Correspondence

Do Apoptotic *Plasmodium*-Infected Hepatocytes Initiate Protective Immune Responses?

To the Editor—In an article recently published in the *Journal of Infectious Diseases*, Leiriao et al. [1] suggested that the uptake by dendritic cells (DCs) of antigens derived from apoptotic *Plasmodium*-infected hepatocytes is a key factor in the induction of sterile protective immunity by inoculation with irradiated sporozoites. This proposal was based on 2 experimental observations: that there is an increase in the occurrence of apoptotic host cells when they are infected with irradiated rather than normal sporozoites and that macrophages and DCs bearing parasite proteins are derived from apoptotic *Plasmodium*-infected hepatocytes in the livers of mice immunized with irradiated sporozoites. The authors implied that presentation by these cells would be responsible for the protection induced by irradiated sporozoites. The data prompted a supportive commentary by James [2], who had previously hypothesized that invasion by pathogens would induce apoptosis of host cells to limit the infection [3]. Given that costly vaccine development programs strive to reproduce and extend the reference standard of sterile protection obtained after immunization with irradiated sporozoites, the elucidation of the events that efficiently prime the immune response is highly desirable, and, thus, the conclusions of Leiriao et al. [1] merit careful scrutiny.

We have identified a major flaw in the experimental design, and it calls into question the series of events that the authors propose as being associated with protection. It is known that the protection induced by immunization with irradiated sporozoites is strictly dose dependent. Only mice immunized with *P. berghei* sporozoites irradiated at doses of 8–12 krad, and not mice immunized with sporozoites irradiated at doses of ⩾15 krad, were completely protected from infection after challenge with normal sporozoites [4]. The dose used by Leiriao et al. was 20 krad, and, therefore, it can be argued that the data presented might not be relevant to the immunological mechanisms leading to protection. That there was an experimental flaw is consistent with the remarkably low numbers of DCs ($n = 2$) and macrophages ($n = 3$) that were bearing vesicular material derived from apoptotic *Plasmodium*-infected hepatocytes and were isolated from whole liver 6 h after mice were immunized with 1×10^6 irradiated sporozoites. It is difficult to envisage that such low numbers of these cells would be sufficient to induce a strong protective immune response. One crucial aspect of these responses is their dependence on the presence of live parasites in the infected hepatocytes, as has been demonstrated for exposure not only to irradiated sporozoites [5] but also to normal sporozoites. Indeed, normal sporozoites induce equally potent protective immune responses [6]. In both types of challenges, treatment of mice with primaquine, a drug that kills liver-stage parasites, abolishes protection.

We suggest that the uptake by DCs of parasite antigens from live rather than apoptotic or dead hepatocytes might instead account for the priming and maintenance of the protective immune response, because these cells represent the major source of liver-stage antigens. We propose that DCs, which are potent stimulators of primary immune responses, are activated after they take up parasite antigens directly from viable infected hepatocytes in a process similar to that recently described for HIV-infected cells [7] and for tumor cells [8]. The role played by DCs, although likely to be dominant, might not necessarily be exclusive. Uptake of parasite antigens by phagocytic cells, such as neutrophils, would also result in their activation and the transport of antigens to the lymphoid organs. These cells could also transfer antigens to resident DCs, leading to their maturation [9]. The animal model of malaria, in which the preerythrocytic stages of the parasites are amenable to detailed investigations, remains the only means to elucidate the basis of protection induced by attenuated or normal sporozoites. The hope is that studies using this model can then guide the development of a truly effective preerythrocytic vaccine.

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Reply to Rénia et al.

To the Editor—In their letter, Rénia et al. [1] argue that immunization with *Plasmodium berghei* sporozoites irradiated with 20 krad does not lead to protection against infection in mice. However, we have previously shown that this dose is sufficient to induce protection in mice immunized with irradiated *P. yoelii* 17XNL sporozoites [2]. Therefore, this is not an experimental flaw, and the data presented in our recent study [3], which used the same dose and parasite species, were generated in conditions under which protective immunological responses are induced.

Variations in the dose of radiation used on sporozoites to induce protection have been reported, and the doses can range from 8 to 20 krad. For *P. falciparum* sporozoites, a minimum dose of 15 krad is necessary, because a 12-krad dose results in insufficient attenuation leading to blood-stage infection in human volunteers [4]. Other investigators routinely use 20 krad, to ensure good stimulation of $CD8^+$ T cells and a protective immune response against *P. yoelii* [5]. Differences among parasite strains or even errors in the calibration of the radiation source in different institutions may account for these differences in doses.

We found low numbers of dendritic cells (DCs) carrying parasite materials in the liver; however, this finding is consistent with the dynamics of the migration of these cells in the body. After activation and uptake of antigens, DCs rapidly leave peripheral organs and travel to lymph nodes. In our study, we screened for DCs bearing parasite antigens 6 h after immunization. With this approach, we could find only the cells that had already phagocytosed parasite antigens but had not yet left the liver. The time between these 2 events is ~1 h [6]. Therefore, it is likely that higher numbers of DCs take up apoptotic hepatocytes during *Plasmodium* infection but that this occurs either before or after the time when we analyzed the livers. Analysis of liver-draining lymph nodes, where migrating DCs carrying parasite antigens accumulate, could provide a more accurate reflection of the numbers of these cells in immunized animals.

Rénia et al. also argue that low numbers of DCs could not initiate protective immune responses. It is well known that DCs are very powerful activators of T cells. T cell activation can be detected when only 100 mature DCs migrate to lymph nodes (after injection of 1.8×10^5 DCs in the footpads of mice) [7]. Therefore, it is possible that low numbers of DCs initiate efficient T cell responses. Because it is not possible to inhibit apoptosis in a live mouse without affecting the normal functions of its immune system, we could not determine the relative contribution of this mechanism of antigen presentation to the generation of protective immunity. However, our data indicate that such a mechanism is activated after immunization with irradiated sporozoites and that it may be important for protection.

As Rénia et al. point out, live sporozoites, either normal or irradiated, are required to induce a protective response against liver-stage malaria. It is believed that the capacity of sporozoites to infect hepatocytes is required to induce a protective response. It is clear that normal sporozoites are also able to generate $CD8^+$ T cells and protective immune responses when blood-stage infection is inhibited [2, 8, 9]. As we mentioned in our study [3], we found a lower, but significant, level of apoptosis in hepatocytes infected with normal sporozoites. It is possible that a similar mechanism is activated during infections with normal sporozoites, in which a fraction of the infected hepatocytes would undergo apoptosis and serve as an antigen source for DCs, thereby contributing to the generation of protective immunity.

After infection with intracellular pathogens, host cells frequently activate the apoptotic machinery in response to microorganism-induced stress. There are numerous examples of pathogens that counteract this host strategy by inhibiting the capacity of host cells to enter into apoptosis [10]. In particular, *Plasmodium*-infected hepatocytes are more resistant to apoptosis than are control ones, indicating that the parasite inhibits apoptosis in host cells [11]. To achieve this inhibition, *Plasmodium* sporozoites take advantage of a host molecule, hepatocyte growth factor, which is secreted not by the infected cell but by neighboring ones that were previously traversed by the parasite. It is likely that other mechanisms mediated by parasite molecules contribute to the inhibition of apoptosis in host cells.

Rénia et al. refer to the effect of primaquine treatment in the protection induced by sporozoites. To study this issue, we injected mice with primaquine 2 or 24 h after immunization with irradiated sporozoites. We confirmed, using real-time polymerase chain reaction to quantify liver-stage parasites, that primaquine effectively eliminated the parasites, and we found a significant reduction in liver infection after the treatment (figure 1*A*). Ten days later, both groups of mice treated with

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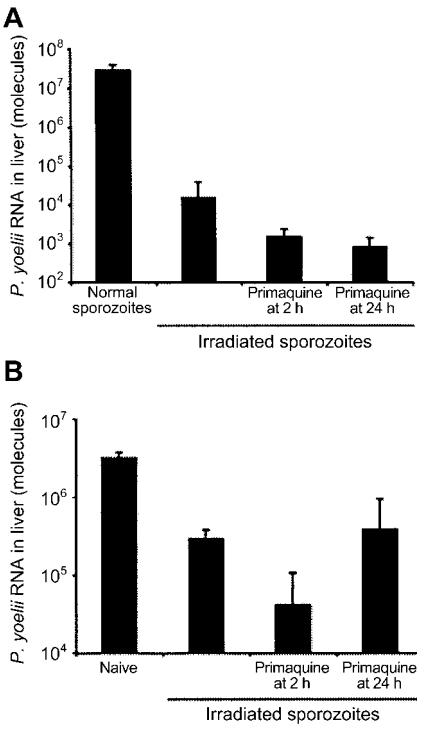

Figure 1. Primaquine treatment in mice. *A,* Efficacy of primaquine treatment. Groups of 3 BALB/c mice were injected intravenously (iv) with 1×10^5 normal or irradiated (20 krad) *Plasmodium yoelii* sporozoites. Mice were then injected iv with primaquine (1.4 mg/mouse) 2 or 24 h after injection of irradiated sporozoites; control mice did not receive primaquine. Livers were collected 40 h after the injection of parasites so that parasite loads could be determined by real-time polymerase chain reaction (PCR). We observed a reduction of 90%–95% in the no. of parasites in the livers of mice that received primaquine, compared with that in the livers of untreated mice. *B,* Effect of primaquine treatment in protection. Groups of 3 mice were injected iv with 1×10^5 irradiated sporozoites and then with primaquine 2 or 24 h later; naive mice were not injected. Ten days later, mice were challenged with 1×10^4 sporozoites iv. Livers were collected 40 h after the injection of parasites so that parasite loads could be determined by real-time PCR. Results are expressed as averages ± SDs for 3 mice analyzed independently.

primaquine at 2 or 24 h after immunization were protected after sporozoite challenge, and the group treated at 2 h after immunization had higher levels of protection (figure 1*B*). It is possible that killing of the parasites by primaquine 2 h after infection actually helps the immune response, because it provides more antigens from dead infected hepatocytes. Because the generation of $CD8^+$ T cells occurs during the early stages of infection with irradiated sporozoites (<8 h after infection [12]), it is likely that only antigens delivered before this time contribute to protection.

Interestingly, Rénia et al. point out the possibility that DCs present antigens taken up directly from live infected hepatocytes. In this recently described mechanism, DCs have close contact with other live cells and take up plasma membrane directly from them. This mechanism, however, would be limited to proteins expressed in the plasma membrane of the hepatocyte or free in their cytosol and would not include the majority of parasite proteins, which are located in the parasitophorous vacuole. During the late stages of infection, a number of parasite proteins are expressed in the plasma membrane of *Plasmodium*-infected hepatocytes [13, 14], suggesting that this mechanism may be activated at this time (>24 h after infection). However, activation of protective responses is very low at this time of the infection [12], which would limit the relevance of this process.

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Rural Kenya: Measles during Pregnancy and Early Infancy

To the Editor—During January 1996 and July 1997 in rural Kenya, 10% of females 14–19 years old were seronegative for measles. Concurrently, 14.5% of neonates born

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to such mothers were seronegative [1]. This would imply that there is a sizeable population of measles-susceptible women of childbearing age in the community. These women might well develop measles during any future outbreak in rural Kenya. Furthermore, a significant proportion of neonates in this population would be susceptible to measles during very early infancy.

In Burkina Faso during a measles outbreak, 16 women (mean age, 20.6 years; gravidity, 2.1; parity, 1.1) were found to have measles. There were 6 cases of laryngitis, 3 cases of pnemonitis, 2 abortions, 3 childbirths, and 1 preterm delivery [2]. Recently in São Paulo City, Brazil, measles virus RNA and IgM antibodies were demonstrated in 2 mothers with measles at a university hospital. In 1 neonate, measles virus RNA persisted in peripheral blood mononuclear cells for 157 days after birth [3]. In Sendai, Japan, during a measles outbreak, 8 pregnant women were hospitalized at the Tohoku University School of Medicine; 3 of 4 pregnancies ended in spontaneous abortion or stillbirth before 24 weeks of gestation, whereas 4 pregnancies ended in live births after 25 weeks. Two of 4 neonates were found to have congenital measles [4].

During 1996, measles virus was isolated from 6 neonates <4 months old during an outbreak in an orphanage in Pune, India. Serum testing showed a positive result for IgM antibodies. During the same period, mothers of 4 IgM-positive neonates were screened for antibodies; 3 of them were negative for measles-specific antibodies [5]. Recently, a measles outbreak affected 41 infants in an orphanage in Bangkok during September–October 2000. The attack rate was 66% among infants 6–12 months old in 2 separate rooms. Sixty-three percent of the 41 children were <9 months old [6].

The efficacy of any mass measles campaigns in Africa targeting children <15 years old [1] would be evident after some years. During the interim phase, future measles mortality or morbidity in HIV and malaria-endemic areas like rural Kenya should be addressed by screening women for measles susceptibility. Simple assay formats that do not require trained personnel or costly equipment would be ideal for measles IgG screening, particularly in physicians' offices. During the routine screening of pregnant women for rubella, a simultaneous screen for measles susceptibility would not be all that difficult. Seronegative women, if they are not planning a pregnancy within a month, could be offered combined mumps-measles-rubella vaccine.

Cord blood from neonates born in areas where HIV and malaria are endemic [1] should be quantified for maternally acquired measles virus IgG. Infants devoid of maternal antibody would be labeled as susceptible and would qualify for an immediate measles vaccination. The ideal time for immunization of neonates with maternally acquired antibody would be linked with the antibody titer. The decline toward titers lower than those compatible with protection in any neonate would be related to the half-life of human gamma-globulin of ~25 days. Simple arithmetic computation would assist in the predetermination of the ideal time of immunization in any neonate.

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Reply to Arya and Agarwal

To the Editor—Arya and Agarwal [1] point out, importantly, the severity of measles in adults and potential harmful consequences of measles in pregnancy to both mother and baby. We agree that it is important to continue to monitor the age distribution of persons with measles, to detect changes over time after population-wide vaccination. These may include a shift in the relative frequency of cases of measles to older persons [2], and, eventually, a reduction in the levels of measles antibody transmitted from mother to neonate via the placenta [3], because vaccine-induced antibody levels are lower than those after measles infection. Screening for measles susceptibility at the level of individual mothers or infants, however, is not likely to be an appropriate public health response to these changes. Screening for measles antibodies costs as much as, if not more than, administering measles vaccine [4]. Because simple "bedside" test kits do not exist that provide instant and reliable results for measles antibodies, in addition to the laboratory resources required, an additional visit to the clinic would be needed to vaccinate seronegative persons once their measles antibody status is known. In most low-income countries, there is no routine rubella screening at antenatal clinics, nor are there arrangements to vaccinate susceptible women after delivery. These programs have been difficult to implement successfully even in industrialized countries [5]. It is not feasible

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for public health programs to tailor the age for measles vaccination to individual children, even if the age at loss of maternal antibody could be predicted from the antibody level in cord blood.

Mass immunization campaigns for the elimination of measles that are conducted in the setting of high ongoing coverage of routine infant immunization are aimed at reducing overall transmission within a population; thus, both young and old persons are protected indirectly by the induction of herd immunity. After the reduction of measles transmission to very low levels, the age for routine infant vaccination can in fact be increased, to achieve a higher efficacy of the first dose of measles vaccine. The success of this strategy has been reported even in areas with high HIV and malaria transmission [6, 7]. Outbreaks can still occur after vaccination campaigns, as was recently reported in Mexico [8], and it is clearly important to determine the population profile of measles susceptibility and to define the age range for campaigns accordingly, as well as to target high-risk groups of adults [9]. Further work should preferably be aimed at determining the optimum age range for campaigns for long-term measles reduction, as well as the optimal age for routine vaccination, especially in areas of high HIV and malaria prevalence, in accordance to the goal of the measles control program.

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