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

Michelle N. Wykes, Yong-Hong Zhou, Xue Q. Liu and Michael F. Good

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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 hyperendemicity, 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 (10⁴) *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 a 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.

Molecular Immunology Laboratory, Queensland Institute of Medical Research, Brisbane, Queensland, Australia

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2. Address correspondence and reprint requests to Dr. Michael F. Good, Queensland Institute of Medical Research, The Bancroft Centre, 300 Herston Road, Brisbane, Queensland, Australia 4029. E-mail address: michaelG@qimr.edu.au

3. The abbreviations used in this paper: MSP119, merozoite surface protein-1; BM, bone marrow; ASC, Ab-secreting cell; LLPC, long-lived plasma cell; PIMSP119, Plasmodium falciparum-MSP-119; pRBC, *P. yoelii* YM-infected RBC; DT, diptheria toxoid; KLH, keyhole limpet hemocyanin; PI, propidium iodide.


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

Michelle N. Wykes, Yong-Hong Zhou, Xue Q. Liu, and Michael F. Good

Malaria is a serious cause of morbidity and mortality for people living in endemic areas, but unlike many other infections, individuals exposed to the parasite do not rapidly become resistant to subsequent infections. High titers of Ab against the 19-kDa C-terminal fragment of the merozoite surface protein-1 can mediate complete protection in model systems; however, previous studies had not determined whether this vaccine generated long-term protection. In this study, we report that functional memory cells generated by merozoite surface protein-1, per se, do not offer any protection. This is because the parasite induces deletion of vaccine-specific 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.
**Immunization protocol for apoptosis studies**

BALB/c mice were immunized with three (instead of four) doses of MSP119, or with two doses of diphtheria toxoid (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 first in Freund’s complete and then in Freund’s incomplete adjuvant as for the MSP119 groups, 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), naive, 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 in the 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 rested mice were challenged with 105 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 LG.3A10-biotin; memory B cells) or CD138 (clone 281-2-biotin; plasma cells) (BD Pharmingen) expression followed by Streptavidin-Quantum Red (Sigma-Aldrich). To identify cells that were apoptosing or activated, cells were labeled to detect cytoplasmic active 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 7-aminoactinomycin D or propidium iodide (PI) excluded dead cells. Between 7.5 × 104 and 1.4 × 106 cells from each sample were analyzed so that at least 5 × 104 (average 9.1 × 104) CD19+/CD27+ memory B cells or CD138+ plasma cells from each sample were used for analysis of expression for active caspase-3 and Ki-67. 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 107 BM cells. Samples were never fixed and left overnight as the flurochromes 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 105 P. yoelii YM pRBC. After 7 days, the BM cells from individual mice were labeled to detect CD138+/annexin V+/PI− cells from uninfected mice as an additional control, due to their extremely low frequency without infection. To measure activation of memory cells following infection, immunized and rested mice were challenged with 109 P. yoelii YM pRBC i.v., and parasitemias were monitored every 2 days. To determine whether apoptosing CD138+ cells were MSP119-specific, 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 measured using Flow cytometric labeling with three to six mice per group.

**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 105 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). To
measure immunity and protection in these mice, parallel cohorts of mice then either were given a small dose (10 μg) of MSP19 to recall memory responses or infected to measure protection. Boosting of these mice with MSP19-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-MSP19 titers were 8 × 10^6 before boosting with MSP19 (Fig. 2a) and increased by ~20% following boosting (Fig. 2b). IgG2a, IgG2b, and IgG3 titers increased by ~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 MSP19, 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-MSP19 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.

**Apoposis of memory B cells**

To determine why MSP19-specific memory B cells were unable to confer any protection, cohorts of three mice (in three repeat experiments) were immunized with MSP19 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 MSP19 to activate MSP19-specific memory cells. After 4 days, when parasitemia was still subpatent (below detectable levels), an ELISPOT assay was used to enumerate the number of MSP19-specific memory cells that had differentiated into MSP19-specific ASC. These studies found a very significant reduction in the numbers of MSP19-specific ASC in the spleen of infected mice compared with mice boosted with MSP19 (p < 0.004; Fig. 3a). Similarly, infected mice had significantly fewer numbers of MSP19-specific ASC in their BM compared with mice boosted with MSP19 (p < 0.03; Fig. 3b). These experiments showed that memory B cells were either lost or not activated following infection. The absence of any MSP19-specific ASC in spleens of PBS-immunized mice showed that new ASC were not generated within 4 days of vaccine-boosting or infection (Fig. 3a and b).
To directly analyze these memory B cells following boosting with the vaccine or infection, we used flow cytometry. The expression of CD27 (in combination with CD19) is an accepted marker of memory B cells in humans (32, 33). In contrast, in mice, CD27 is acquired by Ag-primed B cells at a centroblast stage of development and is expressed on a lower frequency of mouse memory B cells (34). Thus, we hypothesized that a proportion of MSP119-specific memory B cells would also express CD27 and CD19. As such, we labeled spleen cells from mice in experiments related to Fig. 3, a and b, to determine whether CD19+/CD27+ cells were expressing markers of early apoptosis (active caspase-3) or proliferation (Ki67), which indicated activation (c) or early apoptosis (d; active caspase-3).

**Apoptosis of LLPC**

All plasma cells (short lived and LLPC) express CD138+, but after 10 wk, only LLPC survive in significant numbers in the BM (26). To determine whether *Plasmodium* affected LLPC, cohorts of three mice (in two repeat experiments) were immunized with either MSP119 or PBS in adjuvant to generate plasma cells and rested for 13 to 16 wk, after which only LLPC would survive. One half of each cohort of mice was infected, and the other half was boosted with MSP119. After 7 days, when parasitemia was still subpatent (below detectable levels) because of circulating Ab, numbers of MSP119-specific LLPC were quantified in the BM by an ELISPOT assay. Although the numbers of LLPC following boosting with MSP119 were equivalent to numbers before boosting, infection consistently reduced the numbers of MSP119-specific LLPC by >50% (p < 0.02; Fig. 4a). The absence of MSP119-specific LLPC in PBS-immunized mice confirmed that no new vaccine-specific plasma cells had migrated to the BM within 7 days of infection or boosting with the vaccine (Fig. 4a).

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 MSP119-immunized (p < 0.002) and PBS-immunized (p < 0.01) mice compared with mice that were boosted with the vaccine (Fig. 4b). Moreover, because a small proportion of pre-B cells express CD138, we sorted apoptosing CD138+ cells from the BM of infected mice to show that they were vaccine-specific LLPC. For this, we sorted apoptosing (CD138+/annexin V+/PI-) or viable (CD138+/annexin V-/PI-) cells from the BM of MSP119-immunized and rested mice that had then been infected, and tested their MSP119 specificity by an ELISPOT assay. The apoptosing CD138+ cells had nearly 200-fold more MSP119-specific cells than the viable CD138+ cells confirming the loss of vaccine-specific LLPC during a malaria infection (p < 0.0001; Fig. 4c). We could not examine apoptosing (CD138+/annexin V+/PI-) cells in the MSP119-boosted group as their numbers were too low for sorting. Clearly the apoptosing CD138+ cells could not have been Ag-specific pre-B cells that do not secrete Ab. The apoptosis of plasma cells became evident between days 3 and 7 following infection (Fig. 4d).

**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
Another possibility is that the survival of CD4+ ASC, it does not explain why these cells underwent apoptosis. As MSP-119 in whole parasite has to compete with other parasite infection. The PBS-immunized mice had no anti-MSP119-secreting cells indicating specificity of the assay.

apoptosis of CD19pared with boosting with the vaccine. We also found 6-fold more cells are significantly increased during malaria (35) also indicating that these cells were not activated. Further-

of memory B cells) and 6-fold lesser activation of these cells. The disulphide bonds in MSP1 19 can impede processing of the Ag.

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 were deleted, but non-Plasmodium-specific memory cells were unaffected.

**Discussion**

This study shows a novel mechanism by which *Plasmodium* parasites (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.

comparison, apoptosis of CD19+/CD27+ cells (Fig. 5c) and frequencies of DT-specific (Fig. 5d) and KLH-specific memory B cells were similar 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.

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 endemnity (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 naive mice. Moreover, we used MSP119, which has clearly been shown to mediate protection via Ab alone, independent of T cell
mediated protection (7). As such, any protection would be mediated by Ab produced by memory B cells. Furthermore, we also assessed whether memory B cells could produce Ab rapidly 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 reinfection; 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 PMSP-19, although they were exposed to malaria (15). As such, at this point, the apoposis of IgG memory B cells and LLPC following infection is a logical explanation.

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Disclosures

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