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Attrition of *Plasmodium*-specific memory CD8 T cells results in decreased protection that is rescued by booster immunization¹

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Abstract

Sterile protection against infection with *Plasmodium* sporozoites requires high numbers of memory CD8 T-cells. However, infections with unrelated pathogens, as may occur in malaria endemic areas, 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 anti-malarial 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 malaria endemic areas, 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.

Introduction

Malaria causing *Plasmodium* species exert 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 red blood cell infection and malaria 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 definitely proven in humans. However, CD4 T cells, antibodies, 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 amongst 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 antigen-specific CD8 T cell

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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 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 to 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* (LM) (21). Furthermore, in scenarios where 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. Here we addressed 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 (NCI, Frederick, MD) were housed at the University of Iowa. Mice were primed with $0.4-1 \times 10^6$ dendritic cells (DC) coated with circumsporozoite protein (CS)₂₅₂₋₂₆₀ (DC-CS) i.v. and boosted 7 days later with 1×10^7 recombinant *actA*-*inlB*-deficient LM expressing CS₂₅₂₋₂₆₀ (LM-CS) i.v., as described (23). Naïve and (DC-CS +LM-CS) mice were infected with 2×10^5 pfu lymphocytic choriomeningitis virus (LCMV) Armstrong i.p., 5×10^6 *actA*-deficient LM (DP-L1942) (LM) i.v., 1×10^4 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 2×10^7 LM-CS i.v. Mouse experiments were approved by the IACUC at the University of Iowa.

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 Tris-ammonium chloride to lyse red blood cells. PBL 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 hour incubation in brefeldin A (Biolegend, San Diego, CA), in the presence or absence of 200 nM CS₂₅₂₋₂₆₀ peptide. P815 antigen-presenting cells were added to the stimulation to identify CS₂₅₂-specific CD8 T cells in PBL 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 Inc., Louisville, KY) for 30 minutes prior to and during stimulation with CS₂₅₂₋₂₆₀ peptide (25).

Antibodies

The following antibodies were used from eBioscience (San Diego, CA) CD4-APC (RM4-5), CD8-FITC/PE/PE-Cy7 (53-6.7), CD11a-FITC (M17/4), CD27-PE (LG.7F9), CD127-APC (A7R34), KLRG1-APC (2F1), Golden Syrian Hamster IgG-APC, IFN- γ -APC/PE-Cy7 (XMG1.2), TNF-APC (MP6-XT22), IL-2-PE (JES6-5H4), rat IgG2a-PE/APC (eBR2a). The following antibodies were used from BD Pharmingen CD62L-PE (MEL-14).

Sporozoite challenge

P. berghei (*Pb*) ANKA clone 234 sporozoites were isolated from the salivary glands of infected *Anopheles stephensi* mosquitoes. Mice were challenged with 1000 *Pb* sporozoites i.v. Parasitized red blood cells were identified by Giemsa stain 10 days post challenge. 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 coated with CS₂₅₂₋₂₆₀ (DC-CS) and boosted one week later with attenuated *L. monocytogenes* (LM) expressing the CS₂₅₂₋₂₆₀ epitope (LM-CS). This vaccination approach provides essentially life-long protection to BALB/c mice in a CD8 T cell-dependent manner against repeated challenge with *Pb* sporozoites (23,26). Following the LM-CS boost mice were left alone or infected at three week intervals with; LCMV, LM that do not express the CS₂₅₂₋₂₆₀ peptide, VacV, and finally MHV-1 (Fig. 1A). Controls were age matched naïve mice and mice were only infected with LCMV, LM, VacV, and MHV-1 to control for any non-specific resistance against sporozoite challenge. T cell analyses and *Plasmodium* challenge infections were undertaken ~6 weeks after the last unrelated infection (Fig. 1A). DC-CS+LM-CS immunization and infection with multiple pathogens had no impact on the frequency of CD4 T cells amongst the PBL compared to naïve mice (Supplemental Fig. 1A and B). However, the frequency of CD8 T cells in the PBL was higher in mice infected with multiple pathogens compared to naïve mice (Supplemental Fig. 1A and C). The increase in circulating CD8 T cells tracked with increased frequency of antigen-experienced CD8 T cells (Supplemental Fig. 1A, D, and E), which were detected by down-regulation of CD8 α and up-regulation of CD11a (27). Consistent with a previous report (19), these results show that as the number of infections increased so did the frequency of total CD8 T cells and antigen-experienced CD8 T cells amongst the PBL.

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+LM-CS immunized mice subsequently infected with multiple pathogens (Supplemental Fig. 1F and Fig. 1B). 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 PBL in DC-CS+LM-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⁻, CD27^{lo}, IL-2⁻), in contrast to central memory CD8 T cells (Tcm, CD62L⁺, CD27^{hi}, 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. LM-specific memory CD8 T cells that had or had not undergone attrition in response to modified vaccinia virus Ankara 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 and Supplemental Fig. 1G-I). Conversely, attrition increased the percent of memory CS₂₅₂-specific CD8 T cell populations that were CD27^{hi}, CD62L⁺, and IL-2⁺ (canonical Tcm markers (28)) (Fig. 2A and Supplemental Fig. 1G-I). Additionally, attrition decreased the percent of memory CS₂₅₂-specific CD8 T cells that were KLRG1⁺ (Fig. 2A and 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 percent CS₂₅₂-specific Tem was relatively modest. However, coupling the changes in percent 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 numbers 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. 1J and Fig. 2B respectively). This change in subset composition is likely to have functional consequences since 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 Tcm CD8 T cells (24, 29). On the other hand, 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*, where protection may be tightly linked to 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 PBL that were memory CS₂₅₂-specific CD8 T cells. Consistent with our previous analysis (Fig. 1D), there was a decrease in the percent of PBL that were memory CS₂₅₂-specific CD8 T cells in DC-CS+LM-CS immunized mice infected with multiple pathogens compared to DC-CS+LM-CS immunized mice (Fig. 3). We then challenged those same mice and age-matched naïve mice with a stringent dose of 1000 *Pb* sporozoites where 100% of the naïve mice developed blood stage parasitemia (Fig. 3). Consistent with our previous results (23), a high proportion (70%) of DC-CS+LM-CS immunized mice were protected (Fig. 3). However, only 10% of DC-CS+LM-CS immunized mice infected with multiple pathogens were protected (Fig. 3). Finally, 100% of the LCMV+LM+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 non-specific resistance to *Pb* 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 antigen re-encounter and provide increased protection (30). However, it is not known if 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+LM-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 (Supplemental Fig. 2A and Fig. 4B–C) 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+LM-CS immunized mice that had undergone infection-induced attrition were less resistant to *Pb* sporozoite challenge (Fig. 4D). However, booster immunization with LM-CS induced substantial expansion of memory CS₂₅₂-specific CD8 T cells that had undergone attrition (Supplemental Fig. 2A and Fig. 4B–C). Additionally, phenotypic and functional analyses revealed that the resulting memory CS₂₅₂-specific CD8 T cells in the boosted mice exhibited a Tem phenotype (CD62L[−], CD27^{lo}, IL-2[−]) (Supplemental Fig. 2B). Strikingly, booster immunizations completely rescued protection against *Pb* sporozoites 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 infections 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 multi-factorial immune response including CD8 T cells, CD4 T cells, antibodies, and NK cells that contribute to protective immunity (7–13). Of note, it has been shown in both rodent and non-human 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 fact that, due to the paucity of defined epitopes, RAS-induced CD8 T cells can only be tracked using 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 malaria endemic areas. We demonstrate *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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

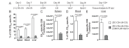
DC	dendritic cells
CS	circumsporozoite protein
LM	<i>Listeria monocytogenes</i>
LCMV	lymphocytic choriomeningitis virus
VacV	vaccinia virus-western reserve
MHV-1	mouse hepatitis virus-1
<i>Pb</i>	<i>P. berghei</i>

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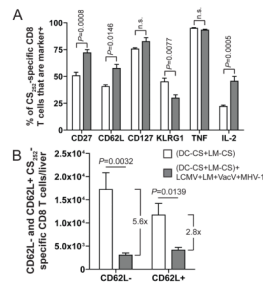
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**FIGURE 1.**

CS₂₅₂-specific CD8 T cells undergo attrition in multiple tissues. *A*, Experimental design. *B*, Frequency of CS₂₅₂-specific of 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*, Percent of PBL that are CS₂₅₂-specific CD8 T cells. *E*, Total number of CS₂₅₂-specific CD8 T cells per liver. *B–E*, Cumulative data (mean \pm S.E.M.) from 6 mice from two experiments. Data were analyzed by unpaired two-tailed t-test.

**FIGURE 2.**

Infection with multiple pathogens alters the phenotype and subset composition of memory CS₂₅₂-specific CD8 T cells. Mice were treated as shown in Fig. 1A. *A*, Percent of CS₂₅₂-specific CD8 T cells as detected ICS for IFN- γ that are marker positive in the liver. *B*, Number of CD62L negative and CD62L positive CS₂₅₂-specific CD8 T cells in the liver. The number to the right of each group represents the fold difference between the means. *A* and *B*, Data (mean \pm S.E.M.) from 6 mice from two experiments. Data were analyzed by unpaired two-tailed t-test.

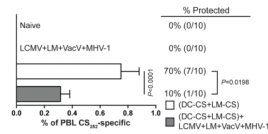
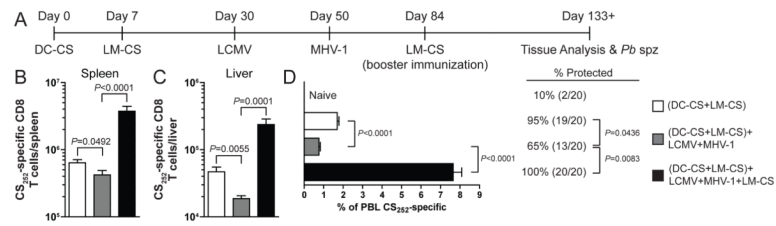


FIGURE 3.

Attrition of CS₂₅₂-specific CD8 T cells results in decreased protection against *P. berghei* sporozoite challenge. Mice were treated as shown in Fig. 1A. Percent of PBL that are CS₂₅₂-specific CD8 T cells as detected ICS for IFN- γ . Data (mean \pm S.D.) are from 10 mice per group. Data were analyzed by unpaired two-tailed t-test. Mice were challenged with 1000 *Pb* sporozoites. Numbers to right of bar graph represents 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.

**FIGURE 4.**

Booster immunization increases memory CS₂₅₂-specific CD8 T cell numbers and rescues protection against *P. berghei* sporozoite challenge. **A**, Experimental design. **B**, Total number of CS₂₅₂-specific CD8 T cells per spleen as detected ICS for IFN- γ . **C**, Total number of CS₂₅₂-specific CD8 T cells per liver. **B** and **C**, Cumulative data (mean \pm S.E.M.) from 5–8 mice from three experiments. **D**, Percent of PBL that are CS₂₅₂-specific CD8 T cells. Data (mean \pm S.E.M.) are from 20 mice per group from two experiments. **B–D**, Data was analyzed by unpaired two-tailed t-test. Mice were challenged with 1000 *Pb* sporozoites. Numbers to right of bar graph represents the percent of mice protected against the development of blood stage parasitemia (number protected/number challenged). Protection results were analyzed by Fisher's exact test.