD8 T cells. These mice were infected with L. monocytogenes-3 depending on the experimental design and left alone or infected with additional pathogens. The resulting data are presented as means ± SEM from six mice per group.

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

Increases in total CD8 T cell numbers after 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, CD62Llo, IL-2–), in contrast to central memory CD8 T cells (Tcm, CD62Lhi, 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–J). Conversely, attrition increased the percentage of memory CS252-specific CD8 T cell populations that were CD27hi, CD62Lhi, and IL-2+ [canonical Tcm markers (28)] (Fig. 2A, Supplemental Fig. 1G–J). In addition, attrition decreased the percentage of memory CS252-specific CD8 T cells that were KLRG1+ (Fig. 2A, Supplemental Fig. 1G–J). 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 (CD62Llo) CS252-specific CD8 T cells decreased in number approximately twice as much as Tcm (CD62Lhi) 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. 1F 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 (Fig. 3). 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<sup>+</sup>, CD27<sup>lo</sup>, IL-2<sup>+</sup>) (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

The authors have no financial conflicts of interest.

References


SUPPLEMENTAL FIGURE 1. Infection of mice with multiple pathogens increases the frequency of CD8 T cells in the blood while attrition and alterations in the phenotype and subset composition of CS₂₅₂⁻ specific CD8 T cells in multiple tissues. Mice were treated and analyzed as shown in Fig. 1A. A, Representative plots depicting the frequency of PBL that are CD4⁺, CD8⁺, and CD8⁺CD₁₁ᵃ⁻. Number in parentheses in the lower panels is the percent of CD8⁺ cells that are CD₈⁺CD₁₁ᵃ⁻. Cumulative data (mean±S.E.M.) are from 18-20 mice/group in two experiments. Frequency of PBL that are CD4⁺ (B), CD8⁺ (C), or CD8⁺CD₁₁ᵃ⁻ (D). E, Frequency of CD8⁺ cells that exhibit the CD₈⁺CD₁₁ᵃ⁻ activated phenotype. Data were analyzed by the Kruskal-Wallis One-way ANOVA test followed by the Dunn’s Multiple Comparison test to compare the indicated group to the naïve group. F, Representative plots showing the frequency of CS₂₅₂⁻ specific of all CD8 T cells as detected by intracellular cytokine staining (ICS) for IFN-γ. The number in parentheses for the blood is the frequency of PBL that are CS₂₅₂⁻ specific. G, Representative plots showing phenotype of CS₂₅₂⁻ specific CD8 T cells. Number represents percent of CS₂₅₂⁻ specific CD8 T cells that are marker positive. Cumulative results showing percent of CS₂₅₂⁻ specific CD8 T cells that are marker positive in the spleen (H) and blood (I). H and I, Cumulative data (mean±S.E.M.) are from 6 mice from two experiments. Data were analyzed by unpaired two-tailed t-test. J, Number of CD62L⁻ and CD62L⁺ CS₂₅₂⁻ specific CD8 T cells in the spleen. Cumulative data (mean±S.E.M.) are from 6 mice from two experiments. The number to the right of each group represents the fold difference between the means. Data were analyzed by unpaired two-tailed t-test.
SUPPLEMENTAL FIGURE 2. Booster immunization increases memory CS\textsubscript{252}-specific CD8 T cell numbers and alters memory phenotype. A, Percent of PBL that are CS\textsubscript{252}-specific CD8 T cells determined by ICS for IFN-γ. Data (mean±S.D.) are from 10 mice per group. B, Percent of spleen CS\textsubscript{252}-specific CD8 T cells that are marker positive. Cumulative data (mean±S.E.M.) are from 5 mice from two experiments. Data were analyzed by unpaired two-tailed t-test.