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CD8 T- cell immunity to *Plasmodium* permits generation of protective antibodies after repeated sporozoite challenge

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Abstract

Individuals living in malaria endemic areas are subject to repeated infections yet fail to develop sterilizing immunity, however, immunization of mice with attenuated sporozoites or sub-unit vaccines has shown the ability to protect mice against a sporozoite challenge. We recently reported that mice primed with dendritic cells coated with the dominant circumsporozoite epitope from *Plasmodium berghei* followed by a boost with recombinant *Listeria monocytogenes* expressing the same epitope exhibited sterile immunity against a sporozoite challenge for more than one year. In this report we show those mice do not contain protective antibodies and that depletion of CD4 T cells in the immunize mice did not affect sterile immunity. In contrast, CD8 T cell depletion eliminated protection. Thus, protective immunity generated by this immunization approach is entirely memory CD8 T cell dependent. We also show here that mice initially protected by circumsporozoite-specific memory CD8 T cells develop sterilizing sporozoite-specific antibodies after repeated asymptomatic challenges with physiologic numbers of viable sporozoites. Therefore, initial protection by a CD8 T cell-targeted liver stage subunit vaccine allows the generation of enhanced sterilizing immune responses from repeated exposure to *Plasmodium* parasites.

Keywords

Plasmodium berghei; memory CD8 T cells; antibodies

1. Introduction

Plasmodium infections are a global health crisis with 300–500 million new cases of malaria each year and the death of about 1 million people annually[1]. Whereas, individuals living in malaria endemic regions develop resistance to severe malaria disease with repeated exposure to the parasite, blood stage parasitemia continues to occur. Thus, multiple infections with *Plasmodium* do not elicit durable sterilizing immune responses, perhaps due to the severe immune dysregulation associated with this parasite infection. However, clear evidence exists that both rodents and humans can be protected from *Plasmodium* infection by vaccination and much effort has been directed toward the development of an effective liver stage vaccine[2]

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that will prevent the parasite from developing into the symptomatic blood stage. Substantial effort has also been directed toward the development of an effective vaccine that targets the parasite during the blood stage of the life cycle[3]. To date the most efficacious immunization approach has been the use of radiation or genetically attenuated sporozoites[4–6]. Effectiveness of attenuated whole-parasite vaccines was attributed to the generation of a multifactorial immune response including antibodies and T cells, with CD8 T cells playing an essential role in the resistance observed in immunized mice[7]. Consistent with those results, it has been shown that sub-unit vaccines can elicit CD8 T cell responses that provide sterile immunity for up to several months[8–14]. These results underscore the ability of the host immune system to generate a protective immune response to *Plasmodium* under the appropriate conditions.

In a recent report we demonstrated the ability of BALB/c mice primed with dendritic cells (DC) coated with the circumsporozoite (CS)_{252–260} (SYIPSAEKI) epitope (DC-CS₂₅₂) from *P. berghei* and boosted a week later with recombinant *Listeria monocytogenes* expressing the same epitope (LM-CS₂₅₂) generated a memory CS₂₅₂-specific CD8 T cell response capable of providing long-term (> 1 year) sterile immunity to multiple *P. berghei* sporozoite challenges [15]. In this report we show that this immunization approach does not generate CS-specific CD4 T cells or antibodies that contribute to protective immunity. On the contrary, DC-CS₂₅₂+LM-CS₂₅₂ immune mice repeatedly challenged with low numbers of sporozoites developed sporozoite-specific antibodies that were conferred protective immunity when transferred to naïve BALB/c mice. These results underscore the ability of the immune system to develop a protective response under the appropriate conditions.

2. Materials and Methods

2.1 Mice and immunizations

BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Mice were housed at the University of Iowa animal care unit under the appropriate biosafety level. Mice were primed with 5×10^5 splenic DC coated with CS_{252–260} (DC-CS₂₅₂) and boosted seven days later with 2×10^7 recombinant *L. monocytogenes* expressing CS_{252–260} (LM-CS₂₅₂) as previously described[15].

2.2 Quantification of antigen-specific T-cells

The percentage of PBL that were CS₂₅₂-specific CD8 T-cells was determined by ICS for IFN- γ (clone XMG1.2) (eBioscience, San Diego, CA) after 5 hrs of incubation in brefeldin A (Biolegend, San Diego, CA) in the presence or absence of CS_{252–260} coated P815 (H-2^d) cells.

2.3 T-cell depletions

Immune mice were injected with 0.4 mg i.p. rat IgG, anti-CD4 (GK1.5), and/or anti-CD8 (2.43) antibodies on day -3 and day -1 prior to challenge with sporozoites. Depletion was verified by analyzing the CD8 and CD4 T-cell populations in the blood prior to challenge.

2.4 Serum adoptive transfers

Blood was collected from individual naïve mice or 44 days after immunized mice were challenged the fourth and fifth times with 1000 viable sporozoites and allowed to clot for 2 hours at room temperature. Serum was collected following centrifugation of blood samples at 14,000 rpm at 4 °C. Serum from individual naïve mice or immune mice previously challenged with sporozoites was pooled together. 100 μ l of serum from naïve or immunized mice was transferred to naïve recipient mice prior to sporozoite challenge.

2.5 Sporozoite-specific antibody titer

The serum sporozoite-specific antibody titer from repeatedly challenged mice was determined by the indirect fluorescent antibody test (IFAT). *P. berghei* sporozoites (~5000/well) were air dried over night on a multiwell microscope slide (Cel-Line Thermo Scientific, Waltham, MA). Wells were blocked with 1% BSA/PBS in a humidified chamber at room temperature. Naïve and immune serum was then added to wells and incubated in a humidified chamber overnight at 4°C. Wells were washed with PBS and sporozoite-specific antibodies were detected by incubating CyTM3-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 hour in a humidified chamber at room temperature. Antibody titer is defined as the recipricol of the lowest serum dilution where fluorescent sporozoites could be detected.

2.6 Sporozoite challenge

Plasmodium berghei (ANKA strain clone 234) sporozoites were isolated from the salivary glands of infected *A. stephensi* mosquitoes. Naïve and immunized mice were challenged with 1000 sporozoites i.v.

2.7 Identification of protected mice

Thin blood smears were performed 7 to 12 days after sporozoite challenge. Parasitized red blood cells were identified by Giemsa stain. Protection is defined as the absence of blood stage parasites. We observed at least 10 high-power (1000x) fields in protected mice.

3. Results

We recently demonstrated that BALB/c mice primed with DC-CS₂₅₂ from *P. berghei* and boosted a week later with recombinant LM-CS₂₅₂ generated a memory CS₂₅₂-specific CD8 T cell response capable of providing long-term sterile immunity to multiple *P. berghei* sporozoite challenges[15]. The DC-CS₂₅₂+LM-CS₂₅₂ immunization approach was designed to generate only a CS₂₅₂-specific CD8 T cell response, however, the possibility that CD4 T cells or antibodies were also involved in the protective response was not formally excluded. To address this issue BALB/c mice were primed with DC-CS₂₅₂ and boosted a week later with LM-CS₂₅₂. As reported previously[15], this immunization approach generated a memory CS₂₅₂-specific CD8 T cell response representing about 2% of all peripheral blood leukocytes (PBL) (Fig. 1A). To determine if CS-specific antibodies or CD4 T cells also contributed to protective immunity we depleted CD8 and/or CD4 T cells in groups of these immunized mice (Fig. 1B). Consistent with our previous report, DC-CS₂₅₂+LM-CS₂₅₂ immunized mice left untreated or treated with control rat IgG exhibited a high degree of sterilizing immunity, with 9/10 mice protected from sporozoite challenge (Fig. 1C). Depletion of CD8 T cells alone or in combination with depletion of CD4 T cells completely abrogated protection whereas depletion of CD4 T cells alone did not compromise protection (Fig. 1C). Furthermore, transfer of serum from DC-CS₂₅₂+LM-CS₂₅₂ immunized mice did not provide sterilizing immunity to naïve recipients (Fig. 1D). Together, these results show that DC-CS₂₅₂+ LM-CS₂₅₂ immunization does not elicit a CS-specific CD4 T cell or antibody response capable of protecting in the absence of CS-specific memory CD8 T cells.

DC-CS₂₅₂+LM-CS₂₅₂ immunization provides sterilizing immunity against sporozoite challenge for greater than one year after immunization[15]. Importantly these immune mice were also protected from three-four additional sporozoite challenges out to 565 days from the initial immunization[15]. Analysis of the CS₂₅₂-specific CD8 T cell response in multiply challenged mice demonstrated that a very large CS₂₅₂-specific CD8 T cell response comprising 2% to 6% of the PBL was still detectable at day 649 (Fig. 2A). Furthermore, these five mice were all protected against their fifth and sixth sporozoite challenges (Fig. 2A). We used a

relatively low, but physiologic challenge dose of 1000 sporozoites in those studies. Thus, it is formally possible that sporozoite-specific CD8 and CD4 T cells and antibodies were contributing to the protective immune response in the repeatedly challenged mice. To address this we depleted CD8 T cells from three of the previously protected immunized mice prior to their final sporozoite challenge. Interestingly, and in contrast to the mice given only DC-CS₂₅₂+LM-CS₂₅₂ (Fig. 1C), the repeatedly challenged immunized mice that had been depleted of CD8 T cells were now protected against the final sporozoite challenge (Fig. 2A). These data suggest that sporozoite-specific CD4 T cells and/or antibodies were primed in the repeatedly challenged mice and contributing to protective immunity.

It has been shown that multiple exposures to high doses of radiation attenuated sporozoites generates an antibody response[16], which is capable of reducing parasite burden in the liver [17]. However, it is not known if exposure to physiological doses of viable sporozoites will induce an antibody response if blood stage malaria is prevented by memory CD8 T cells. To determine if protective antibodies were generated in the repeatedly challenged mice we analyzed the sporozoite-specific antibody response at day 609, which was 44 days after mice were challenged the fourth and fifth time respectively. We detected sporozoite-specific antibodies in the serum from the repeatedly challenged immunized mice at an indirect fluorescent antibody test (IFAT) titer of 800 whereas sera obtained from naïve mice or DC-CS₂₅₂+LM-CS₂₅₂ immunized mice failed to react with sporozoites (Fig. 2B). Importantly, the sporozoite-specific antibodies were protective as five out of six naïve mice that received sera from the repeatedly challenged immunized mice exhibited sterile immunity. Transfer of naïve sera failed to protect naïve mice from developing blood stage parasitemia (Fig. 2C). Together the serum transfer and CD8 T cell depletion results clearly indicate that repeated sporozoite exposure of DC-CS₂₅₂+LM-CS₂₅₂ immunized mice generates a sporozoite-specific antibody response capable of protective immunity. While we did not analyze the potential contribution of CD4 T cells due to the low numbers of available repeatedly challenged mice, it is likely that CD4 T cells were engaged and at a minimum, provided help for generation of the sporozoite-specific antibody response. It will be interesting to determine when sporozoite-specific antibodies develop and the minimum number of sporozoite challenges necessary for the development of sporozoite-specific antibodies capable of conferring sterile protection.

4. Discussion

These results are compelling for several reasons. First, protective immunity after subunit vaccination wanes with time. Yet, our results demonstrate that if sterile protection can be conferred through a subunit vaccine, even if only for a limited period, it is possible for additional protective immune responses to be generated by repeated exposures to viable sporozoites that are cleared by the existing memory CD8 T cell memory response. Thus, even if the initially protective CD8 T cell response wanes with time, the host may remain protected. This issue has particular relevance to *Plasmodium* infections of humans, which can occur with high frequency in endemic areas. Secondly, our results show that protective antibody responses can be generated after repeated exposure to physiologic numbers of viable sporozoites in hosts that are protected against liver stage infection. These results underscore the notion that immune dysregulation during blood stage *Plasmodium* infection may negatively impact the generation of protective sporozoite-specific antibodies in multiply exposed humans.

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Fig. 1. Protective immunity in DC-CS₂₅₂+LM-CS₂₅₂ immunized mice is CD8 T cell-dependent. Mice were primed with 5×10^5 DC-CS₂₅₂ and boosted a week later with 2×10^7 LM-CS₂₅₂. (A) Frequency of PBL that are CS₂₅₂-specific CD8 T cells as determined by ICS at day 101 post immunization. (B) Frequency of total CD8+ and CD4+ cells in the PBL following the indicated antibody treatment. (C) Protection of the indicated groups of mice following challenge with 1000 viable *P. berghei* sporozoites. (D) Serum from naïve mice or DC-CS₂₅₂+LM-CS₂₅₂ immunized mice day 95 post immunization (Immune Serum) was transferred to naïve mice. Mice were then challenged with 1000 viable *P. berghei* sporozoites.

(B)

	IFAT Titer
Naive Serum	None Detected
Immune Serum	None Detected
Immune + Spz Exposure Serum	800

(C)

	Challenged	Protected	%Protected
Naive Serum	4	0	0%
Immune + Spz Exposure Serum	6	5	83%

Fig. 2.

Repeatedly challenged DC-CS₂₅₂+LM-CS₂₅₂ immunized mice develop a protective antibody response. Mice were primed with 5×10^5 DC-CS₂₅₂ and boosted a week later with 2×10^7 LM-CS₂₅₂. Mice were challenged with 1000 *P. berghei* sporozoites four and five time previously between day 210 and 565 post DC-CS₂₅₂ prime. (A) Frequency of PBL at day 649 after immunization that are CS₂₅₂-specific CD8 T cells as determined by ICS prior to the last sporozoite challenge. Three of the previously challenged immunized mice were depleted of their CD8 T cells prior to challenge. n.d. = none detected. Naïve and immunized mice were challenged with 1000 viable *P. berghei* sporozoites. (B) Serum sporozoite-specific antibody titer from naïve mice (Naïve Serum), DC-CS₂₅₂+LM-CS₂₅₂ immunized mice, or DC-

CS₂₅₂+LM-CS₂₅₂ immunized mice previously challenged with sporozoites four or five times (Immune+Spz Exposure Serum) was determined by IFAT. (C) Serum from naïve mice or from DC-CS₂₅₂+LM-CS₂₅₂ immunized mice previously challenged four or five times (Immune +Spz Exposure Serum) was transferred into naïve mice. Mice were then challenged with 1000 viable *P. berghei* sporozoites.