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Plasmodium yoelii Can Ablate Vaccine-Induced Long-Term Protection in Mice

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Malaria is a serious cause of morbidity and mortality in millions of individuals each year. People living in endemic areas build up partial immunity only after repeated attacks of malaria over several years (1–3). As such, it is clear that a vaccine is required, and a number of vaccine candidates are being developed. The 19-kDa C-terminal fragment of the merozoite surface protein-1 (MSP119) and the related molecule, MSP142, are vaccines that can protect monkeys and mice from infection (4–13).

In humans, studies on natural immunity to MSP119 have found that despite multiple infections, <20% of children in Sierra Leone and only 60% of adult Gambians possess Abs to Plasmodium falciparum-MSP-119 (PfMSP-119) indicating that even lifelong exposure to this protein may be insufficient to induce an Ab response (14). The reason why 40% of adult donors from cross-sectional studies do not possess Abs to PfMSP-119 is not known as all donors were known to have been exposed to malaria over many years (15). Other studies on natural immunity to MSP119 found a scarcity of IgG anti-MSP119 responses in areas of hyperendemity, both in terms of the number of responders and intensity of the response, while IgM responses were elevated (16, 17). Similarly, IgM responses to other Plasmodium Ags, including the circumsporozoite protein, have also been found to be elevated in areas of endemicity (18–21). IgM Ab is a sign of a primary Ab response, usually expected in previously unexposed individuals. Moreover, studies in the nonendemic area of Ndiop and the endemic area of Dielmo (in Senegal), found that few individuals had IgG3 anti-MSP119 Ab, even 3–4 mo after an epidemic of malaria when the infection had cleared (22). Although the parasite could have absorbed the Ab during infection, it was expected that infection would boost the number of memory and plasma cells and that the number of patients accumulating IgG3 anti-MSP119 would increase with time. Furthermore, there are anecdotal data that individuals lose immunity when they leave endemic areas (23). In any case, taken together, the data suggest that immunological memory of the parasite is not consistent in endemic areas (24).

Thus, the current study was initiated to assess whether MSP119 generated memory B cells and long-lived plasma cells (LLPC) in mice, and to determine whether these cells protected against lethal, Plasmodium yoelii YM infection. This study has shown formally for the first time that the vaccine generates memory B cells and LLPC. However, these functional memory B cells, per se, do not protect mice against infection. We then showed that Plasmodium ablates immunological memory of the vaccine and LLPC.

Materials and Methods

Animals

SPE, 6–8 wk old, BALB/c mice, and SCID mice on a BALB/c background were obtained from the Animal Research Centre (Perth, Western Australia). These studies have been reviewed and approved by The Queensland Institute of Medical Research Animal Ethics Committee.

Infectious challenge and monitoring parasitemia

Mice were always challenged with a lethal dose of (106) P. yoelii YM-infected RBC (pRBC) given by i.v. injection. To measure the level of infection in the blood (parasitemia), drops of blood were taken by tail snips and smeared on a glass slide. The slides were stained with Giemsa stain, and the numbers of infected cells in at least 400 red cells or 20 fields were counted. To confirm sterile immunity (complete clearance of parasite) in immunized mice, 50 μl of blood was transferred from these mice to naive mice and parasitemia was monitored for 3 wk.

Immunization protocol for protection studies

BALB/c mice were immunized with 50 μg of MSP119 in Freund’s complete adjuvant per mouse, given s.c., followed by three additional doses of 20 μg of MSP119 in Freund’s incomplete adjuvant. The second dose was given s.c. and then the last two doses were given i.p., with at least 3-wk intervals between each dose. This protocol was shown previously to induce complete protection against a lethal infection (7, 8). To control for the effects of the adjuvant, parallel cohorts of mice were given PBS in adjuvant using the same four-dose protocol. The mice were rested for rested for 2–12 wk as stated in specific experiments.

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Malarial immunization is an attractive approach to control malaria because it would be administered to asymptomatic individuals who do not show any clinical signs of infection, providing a mass prophylactic measure to many millions of people. However, malaria is a complex disease for which a single vaccine might not be sufficient. In this study, we report that functional memory B cells as well as long-lived plasma cells including those specific for bystander immune responses. Our study demonstrates a novel mechanism by which Plasmodium ablates immunological memory of vaccines, which would leave the host immuno-compromised. The Journal of Immunology, 2005, 175: 2510–2516.

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1 Abbreviations used in this paper: MSP1 19, merozoite surface protein-1; BM, bone marrow; ASC, Ab-secreting cell; LLPC, long-lived plasma cell; PIMSP-119, Plasmodium falciparum-MSP-119; pRBC, P. yoelii YM-infected RBC; DT, diptheria toxoid; KLH, keyhole limpet hemocyanin; PI, propidium iodide.
Immunization protocol for apoptosis studies

BALB/c mice were immunized with three (instead of four) doses of MSP119 or with two doses of diethytoxoid (DT) or keyhole limpet hemocyanin (KLH), (100 µg/mouse) as described above. To control for the effects of the adjuvant, mice were immunized with PBS in Freund’s complete and then in Freund’s incomplete adjuvant as for the MSP119 group, and rested for 10–16 wk.

Cell transfer studies

To define protection by memory B cells, lymphocytes were enriched from spleens of immunized or PBS-immunized mice (after >10 wk) by removal of stromal and APCs that adhere to plastic at 37°C within 30 min. The lymphocytes were then transfused i.v. into SCID or sublethally irradiated (550 Gy), naïve, BALB/c mice to separate memory cells from serum Ab in immunized mice.

In vivo activation of memory responses

To measure memory Ab responses in vivo, immunized mice with memory cells or recipient mice with transfused memory cells were boosted i.v. with 10 µg of MSP119. The memory IgG anti-MSP119 responses were measured in the serum 7 days after boosting with the vaccine by an Ag-specific ELISA or after 4 days by an Ag-specific ELISPOT assay of spleen and bone marrow (BM) cells. To measure activation of memory cells following infection, immunized and rest mice were challenged with 10^7 P. yoelii YM pRBC i.v., and parasitemias were monitored every 2 days.

Flow cytometric labeling

To identify memory B cells and plasma cells, spleen or BM cells were labeled to detect surface CD19 (clone 1D3-FITC) and CD27 (clone BD Pharmingen) expression followed by Streptavidin-Quantum Red (Sigma-Aldrich). To identify cells that were apoptosing or activated, cells were labeled to detect cytoplasmic caspase-3 (PE: early apoptosis marker) or Ki-67 Ag (PE: marker of proliferation indicating activation). The latter were purchased from BD Biosciences as kits. Nonspecific labeling was blocked by preincubation with purified rat Ig in 5% BSA/PBS. Data were acquired and analyzed using CellQuest software, and labeling with isotype control Abs was used to set gates. The absolute number of apoptosing or proliferating cells were calculated by multiplying the percentage of caspase-3^+ or Ki-67^+ cells by the percentage of memory B cells or plasma cells in each sample and adjusting for either the total number of splenocytes or 10^7 BM cells. Samples were never fixed and left overnight as the fluorochromes were affected by fixation.

Flow cytometric sorting

To determine whether apoptosing CD138^+ cells were MSP119-specific, BM was taken from MSP119-immunized mice that had been rested for 13 wk and then infected i.v. with 10^5 P. yoelii YM pRBC. After 7 days, the BM cells from individual mice were labeled to detect CD19, annexin V^+ and PI (all from BD Biosciences) following preincubation with 5% rat Ig, respectively. Cells from each mouse that were CD138^+annexin V^+/PI^+ or CD138^+annexin V^−/PI^− were sorted into subpopulations on a Dako Cytomation MoFlo cell sorter. Data were acquired and analyzed using Summit software. Four replicates of 1–2 x 10^4 sorted cells or total BM cells were then placed on ELISPOT plates previously coated with 10 µg/ml MSP119 to count numbers of vaccine-specific LLPC. It was not possible to isolate apoptosing CD138^+/annexin V^−/PI^− cells from uninfected mice as an additional control, due to their extremely low frequency without infection.

ELISPOT assay (25, 26)

Multiscreen-HA plates were coated with 10 µg/ml MSP119, DT, or KLH. In most experiments, isolated spleen or BM cells were directly tested for Ab-secreting cells (ASC) using the published method (25, 26). However, for data in Fig. 4c, apoptosing CD138^+ cells were sorted and then tested for MSP119 specificity. Four replicates were used for each BM or spleen sample with three to six mice per group.

Limiting dilution assay

Frequencies of KLH- or DT-specific memory B cells were assessed using an ELISPOT based limiting dilution assay (25, 26).

ELISA

Ab titers were determined as previously described using ELISA plates coated with 0.5 µg/ml MSP119 (27). Curves of absorbance against serum dilution were plotted and the reciprocal dilution of the end titer determined based on the absorbance of serum from naive mice.

Statistics

Error bars shown are means ± SEM. Values of p were calculated using the Mann-Whitney nonparametric t test, with a two-sided tail, based on pooled data from two to four replicate experiments.

Results

Protection by MSP119

To determine whether MSP119 generated memory responses, cohorts of six mice (in two repeat experiments) were immunized with either MSP119 (7, 8) or with PBS in adjuvant. After 2 wk, anti-MSP119 titers were assessed in these mice to confirm that vaccination with MSP119 had generated high titers of vaccine-specific Ab (Fig. 1a). Other cohorts of MSP119- or PBS-immunized mice were infected i.v. with 10^3 pRBC to assess protection by the vaccine. The progression of infection was monitored in the blood (parasitemia) by taking blood smears. Although all PBS-immunized mice died within 8 days, all MSP119-immunized mice survived for >48 days (when testing by blood-transfer showed complete clearance of the parasite; Fig. 1b). These studies clearly showed that vaccination with MSP119 conferred excellent protection against infection, 2 wk after the completion of immunization. The death of all PBS-immunized mice showed that the adjuvant had no effect on protection or survival of mice.

To study long-lived protection, all the other cohorts of immunized mice were rested for 10 wk, as vaccine-specific primary immune cells subside in 4–6 wk (27, 28). Anti-MSP119 titers were then measured in the serum of these mice and they had extremely high titers of anti-MSP119 Ab (Fig. 2a). Because the half-life of IgG Abs are generally only 7–23 days long (depending on the isotype), these high titers reflected accumulation of Ab produced by LLPC and possibly some residual Ab from the short-lived plasma cells that could not be differentiated. LLPC have been shown to survive and secrete Ab for at least 1 year (26, 29, 30).

FIGURE 1. Anti-MSP119 titers and protection from infection following MSP119 or PBS immunizations. Cohorts of mice were immunized with MSP119 or PBS. After 2 wk, data show anti-MSP119 titers in serum (a) and parasitemias following challenge with a lethal dose of P. yoelii YM (b). Mean titers are indicated within the bars in the figure. The data represent one of two experiments with groups of six mice, which had similar results.
measure immunity and protection in these mice, parallel cohorts of mice then either were given a small dose (10 μg) of MSP119 to recall memory responses or infected to measure protection. Boosting of these mice with MSP119 activated memory B cells and increased IgG vaccine-specific Ab titers within 7 days (Fig. 2b) compared with levels before boosting (Fig. 2a). Average IgG1 anti-MSP119 titers were \(8 \times 10^6\) before boosting with MSP119 (Fig. 2a) and increased by \(\sim 20\%\) following boosting (Fig. 2b). IgG2a, IgG2b, and IgG3 titers increased by \(\sim 60\, 10\), and 600\%, respectively, following boosting (Fig. 2b). When parallel cohorts of these immunized and rested mice were infected (with no prior boosting), all the mice survived infection but did develop patent parasitemia for \(>22\) days (Fig. 2c).

To measure protection by memory cells, per se, spleen cells from parallel cohorts of immunized and rested mice (a source of memory cells) were transferred to SCID mice (one spleen equivalent per mouse) to eliminate accumulated, circulating Ab. We further defined memory B cells by the production of high titers of IgG Ab within 7 days of exposure to the immunogen or infection (31). A new immune response would take \(9–14\) days to produce significant quantities of IgG Ab in serum. As expected, when recipient mice were boosted with MSP119, very high titers of memory Ab, specific for the vaccine were produced (Fig. 2d). However, when memory cells from an identical group of immunized and rested mice were transferred to SCID mice, which were then infected, the protection was equivalent to that mediated by cells from PBS-immunized mice (Fig. 2e). Although the memory cells were capable of producing Ab in uninfected mice (Fig. 2d), these cells did not even delay onset of parasitemia in parallel-infected mice.

Because mice with memory cells and accumulated Ab had survived a lethal infection (Fig. 2c), naive mice were given hyperimmune anti-MSP119 serum to determine the role of Ab in clearing infection independent of memory B cells. All mice cleared parasitemia (Fig. 2f), further indicating that while Ab accumulated from LLPC could protect mice, memory cells did not contribute to control of the parasite.

**Apopoptosis of memory B cells**

To determine why MSP119-specific memory B cells were unable to confer any protection, cohorts of three mice (in three repeat experiments) were immunized with MSP119 or PBS and after 10–12 wk, when primary immune cells had subsided, spleen cells containing memory cells were transferred to sublethally irradiated naive mice. Recipients then either were infected or given MSP119 to activate MSP119-specific memory cells. After 4 days, when parasitemia was still subpatent (below detectable levels), an ELISPOT assay was used to enumerate the number of MSP119-specific memory cells that had differentiated into MSP119-specific ASC. These studies found a very significant reduction in the numbers of MSP119-specific ASC in the spleen of infected mice compared with mice boosted with MSP119 (\(p < 0.004\); Fig. 3a). Similarly, infected mice had significantly fewer numbers of MSP119-specific ASC in their BM compared with mice boosted with MSP119 (\(p < 0.03\); Fig. 3b). These experiments showed that memory B cells were either lost or not activated following infection. The absence of any MSP119-specific ASC in spleens of PBS-immunized mice showed that new ASC were not generated within 4 days of vaccine-boosting or infection (Fig. 3, a and b).
The numbers of memory B cells that had differentiated into anti-MSP19-specific ASC and were resident in spleen (a) or had migrated to BM (b) were quantified by ELISPOT assays. c and d. Numbers of CD19+/CD27+ cells per spleen that expressed markers of proliferation (Ki67+) indicating activation (c) or early apoptosis (d; active caspase-3+).

To determine whether LLPC were deleted by apoptosis, BM cells from the above cohorts of mice were labeled to determine whether CD138+ plasma cells were expressing markers of early apoptosis (active caspase-3). Significant apoptosis of CD138+ plasma cells was noted following infection of both MSP19-immunized (p < 0.002) and PBS-immunized (p < 0.01) mice compared with mice that were boosted with the vaccine (Fig. 4b).

FIGURE 3. Analysis of memory B cells following infection. Cohorts of three mice (in three repeat experiments) were immunized with MSP19 or PBS in adjuvant and then rested for 10 wk. The spleen cells from these mice were then transferred to irradiated mice, which were then either infected with P. yoelli YM or given MSP19 to activate memory B cells. The spleen and BM cells from the recipient mice were then studied in vitro after 4 days. a and b. The numbers of memory B cells that had differentiated into anti-MSP19-specific ASC and were resident in spleen (a) or had migrated to BM (b) were quantified by ELISPOT assays. c and d. Numbers of CD19+/CD27+ cells per spleen that expressed markers of proliferation (Ki67+) indicating activation (c) or early apoptosis (d; active caspase-3+).

Effects of Plasmodium on bystander immune responses

Because we had noted apoptosis of CD138+ cells in PBS-immunized mice (Fig. 4b) that did not have vaccine-specific cells, we then investigated whether LLPC specific for other immunogens were also apoptosing during infection. Cohorts of five mice (in two repeat experiments) were immunized with DT or KLH to generate memory B cells and LLPC, specific for these immunogens. These mice were then rested for 17 and 13 wk, respectively, so that primary cells could die and only memory cells and LLPC survived. One half of these cohorts of mice were infected and all mice were studied after 4 days.

These studies found significantly more apoptosis of CD138+ cells in the BM of mice immunized with DT (p < 0.03) or KLH (data not shown) following infection compared with uninfected control mice (Fig. 5a). ELISPOT assays confirmed that infected mice had ~50% fewer DT-specific (Fig. 5b) and KLH-specific (data not shown) LLPC than uninfected control mice in the BM (p < 0.003) and ~30% fewer LLPC in the spleen (p < 0.035). By
comparison, apoptosis of CD19⁺/CD27⁺ cells (Fig. 5c) and frequencies of DT-specific (Fig. 5d) and KLH-specific memory B cells (data not shown) per spleen were equivalent between infected and uninfected mice. We thus concluded that following infection, significant numbers of LLPC of various specificities were deleted, but non-Plasmodium-specific memory cells were unaffected.

**Discussion**

This study shows a novel mechanism by which *Plasmodium* evades immunological memory of vaccines. We initially established that the vaccine MSP1₁₉ could generate memory B cells and LLPC. Studies on protection then found that while these cell were functional, memory B cells per se did not offer any protection. We thus studied memory B cells directly and have evidence that these cells apoptose following infection. Finally, we found that LLPC of various specificities, including those specific for diphtheria toxoid, were also deleted following infection.

Our studies had observed that following infection, 14-fold fewer MSP₁₁₉-specific memory B cells differentiated into ASC, compared with boosting with the vaccine. We also found 6-fold more apoptosis of CD19⁺/CD27⁺ B cells (which represent a proportion of memory B cells) and 6-fold lesser activation of these cells. The significant reduction in numbers of vaccine-specific ASC suggested that either memory B cells received direct apoptotic signals during activation or that these cells were not activated. Furthermore, another study has also found that numbers of apoptotic B cells are significantly increased during malaria (35) also indicating that *Plasmodium* affects B cell survival. There are suggestions that the disulphide bonds in MSP₁₁₉ can impede processing of the Ag as MSP-1₁₉ in whole parasite has to compete with other parasite Ags for presentation (36). Although problems with processing of MSP₁₁₉ may explain why fewer memory B cells differentiated into ASC, it does not explain why these cells underwent apoptosis. Another possibility is that the survival of CD4⁺ T cells may be compromised during infection (37–39), and this affects the survival or activation of memory B cells. Memory B cells require CD4⁺ T cells for their survival (40) and activation (28). As a final point, a very recent study of memory B cells in endemic areas found that exposure to malaria did not result in the establishment of stable populations of circulating Ag-specific memory B cells (41). In our studies, we have only investigated the deletion of MSP₁₁₉-specific memory B cells in mice but there is evidence that IgM responses to other *Plasmodium* Ags are elevated in areas of endemicity (18–21), suggesting a possible problem with memory responses to the parasite. We thus speculate that repeated infection of humans could delete circulating parasite-specific memory B cells but further studies are required.

The current study also found that LLPC, specific for the vaccine as well as other immunogens, underwent apoptosis following infection. The apoptosis of LLPC in the BM suggests that deletion is initiated by inflammatory mediators that are released in response to infection, as these plasma cells are generally not known to interact with other cell types. The apoptosis of these LLPC in the BM was not likely to be a result of changes in homeostasis (42), as few parasite-specific plasma cells would develop in the spleen and migrate to the BM within 7 days of infection. In fact none were noted following infection of PBS-immunized mice (Fig. 4a). Most new plasma cells would still predominate in the spleen (Fig. 3, d and e). Nonetheless, if an acute malaria infection does cause such severe homeostatic changes that result in the apoptosis of LLPC in the BM, these observations have significant implications for general immunity of individuals with clinical malaria.

Although our studies suggest that long-term protection by vaccines that mediate protection via Abs may not be viable, other studies to date on immunological memory following malaria have not seen significant problems. Those studies have focused on memory responses to the whole parasite, in many cases using an infection and cure regime to generate immunological memory (43, 44). In this case, the parasite would generate memory T cells and B cells and it is not easy to differentiate the contribution of each cell type in long-term protection. These protocols could also generate LLPC, which could secrete Ab for years to maintain protective Ab levels. Thus, we examined the function of memory cells independent of circulating Ab by transferring memory B cells into naïve mice. Moreover, we used MSP₁₁₉, which has clearly been shown to mediate protection via Ab alone, independent of T cell
B cells and LLPC specific for DT were either infected with P. yoelii YM that expressed active caspase-3, a marker of early apoptosis. The explanation of IgG memory B cells and LLPC following infection is a logical approach. As such, at this point, the apoptosis of memory B cells is not direct enough to offer protection and found that this was not the case.

Our study used Freund’s complete adjuvant, as MSP119 had previously been shown with this adjuvant to mediate complete protection and we wanted to determine whether memory responses generated with this adjuvant would also mediate protection. We found that all PBS (in adjuvant)-immunized mice died, indicating that the adjuvant alone had no effect on protection or survival of mice. Furthermore, the memory responses were not protective. As such, the adjuvant selected probably determines the quality and quantity of the immune response but otherwise does not directly affect protection.

Finally, in the context of human disease, our observations have two very significant implications for human vaccines: 1) while an immunized individual may control an initial infection due to accumulated Ab, the deletion of memory B cells and LLPC would leave the host susceptible to re-infection; 2) the deletion of bystander LLPC renders the host susceptible to other infections. Of course, not all cells may be deleted before new protective immune responses are generated and the infectious process could produce new memory B cells and LLPC. Nonetheless, while there are numerous valid arguments indicating that memory B cells persist in humans in the absence of Ag (24), there is no explanation as to why there is a scarcity of IgG responses against Plasmodium in areas of hyperendemicity (16), or why a significant proportion of West Africans do not possess IgG Abs to PIMSP-119, although they were exposed to malaria (15). As such, at this point, the apoptosis of IgG memory B cells and LLPC following infection is a logical explanation.

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Disclosures
The authors have no financial conflict of interest.

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