

Uninfected Mosquito Bites Confer Protection against Infection with Malaria Parasites[∇]

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Despite decades of research and multiple initiatives, malaria continues to be one of the world's most debilitating infectious diseases. New insights for malaria control and vaccine development will be essential to thwart the staggering worldwide impact of this disease (A. Bjorkman and A. Bhattacharai, *Acta Trop.* 94:163–169, 2005); ultimately successful vaccine strategies will undoubtedly be multifactorial, incorporating multiple antigens and targeting diverse aspects of the malaria parasites' biology (M. F. Good et al., *Immunol. Rev.* 201:254–267, 2004). Using a murine model of malaria infection, we show here that exposure to bites from uninfected mosquitoes prior to *Plasmodium yoelii* infection influences the local and systemic immune responses and limits parasite development within the host. In hosts preexposed to bites from uninfected mosquitoes, reduced parasite burdens in the livers were detected early, and during the blood-stage of the life cycle, these burdens remained lower than those in hosts that received mosquito bites only at the time of infection. Repeated exposure to bites from uninfected mosquitoes skewed the immune response towards a T-helper 1 (Th1) phenotype as indicated by increased levels of interleukin-12, gamma interferon, and inducible nitric oxide synthase. These data suggest that the addition of mosquito salivary components to antimalaria vaccines may be a viable strategy for creating a Th1-biased environment known to be effective against malaria infection. Furthermore, this strategy may be important for the development of vaccines to combat other mosquito-transmitted pathogens.

Malaria continues to be a major public health threat and has an enormous economic impact, resulting in nearly 3 million deaths annually and ranking eighth as a contributor to the loss of global disability-adjusted life years (7). Emerging drug resistance in the *Plasmodium* spp. parasites that cause malaria and insecticide resistance in mosquito vectors that transmit these pathogens emphasize the urgent need for developing an effective malaria vaccine to control the devastating burden of malarial disease. The complex biology of malaria parasites coupled with antigenic polymorphism, poor antigen immunogenicity, and parasite-induced immunosuppression distinguishes the quest for a malaria vaccine as extraordinarily daunting.

Malaria parasites are transmitted to humans via the bite of their insect vector, a female anopheline mosquito. During blood feeding, mosquitoes inject infective *Plasmodium* sporozoites into the avascular skin tissue of their hosts, where the sporozoites eventually migrate into the circulation (53); simultaneously, a plethora of pharmacologically active compounds in mosquito saliva are introduced into the hosts. These compounds have substantial antihemostatic, anti-inflammatory, and immunosuppressive activities that aid the mosquito in the blood-feeding process (40). Furthermore, many of the salivary components are immunogenic and elicit strong immune re-

sponses, evidenced by the swelling and itching that accompany a mosquito bite (38). This substantial effect of immune activation by saliva creates an inflammatory context for further responses to coinjected pathogens.

A role for arthropod saliva in modifying the outcome of infection is not a novel idea introduced in the context of mosquitoes and malaria parasites; increased pathogen infectivity in association with ticks, sand flies, and mosquitoes has been described previously (for a review, see reference 50). While previous studies have focused primarily on the enhancement of transmission and disease when pathogens are introduced in the presence of vector saliva, some studies have explored the effect of repeated exposure to vector saliva on the outcome of infection. Although the mechanism has yet to be completely elucidated, repeated infestation with pathogen-free *Ixodes scapularis* ticks induces resistance to *Borrelia burgdorferi* transmission (56). The most striking host-parasite vector system to be studied to date is that involved in infection with *Leishmania* spp. through the bites of phlebotomine sand flies. Interestingly, multiple exposures to bites from uninfected sand flies prior to infection confer resistance to *Leishmania major* due to increases in the cytokines responsible for cell-mediated immunity (24). Mosquito bites have also been shown to influence immunity and potentiate viral disease in mouse models (18, 26, 45, 46), possibly through the modulation of host systemic cytokine responses (46, 58).

Mosquito bites induce immediate, delayed, and systemic hypersensitivity reactions in hosts (38); consequently, we hypothesized that the local tissue and systemic environments in hosts "immunized" by using mosquito salivary components could

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enhance malaria immunity. We tested this hypothesis by using the prototypic murine model of malaria infection: sporozoite infection of mice via *Plasmodium yoelii*-infected *Anopheles stephensi* mosquitoes.

MATERIALS AND METHODS

Mice. BALB/c mice aged 6 to 8 weeks were bred at the Friemann Life Sciences Center at the University of Notre Dame, Notre Dame, IN, under approved Institutional Animal Care and Use Committee protocols. All mice were female and were age matched for all experiments. Gamma interferon (IFN- γ)-deficient knockout (KO) mice and their BALB/c wild-type (WT) counterparts were purchased (Jackson Labs, Bar Harbor, ME) and used at 6 to 8 weeks of age.

Pre-sensitization. Mice were anesthetized, and their ears were exposed for 20 min to a screened vial containing 15 to 20 fully matured female adult mosquitoes every 2 weeks for 6 weeks. A control group of age-matched mice were only anesthetized at each time point. Mice were challenged 2 weeks following the last pre-exposure. Twenty-four hours (for the analysis of cytokine responses) or 40 h (for parasite quantification) after the final exposure, ear, liver, and spleen samples were harvested and stored in RNAlater (QIAGEN, Valencia, CA).

Mosquito infections. *P. yoelii* (17XNL) parasites were maintained by alternating passage of parasites through *A. stephensi* mosquitoes and BALB/c mice. Murine parasitemia was assessed by using thin-layer blood smears. Smears were fixed in 100% methanol and stained with Giemsa. Once gametocytes were present, infected animals were anesthetized and *A. stephensi* mosquitoes were allowed to feed. Four to eight days after infection, *A. stephensi* mosquitoes were anesthetized, midguts were dissected and stained with mercurochrome (Fisher Scientific, Chicago, IL), and the number of oocysts per midgut was determined. Average infection rates were between 75 and 100%. After the parasite matured to the salivary gland sporozoite stage (14 days postinfection), appropriate groups were exposed to *P. yoelii*-infected *A. stephensi* mosquitoes. The right ears of mice were exposed to vials containing 10 infected mosquitoes for 15 min, and the same vials of mosquitoes were transferred to the left ears for an additional 15 min. Feeding success was assessed by visual observation to detect blood in the midgut; no obvious differences between feedings on naïve and pre-sensitized mice were detected. Sporozoites were isolated as previously described (17).

Blood-stage quantification. Mice were pre-sensitized as described above. Two weeks after three exposures to uninfected mosquitoes, mice were exposed to bites from *P. yoelii*-infected *A. stephensi* mosquitoes. Twenty-four hours post-exposure, blood samples were taken and thin-layer blood smears were made and stained with Giemsa. Subsequently, samples were taken and parasites were quantified each day until mice were euthanized on day 7 postinfection. For the determination of parasitemia levels, 1,000 cells from each sample were counted.

RNA isolation and generation of cDNA. RNA was isolated using an RNeasy mini kit (QIAGEN, Valencia, CA) for ears and TRIzol reagent (Invitrogen, Carlsbad, CA) for spleens and livers. The entire harvested organ was homogenized in the respective lysis buffer. Contaminating DNA was removed from 1 μ g of RNA via DNase I (Invitrogen, Carlsbad, CA) treatment by using 1 U of DNase I and a final concentration of DNase buffer containing 20 mM Tris-HCl (pH 8.4), 2 mM MgCl₂, and 50 mM KCl. DNA-free RNA was used to generate cDNA with oligo(dT) (for cytokine analysis; Invitrogen, Carlsbad, CA) or random primers (for infection studies; Invitrogen, Carlsbad, CA) at a final concentration of 10 ng or 15 ng, respectively, in addition to 500 nM deoxynucleoside triphosphates (Invitrogen, Carlsbad, CA), 200 U of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), 40 U of RNase Out (Invitrogen, Carlsbad, CA), and 5 mM dithiothreitol.

Quantitative PCR and analysis. cDNA was used for quantitative real-time PCR analysis using the 2 \times SYBR green kit (Applied Biosystems, Foster City, CA). Reactions were run on the ABI 7700 sequence detector machine. The SYBR green was used at a 1 \times proportion, along with 300 nM forward and reverse primers for each reaction. Primers (IDT, Coralville, IA) used were hypoxanthine phosphoribosyltransferase (HPRT) primers 5'-GTT GGA AGG CCA GAC TTT GTT-3' and 5'-GAT TCA ACT TGC GCT CAT CTT AGG C-3', IFN- γ primers 5'-AGA GCC AGA TTA TCT CTT TCT ACC TCA-3' and 5'-CCT TTT TCG CCT TGC TGT TG-3', interleukin-4 (IL-4) primers 5'-ACG AGG TCA CAG GAG AAG GA-3' and 5'-AGC CCT ACA GAC GAG CTC ACT C-3', IL-12p40 primers 5'-AACCAT CTC CTG GTT TGC CA-3' and 5'-CGG GAG TCC AGT CCA CCT C-3', and inducible nitric oxide synthase (iNOS) primers as previously described (39). Cytokine primers were designed to overlap introns to help ensure that no DNA amplification occurred. In order to ensure proper amplification, a melting-curve analysis of each product was performed. We employed the comparative cycle threshold method to determine

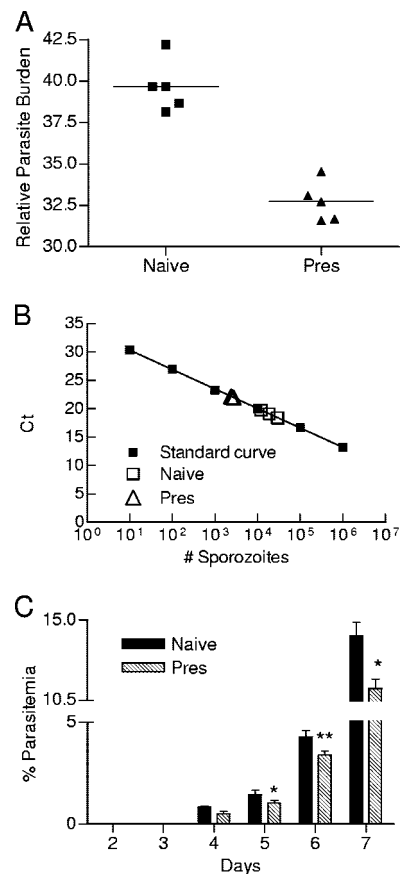


FIG. 1. Lower parasite burdens in mice pre-sensitized with *A. stephensi* saliva. Mice in naïve and pre-sensitized (Pres) groups were exposed to the bites of *P. yoelii*-infected *A. stephensi* mosquitoes. (A) The parasite burdens in the livers were detected by real-time reverse transcriptase PCR, and levels of infection were normalized to levels of mouse HPRT mRNA and expressed as relative parasite burdens ($40 - \Delta\Delta ct$). (B) Naïve and pre-sensitized RNA isolated from the livers was also analyzed by comparison to a standard curve generated using RNA harvested from known numbers of salivary gland sporozoites. Ct, cycle threshold value. (C) Blood-stage infection was monitored each day for 7 days via blood smears from both naïve and pre-sensitized mice. These data are representative of results from three independent experiments (n , 4 to 5 mice per experiment).*, $P < 0.05$; **, $P < 0.10$; Student's t test.

relative differences in parasite burdens. After the generation of cycle threshold values (the cycle numbers at which the reactions crossed the threshold), relative copy numbers were determined according to the following equation: number of copies = $2^{-\Delta\Delta ct}$, where ct is the cycle at which a statistically significant increase in the emission intensity over the background level occurred; $\Delta ct = \Delta ct$ for the sample - Δct for HPRT; $\Delta\Delta ct = \Delta ct$ for the sample - Δct for the calibrator; and Δct for the calibrator is the mean Δct for the naïve control. Parasite loads in livers were determined using primers for *P. yoelii* 18S rRNA 40 h after infection, as previously described (9). The amounts of RNA in the different samples were normalized based on the measurement of the mRNA levels from the mouse housekeeping gene *HPRT* as described above. Cytokine graphs express the means of induction levels that have been normalized to the mean values for naïve tissues. Levels of infection were expressed as relative parasite burdens, represented by the term ($40 - \Delta\Delta ct$) as previously described (51). Significance levels were determined using Student's t test with a confidence level of 95%.

Enzyme-linked immunosorbent assay analysis. Serum was collected from each mouse at the time of organ harvest, and IFN- γ was quantified by enzyme-linked immunosorbent assay according to the instructions of the manufacturer (Pierce Biotechnology, Rockford, IL).

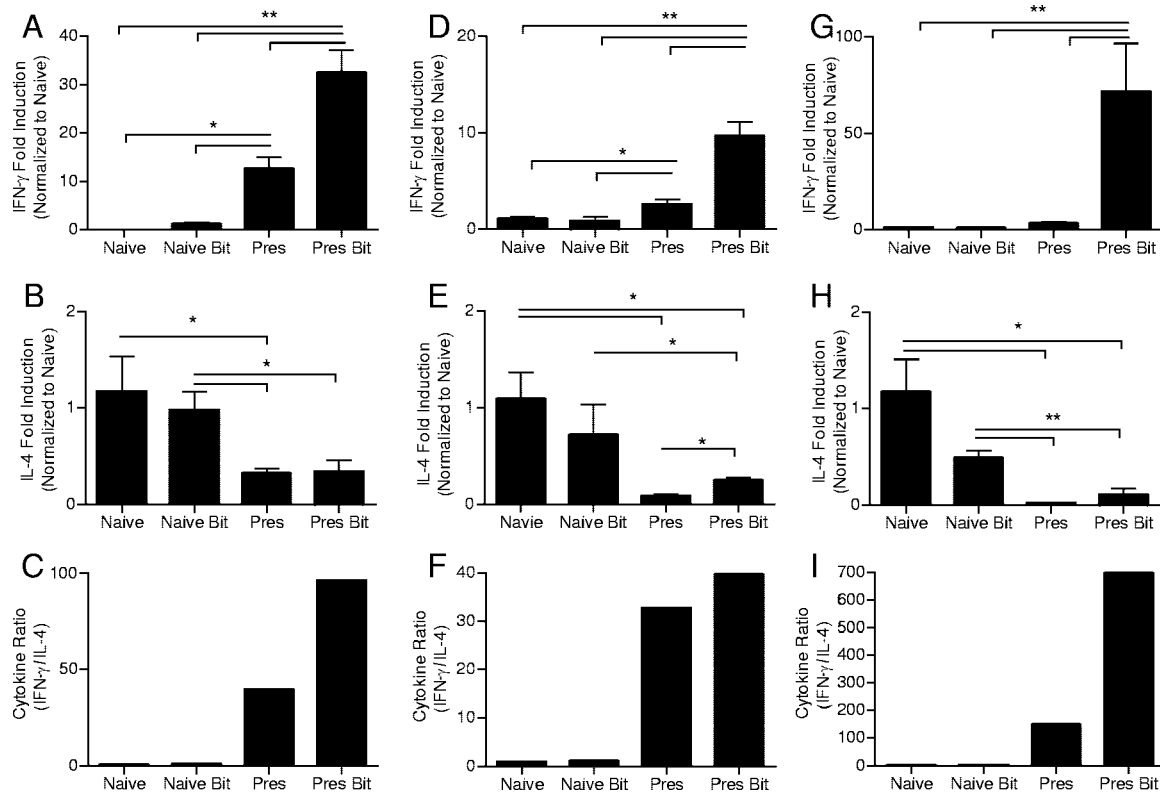


FIG. 2. Presensitization skews the response towards that of the Th1 phenotype. Cytokine levels in naïve and presensitized (Pres) mice and naïve (Naive Bit) and presensitized (Pres Bit) mice that received one final exposure to *A. stephensi* mosquito bites 24 h prior to analysis were quantified. Local (ear, panels A to C) and systemic (liver, panels D to F, and spleen, panels G to I) tissue IFN- γ (A, D, and G) and IL-4 (B, E, and H) mRNA levels were quantified by real-time reverse transcriptase PCR. (C, F, and I) Levels of IFN- γ and IL-4 expression were used to create a cytokine ratio (IFN- γ /IL-4). Data are representative of results from four independent experiments (n , 5 mice per experiment). Error bars represent standard errors of the means. *, $P < 0.05$; **, $P < 0.001$; Student's t test.

RESULTS AND DISCUSSION

In some areas, a single individual can receive nearly 200 mosquito bites/day (52), or more than 10,000 bites/year (22). Malaria infection rates in these vectors range from below 0.1% to 10% (22, 28), indicating that even in the areas with the highest levels of transmission, a single individual is exposed to drastically more mosquito saliva than malaria parasites. To assess the impact of previous exposure to bites from uninfected mosquitoes on *Plasmodium* development, we compared *P. yoelii* burdens in mice preexposed to bites from uninfected *A. stephensi* mosquitoes (presensitized mice) to those in unexposed (naïve) mice. We utilized a system in which bites from infected and uninfected mosquitoes were limited to the ears so that local and systemic responses could be distinguished easily. Forty hours postinfection, after parasite differentiation and amplification but prior to parasite release into the circulation, presensitized mice exhibited significantly reduced *P. yoelii* burdens in the livers compared to naïve mice (Fig. 1A), corresponding to a ninefold reduction in parasite numbers following natural infection (Fig. 1B). These reduced parasite burdens were also evident when blood parasitemia levels were assessed (Fig. 1C). While the prepatent period (i.e., the time it takes to visually detect blood parasites) was not affected by presensitization, the parasitemia levels were reproducibly lower in mice preexposed to bites from uninfected mosquitoes.

The hepatic stage is the stage of the *Plasmodium* life cycle most vulnerable to intervention, and substantial research on liver-stage immunity, using primarily murine models, exists. The predominant effector mechanism mediating this preerythrocytic immunity is the production of IFN- γ that inhibits parasite development within hepatocytes via nitric oxide (16). To investigate the effect that repeated exposure to mosquito saliva has on cytokine profiles, both local tissue and systemic IFN- γ and IL-4 mRNA levels were assessed. Local levels of IFN- γ expression were relatively low in naïve animals and mice that were exposed a single time, while presensitized animals readily produced IFN- γ in response to *A. stephensi* bites (Fig. 2A). Furthermore, presensitized mice expressed reduced levels of IL-4 compared to naïve animals (Fig. 2B). The presensitized mice were Th1 biased, as illustrated by the cytokine ratio (IFN- γ /IL-4), with much more IFN- γ than IL-4 being expressed (Fig. 2C). Therefore, *A. stephensi* saliva significantly changes the local cytokine environment at the tissue site where parasites are introduced; Th1 bias following mosquito presensitization also occurs systemically in the liver (Fig. 2D to F), spleen (Fig. 2G to I), and serum (data not shown), indicating a systemic cytokine shift to a Th1 profile. These results suggest that the up-regulation of IFN- γ is part of the phenotype of protection against *P. yoelii* infection associated with mosquito saliva presensitization.

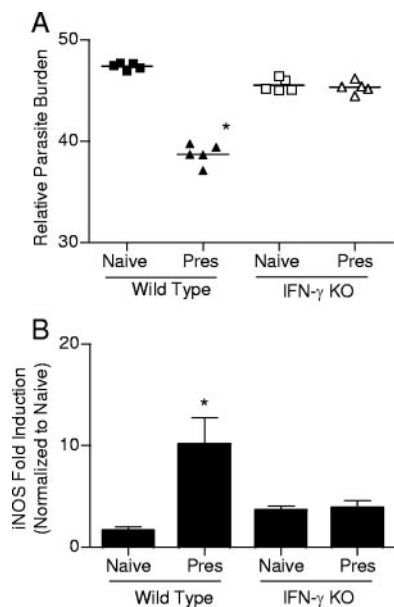


FIG. 3. Lower levels of hepatic parasitemia are IFN- γ dependent. BALB/c WT (closed symbols) and IFN- γ KO (open symbols) animals in both naïve (squares) and presensitized (Pres; triangles) groups were exposed to the bites of *P. yoelii*-infected *A. stephensi* mosquitoes. Parasite burdens (A) and iNOS mRNA levels (B) were quantified by real-time reverse transcriptase PCR 40 h postinfection. Levels of infection were normalized to mouse HPRT mRNA levels and expressed as relative parasite burdens (40 - $\Delta\Delta$ ct). Data are representative of results from two independent experiments (n , 5 mice per experiment). Error bars represent standard errors of the means. *, $P < 0.05$.

To further evaluate the role of IFN- γ , WT and IFN- γ KO BALB/c mice were presensitized to bites from uninfected mosquitoes or were left naïve prior to natural infection with *P. yoelii*. Presensitization-associated protection against *P. yoelii* infection was abolished in the absence of IFN- γ (Fig. 3A), indicating that IFN- γ is essential for the protective response. Because nitric oxide is required for immunity to *P. yoelii* liver infection (5), we measured iNOS mRNA levels in the livers of naïve and presensitized WT and IFN- γ KO mice (Fig. 3B). The 10-fold up-regulation of iNOS levels in response to *A. stephensi* saliva preexposure was not observed in IFN- γ KO mice, suggesting that the IFN- γ induced following presensitization to mosquito bites leads to nitric oxide-induced killing of malarial parasites. Interestingly, iNOS mRNA levels in the local ear environment were also increased by presensitization as early as 5 h postinfection (Fig. 4).

As IL-12 is necessary for immunity against *P. yoelii* (17) and has been proposed as a potential adjuvant for antimalaria vaccines (44, 47), we evaluated IL-12p40 mRNA levels in the livers (Fig. 5A) and spleens (Fig. 5B) of presensitized animals. We detected significantly higher levels of IL-12p40 mRNA in the organs of presensitized animals than in those of naïve mice.

Here, we show that the immunity induced following *A. stephensi* saliva presensitization involves local and systemic up-regulation of IFN- γ and iNOS (Fig. 2 to 4; data not shown). These results raise the question of what tissue is primarily affected by this protective mechanism and the timing of this response. The majority of infectious malaria sporozoites released during mosquito blood feeding do not immediately en-

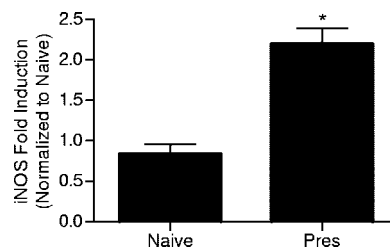


FIG. 4. Local induction of iNOS occurs 5 h after exposure to infected *A. stephensi* mosquitoes. BALB/c animals in both naïve and presensitized (Pres) groups were exposed to the bites of *P. yoelii*-infected *A. stephensi* mosquitoes, and iNOS mRNA levels were quantified by real-time reverse transcriptase PCR 5 h postinfection. Error bars represent standard errors of the means. Data are representative of results from two independent experiments (n , 5 mice per experiment). *, $P < 0.05$; Student's t test.

ter the circulation; instead, sporozoites are deposited into the skin, where they eventually move into dermal vessels (53). Although the timing of vascular entry remains a matter of debate, it is clear that some sporozoites remain in the skin and may take substantially longer than others to enter the bloodstream (53). This delayed timing coupled with the suggestion that sporozoites are directly susceptible to nitric oxide-mediated killing (29) invokes a model of reduction in *P. yoelii* burdens in the liver due to lower numbers of parasites entering the circulation. To determine the chronology and location of parasite killing in presensitized mice, we evaluated the relative parasite burdens in both the ears and the livers at various time points (5 to 30 h) following the natural infection of naïve and presensitized mice (Fig. 6A and B). By 10 h postinfection, parasite burdens in the ears of both groups of mice had re-

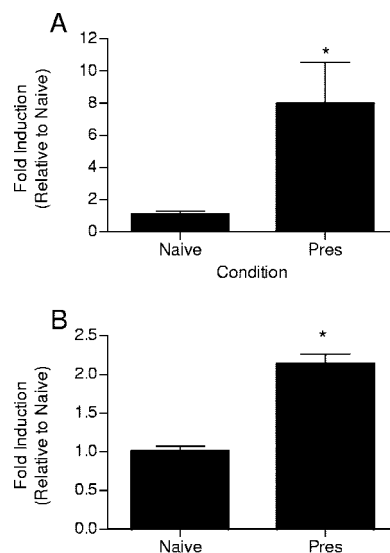


FIG. 5. IL-12p40 is induced by presensitization. BALB/c animals in both naïve and presensitized (Pres) groups were exposed to the bites of *P. yoelii*-infected *A. stephensi* mosquitoes, and IL-12p40 mRNA levels in livers (A) and spleens (B) were quantified 40 h postinfection by real-time reverse transcriptase PCR. Error bars represent standard errors of the means. Data are representative of results from two independent experiments (n , 4 mice per experiment). *, $P < 0.05$; Student's t test.

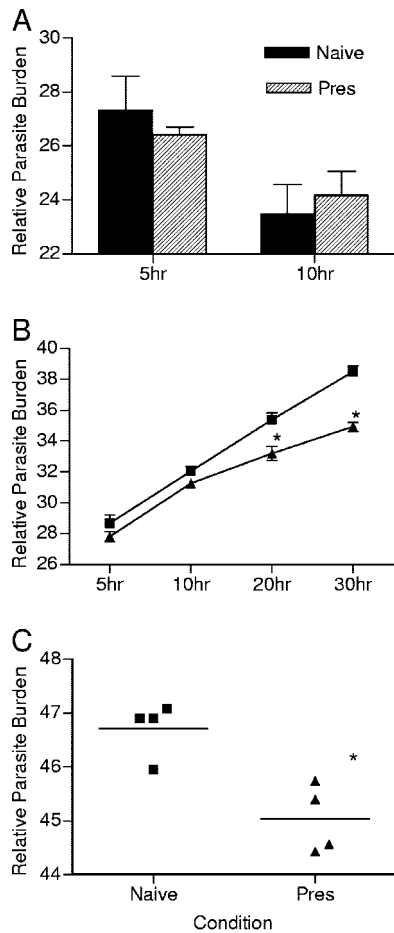


FIG. 6. The protective phenomenon associated with presensitization to mosquito saliva is evident at 20 h postinfection and is localized to the liver. Presensitized (Pres) and naïve animals were subjected to the bites of *P. yoelii* 17XNL-infected *A. stephensi* mosquitoes. Animals were sacrificed at 5, 10, 20, and 30 h postinfection, and 18S rRNA levels in ears (A) and livers (B) were quantified. Squares, naïve mice; triangles, presensitized mice. (C) Presensitized and naïve animals were infected with 1,000 *P. yoelii* 17XNL sporozoites intravenously through the tail vein immediately following a fourth presensitization to uninfected bites and euthanized 40 h postinfection; subsequently, parasite 18S rRNA levels were quantified by real-time reverse transcriptase PCR. Levels of infection were normalized to mouse HPRT mRNA levels and expressed as relative parasite burdens ($40 - \Delta\Delta\text{Act}$). Data are representative of results from two independent experiments ($n = 4$). Error bars represent the standard errors of the means. *, $P < 0.05$; Student's *t* test.

turned to background levels, indicating that the same numbers of sporozoites left the bite sites (Fig. 6A) and entered the livers (Fig. 6B) in naïve and presensitized mice. The difference in liver parasite burdens was not evident until 20 h postinfection. As increases in parasite burdens during the first 5 to 40 h of infection are due primarily to parasite multiplication, our data suggest that the protective mechanism due to presensitization operates in the liver. To conclusively address whether liver protection could be occurring, we exposed naïve and presensitized mice to bites from uninfected mosquitoes and immediately challenged them intravenously through the tail vein rather than exposing the mice to infected mosquitoes. Forty

hours post-intravenous challenge, *P. yoelii* burdens in the livers of presensitized mice remained significantly lower, demonstrating that the protection was due, in part, to the response in the liver (Fig. 6C).

The substantial effort towards the production of an efficacious malaria vaccine has yet to yield a suitable product. Following the initial report that vaccination with radiation-attenuated sporozoites can protect against malaria challenge (36), extensive research has elucidated the immunological parameters that confer such protection. The protective mechanisms identified for malaria sporozoite and liver stages are remarkably similar in rodent models and human volunteers (30). It is possible that residual salivary components remaining from the isolation of sporozoites can partially explain the protection observed in the studies involving radiation-attenuated sporozoites. One original study investigating irradiated sporozoites as vaccine components used repeated vaccination with large amounts of mosquito salivary gland homogenate (SGH; 70 glands) as a control and demonstrated that this procedure confers partial protection to *Plasmodium berghei* infection in mice (1, 2). More recently, it has been demonstrated that *Plasmodium gallinaceum* parasitemia levels are increased in the presence of *Aedes fluviatilis* saliva in a chicken malaria model, a response that was reversed with prior exposure to mosquito saliva (43).

It is well established that naïve travelers and children are at increased risk for severe malarial disease compared to native adult populations in regions where malaria is endemic and that the native adults do not exhibit sterile immunity; rather, their degree of natural immunity results in asymptomatic infections with lower parasite burdens in the circulation (42). Historically, this phenomenon has been attributed to the gradual onset of immunity due to repeated parasite infections (48). We propose that the extensive exposure to mosquito saliva that accrues over time in regions where malaria is endemic results in a Th1 climate that influences pathogen establishment. Coupled with specific immunity to malaria parasites, this inhospitable environment may contribute to lower malarial burdens in adults in regions of endemicity. Here, we utilized a model in which we infected animals by using 10 mosquitoes with infection rates ranging from 75 to 90% and oocyst counts of 24 to 39 per mosquito to ensure parasite numbers adequate for detection. The majority of mosquitoes in the field harbor only one oocyst (12), with infection rates never greater than 10% (22, 28). We did not observe sterile immunity in our model system; however, it is possible that the cytokine shift that we detected may be adequate to control the small numbers of parasites an individual encounters in a natural setting.

The IFN- γ up-regulation due to presensitization that we detected diminished over time, as was evident by the lower levels of IFN- γ mRNA that were present in ears and livers in presensitized mice that did not receive one final bite prior to analysis. This observation is consistent with the typical rapid loss of antimalarial immunity when an individual leaves a region where malaria is endemic (48). We suggest that this loss of immunity can be explained partially by an absence, or a drastically lower level, of boosting with mosquito saliva. In our study, mosquito exposure ceased after the infection was initiated. It is intriguing to postulate that increased resistance may be observed if hosts continued to be exposed to mosquito bites

throughout the duration of the infection, as would be the case in a region where the disease is endemic. IFN- γ production is associated with primary immune responses to blood-stage *Plasmodium* infections in mice (14, 34) and humans (31) and appears to be crucial for the development of protective immunity (15, 49). Although in this study we did not explore blood-stage immunity, the increased levels of IFN- γ in the sera induced by repeated mosquito exposures suggest that blood-stage immunity may be influenced and that continued boosting with mosquito saliva may increase this response.

The systemic shift in cytokine balance that we detected was not unique, as bite-induced systemic responses involving *Rhipicephalus sanguineus* (tick) (19) and *Culex pipiens* and *Aedes aegypti* (58) have been documented previously. The majority of studies assessing arthropod modulation of host immune responses have focused on one-time exposures to bites or salivary gland components (for reviews, see references 6, 8, and 50). Combined, these studies engender a model in which an initial exposure to arthropod saliva induces a Th2 immune response, potentiating infectivity of a variety of vector-borne pathogens. Among mosquito-transmitted pathogens, infectivity of *P. berghei* (54), Cache Valley virus (18), La Crosse virus (37), and vesicular stomatitis virus (26, 27) is enhanced in the presence of saliva. In vesicular stomatitis virus infection, increased viral loads are associated with an SGH-dependent decrease of type I interferons in vitro (26). Feeding of both *Culex pipiens* and *Aedes aegypti* mosquitoes on mice induces increased levels of systemic IL-4 and IL-10, with a concomitant decrease in IFN- γ production (58). Similarly, the inoculation of *Aedes aegypti* SGH along with Sindbis virus results in higher levels of Th2 cytokines and reduced expression of interferons (46).

While we detected increased Th2 responses in naïve mice compared to presensitized hosts, we did not observe an increase in IL-4 upon one exposure to *A. stephensi* bites. Our results are in agreement with those of previous studies investigating skin and lymph node cytokine production in response to *A. stephensi* bites (13). In contrast to our observations, studies exploring splenic cytokine production in response to exposure to *Aedes aegypti* or *Culex pipiens* mosquitoes detected an increase in Th2 cytokine production in C3H/HeJ mice; however, in a congenic host strain (C3H/RV), Th1 cytokines predominated (58). In vitro studies suggest that mosquito species may differ in their abilities to modulate host immune responses (55); the discrepancies between our results and those of previous work investigating in vivo immune modulation by mosquito exposure may be attributed to variations in the experimental approaches or may reflect actual differences among mosquito species.

Data from explorations of the effect of multiple, repeated exposures to arthropod saliva are scant. While single exposures to sand fly bites (24) or SGH (4, 32, 35) are associated with increased levels of Th2 cytokines, repeated exposure leads to a switch to Th1 immunity (4, 24). This switch to the production of primarily IFN- γ at the bite site induces resistance to *L. major* infection. In contrast, repeated exposure to *Aedes aegypti* bites results in the elevated production of antigen-specific IL-4 in cultured spleen cells, although this reaction is not detected in response to concanavalin A stimulation (10). Multiple infestations of ticks are generally thought to lead to heightened levels of Th2 cytokines. As the information in the literature

conflicts as to whether tick infestation blocks *B. burgdorferi* transmission (41, 56) and what type of cytokine response is favorable or detrimental for spirochete transmission (25, 57), the role that the IL-4 induced by repeated tick infestation plays in resistance to Lyme disease is unclear. In contrast to sand flies and mosquitoes, ticks take several days to complete feeding. The modulation of host immunity towards Th2 cytokine expression may provide an evolutionary advantage to ticks to avoid host sensitization to tick feeding; rapid-feeding arthropods may not require such immunomodulation. Interestingly, animal species that have acquired immunity to tick feeding express cutaneous basophil hypersensitivity reactions at attachment sites (3), and these reactions are mediated by Th1 responses (20). Furthermore, even though the immune response of mice to repeated tick infestations is dominated by IL-4, IFN- γ (21) and immunoglobulin G2a (11) levels increase with multiple exposures. In conjunction with our observations, a model indicating that repeated exposure to rapid-feeding arthropods induces Th1 profiles that lead to increased resistance to pathogen transmission is beginning to emerge.

It has been demonstrated previously that the treatment of BALB/c mice with recombinant IL-12 prior to sporozoite challenge protects against *P. yoelii* infection (47), suggesting that short-term prophylaxis with recombinant IL-12 could be used to combat malaria. The increased levels of IL-12p40 mRNA detected in presensitized animals suggest that it is possible that "vaccination" with saliva from uninfected mosquitoes may be an efficient method to induce IL-12 and avoid the toxicity associated with treatment with recombinant proteins.

It is thought that individuals living in mosquito-dense areas naturally become desensitized to mosquito bites. The particular mechanism of this tolerance remains to be defined but is known to be associated with a loss of the wheal and flare reactions of type I hypersensitivity as well as delayed reactions (38). Although cytokine responses in desensitized individuals have not been evaluated, the hypersensitivity reactions in response to mosquito saliva are Th2 mediated (i.e., immunoglobulin E mediated for type I and eosinophil mediated for type IV). Therefore, it is possible that desensitization may cause a reduction in Th2 cytokines, thus promoting an even greater Th1 environment than we detected in our model system.

New vaccine targets and novel strategies will be essential for the ultimate success of malaria vaccine development, and data suggest that any measure that limits parasite densities in the liver will reduce the morbidity and mortality associated with malaria infection (33). Our findings imply that mosquito salivary constituents may be effective components in such a vaccine. In this context, saliva can be thought of as a nonspecific potentiator; as long as vaccinated individuals encounter malaria parasites together with mosquito saliva, the potentiator will be effective at inducing a Th1-biased environment that is known to be effective against malaria infection.

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