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The innate and adaptive response to mosquito saliva and *Plasmodium* sporozoites in the skin

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Abstract

A malaria infection begins when an infected mosquito takes a blood meal and inoculates parasites into the skin of its mammalian host. The parasite then has to exit the skin and escape the immune cells that protect the body from infection and alert the system to intruding pathogens. It has become apparent that this earliest stage of infection is amenable to vaccine interventions. Here, we discuss how the innate and adaptive host response to both mosquito saliva and the parasite may interfere with the infection, as well as possible mechanisms the parasite might use to circumvent the host defense.

Keywords

malaria; sporozoite; mosquito saliva; dermal immune system; human innate immunity; human adaptive immunity; rodent malaria model

Introduction

As a female *Anopheles* mosquito probes for blood, it injects a few nL of saliva into the dermis, which in the case of mosquitoes infected with the parasite *Plasmodium* can carry infectious sporozoites. Once deposited in the skin, the motile sporozoites migrate through the dermis to find and invade blood vessels. The blood circulation carries the parasite to the liver, where the parasite establishes the exoerythrocytic stage of malaria infection. Importantly, mosquito saliva, sporozoites, and tissue damage caused by the probingFIG process initiate local innate and adaptive immune responses that are activated within a time frame similar to that during which the sporozoites are attempting to enter dermal blood vessels.[1,2] Thus, this dermal reaction is likely relevant for sporozoite infectivity. Mosquito saliva is known to have anticoagulant, vasodilatory, and immunomodulatory activities and is detectable in the skin for up to 18 h after the bite.[3] Depending on previous exposure to mosquito bites and the allergic history of the host, the inflammatory response can range from a small wheal and flare to large swellings. An immediate local cutaneous response within 10–15 min after the mosquito bite is especially common after repeated exposure.[4] It

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Conflict of interest

The authors declare no conflicts of interest.

has been observed that the rate of sporozoite exit into blood and lymphatic vessels drops over the first hour, with highest exit rates in the first 20 min after inoculation.[2] Of note, aside from the possible impact on sporozoite infectivity, the local response at the mosquito bite site[5,6] likely affects the immunological environment in the skin-draining lymph nodes, which is the primary site of priming of parasite-specific $CD8^+$ T lymphocytes.[7] This article attempts to review the dermal immune response to mosquito saliva and the *Plasmodium* sporozoite. Given that our current knowledge is based on studies that used mice without prior exposure to mosquito saliva or the parasite, our understanding of the effects of the adaptive response on sporozoite exit from the skin is very limited.

The dermal immune system

As the primary interface between the body and the outside environment, the skin protects the host against injuries and microbial pathogens and harbors cells of the innate and adaptive immune responses. The outermost layer of the skin, the epidermis, consists of terminally differentiated keratinocytes and Langerhans cells, the major epidermal dendritic cell (DC) subset. Separated from the epidermis by a basement membrane, the underlying dermis is less densely packed with cells and is composed of extracellular matrix such as collagen and elastin fibers. Capillary beds and lymphatic vessels support and drain the dermis, allowing for a constant flux of immune cells in and out of the dermis. A vast range of immunologically relevant cell types populates the dermis, including mast cells, macrophages, subsets of DCs, innate lymphoid cells, and T cells (including $CD4^+$ T_H1 and T_H2, T_H17, γδ T cells, and natural killer (NK) T cells). A comprehensive review describing the cutaneous cell populations was recently published.[8]

Innate dermal response to the mosquito bite and *Plasmodium* sporozoites

Mast cells

Mast cells are tissue-resident cells, and as first responders against pathogens are strategically positioned at the interface of the host and environment. Within minutes of exposure to pathogens, mast cells are able to respond with the release of previously synthesized mediators from cytoplasmic granules, as well as through initiation of *de novo* synthesis.[9] A study examining mast cell–dependent responses to the bites of *A. stephensi* mosquitoes reported cutaneous mast cell degranulation leading to local fluid and neutrophil influx and lymph node hyperplasia as a result of recruitment of lymphocytes, DCs, and monocytes.[5] This inflammatory influx in response to mosquito bites was found to be absent in mast cell–deficient mice, unless reconstituted with mast cells.[5] Although a glycoprotein present in *A. stephensi* saliva has previously been reported to have neutrophil chemotactic activity *in vitro*,[10] the mast cell dependency of the local neutrophil influx following mosquito bite found by Demeure *et al*. suggests that neutrophils are attracted by mast cell mediators, rather than being directly recruited by chemotactic components of the mosquito saliva. Mast cell–derived tumor necrosis factor (TNF)-α, a pleiotropic inflammatory cytokine, and MIP-2, the functional analogue of human interleukin 8, are known to recruit neutrophils,[9] and while a previous study found that mosquito salivary gland extracts reduce TNF-α release from mast cells,[11] a more recent report showed that mosquito saliva elevates the levels of MIP-2 in the skin in a mast cell–dependent manner.[6] The effect of mast cell activation on the *Plasmodium*

sporozoite remains unknown, but mast cells have been found in association with sporozoites in the dermis after an infected mosquito bite.[3] It has previously been found that activation of mast cells promotes a breakdown of endothelial cell junctions and causes an increase in blood flow,[12] effects that the sporozoite might exploit in its attempts to enter dermal blood vessels.

Neutrophils and monocytes

Upon activation of mast cells and other tissue-resident sentinel cells by the mosquito saliva and tissue damage, these cells release mediators, many of which will activate the local endothelium. The ensuing upregulation of endothelial cell adhesion molecules initiates the leukocyte adhesion cascade and results in the recruitment of immune cells to the inflammatory site. Neutrophils circulate in the peripheral blood and are believed to be the first leukocytes recruited to inflammatory sites.[13] Once extravasated into the site of infection, neutrophils are capable of eliminating pathogens by phagocytosis and intracellular killing, as well as via the extracellular release of antibacterial peptides, peroxidases, and proteases. A third mechanism involves the breakdown of the neutrophil and release of its DNA and histones, which form neutrophil extracellular traps (NETs) that can immobilize pathogens.[14]

Using a transgenic mouse with enhanced green fluorescent protein (EGFP) under the control of the lysozyme promoter, which r fluorescently labels neutrophils and monocytes,[15] rapid and sustained neutrophil influx into the site of the sand fly bite was observed.[16] With the same mouse model, the influx of $EGFP^+$ cells into the mosquito bite site has been imaged, and fluorescent cells were observed as soon as 25 min after the bite of a mosquito.[17] Histological analyses of mouse skin also identified an influx of polymorphonuclear cells 3 h after the mosquito bite.[3] A recent study used flow cytometry to investigate the local inflammatory response after intradermal injection of salivary gland extracts and sporozoites.[18] Interestingly, while 2 h after injection the influx of neutrophils and inflammatory monocytes was comparable in the presence or absence of sporozoites, it was found that after 4 h, the number of infiltrated neutrophils was approximately fivefold higher, and 24 hours after injection, both the numbers of neutrophils and inflammatory monocytes were increased if sporozoites had been injected.[18] While this points to a parasite-dependent recruitment or retention of neutrophils and monocytes, this work used the surrogate of intradermal injection, and it remains to be determined whether the same is true for infected and uninfected mosquito bites. Importantly, the same study suggested that the impact of neutrophils on *Plasmodium* sporozoite exit from the skin and infection of the liver is likely negligible, since it was found that neutrophil depletion, which resulted in a 90% decrease of the dermal neutrophil infiltrate, had no effect on the number of parasites developing in the liver after intradermal inoculation of sporozoites.[18] Of note, the aforementioned studies[3,17,18] were performed in naive mice, and a more rapid neutrophil infiltration and, as a result, a possibly larger impact of neutrophils on sporozoite infectivity in mosquito saliva– or sporozoite-immunized mice cannot yet be ruled out.

Dermal T_{reg} cells and DCs

In normal skin, approximately 10% of dermal $CD4^+$ cells have a $Foxp3^+$ regulatory T cell (T_{reg}) cell phenotype,[19] and, as a result, the dermis is intrinsically an immunotolerant environment, a quality that the sporozoite might use to its advantage. Interestingly, a recent study investigating motility of T_{reg} cells and DCs in the skin of naive mice following uninfected and *P. berghei*–infected mosquito bites found that $CD11c^+$ and $Foxp3^+$ cells had a higher mobility in the skin after exposure to infected bites, resulting in a tolerogenic response.[20] This result points to a direct impact of the parasite material on T_{reg} cells and DCs in the skin and, together with the data, by Mac-Daniel *et al*., who found a parasite-specific neutrophil response, suggests that not only the mosquito saliva, but also the parasite itself, can affect the dermal immune response. However, since two studies have previously shown *P. berghei*–infected mosquitoes to be more willing to probe compared to uninfected mosquitoes,[3,21] an increased probing time and concomitant increase in tissue damage and exposure to saliva may explain the higher mobility of T_{reg} cells and DCs.

Adaptive dermal response to the mosquito bite and *Plasmodium* sporozoites

The effect of parasite- and saliva-specific adaptive responses in the skin on the infectivity of inoculated sporozoites has not been studied in great detail. Adaptive immunity to mosquito saliva may be affecting the sporozoite in its journey through the dermis, and two studies using murine models have investigated the effect of sensitization to mosquito saliva on sporozoite infectivity, with disparate results. One study found that pre-exposure of mice to uninfected mosquito bites moderately reduced the parasite burden in the liver after infection with *P. yoelii* sporozoites.[22] This effect was correlated with increased expression of interferon γ (IFNγ), a key player in the innate response to the malaria parasite, in both skin and liver tissue. Importantly, the difference in infectivity was abolished in *IFNγ* knockout mice, suggesting that upregulation of IFNγ is responsible for the protective effect of presensitization to mosquito bites.[22] A subsequent study found no effect of presensitization to mosquito bites on the infectivity of *P. berghei* and *P. yoelii* sporozoites injected intradermally.[23] While slight variations in the presensitization protocols used in the two studies may account for the difference in the observed effect, the main difference was that Donovan *et al.* used mosquito bite as route of sporozoite challenge, whereas Kebaier *et al.* performed the challenge by intradermal injection of homogenized mosquito salivary glands containing sporozoites. Thus, the environment in which sporozoites were introduced into the host was drastically different. These marginal effects are in contrast to the powerful protection against the protozoan parasite *Leishmania major* conferred by pre-exposure of mice to uninfected sand fly salivary gland extract[24] or sand fly bites.[25] However, *L. major* is known to establish infection in the early infiltrate of innate immune cells, which capture but are unable to destroy the parasites.[16] The observed increased IFNγ production in mice pre-exposed to sand fly saliva may thus lead to activation of macrophages and result in enhanced parasite clearance.[25] The *Plasmodium* sporozoite, however, is not known to require uptake by host innate immune cells and instead rapidly exits the dermis, and as a result may be unaffected by a heightened dermal immune response in mosquito bite–presensitized mice. Naturally acquired immunity induced by cumulative malaria exposure in endemic areas does

not appear to lead to sterile protection,[26] and it has been hypothesized that this might be due to the low number of sporozoites inoculated per mosquito bite, resulting in an incomplete adaptive response against sporozoites. However, sterile protection against malaria can be achieved through immunization with irradiated sporozoites in rodents and humans, an immunity that involves humoral and $CD8^+$ T cell–based responses, and the sporozoite surface protein CSP has been implicated as one of the key antigens in this response. Indeed, in the rodent malaria model, an antibody targeting CSP is known to protect against sporozoite challenge upon passive transfer.[27] In humans immunized with RTS,S, a malaria vaccine candidate based on CSP, increasing titers of CSP-specific antibody are correlated with a reduced risk for clinical malaria; however, this protection is only seen at titers above 100 EU/ml.[28] High titers of antibodies against CSP are able to inhibit the motility of *P. berghei* sporozoites in the skin,[29] and it was recently found that *P. yoelii* sporozoites inoculated by mosquito bites are more sensitive to passive transfer of CSP-specific antibodies compared to a similar, but slightly larger number of sporozoites inoculated intravenously,[30] suggesting that antibodies are effective in the skin. However, the drawback of targeting sporozoites with antibodies is that a single breakthrough sporozoite can cause infection. It remains undetermined whether the antibody titers found in immunized individuals are capable of completely neutralizing sporozoites in the dermis.

While healthy skin is home to few B cells, it has been shown that, even in its steady state, there are twice the number of T cells in the skin than in peripheral blood.[31] Early studies of protective immunizations with sporozoites in mice found that $CD8^+$ T cells are induced[32] and active against the liver stage of the parasite.[33] Further efforts identified the skin-draining lymph nodes as the primary location for the priming CSP-specific IFNγ-producing $CD8^+$ T cells.[7] In other systems, following priming by tissue-derived DCs, T cells upregulate skin-homing receptors[31] and migrate into the dermis, where they are found in close proximity to postcapillary venules and near the dermal–epidermal interface.[34] Rather than the traditional memory T cell population that was described circulating between skin-draining lymph nodes and the periphery, these cells are tissue-resident memory T cells (T_{RM} cells)[8] that are functional immune sentinels, strategically positioned where a secondary challenge with a previously encountered pathogen is likely. How parasite- or mosquito saliva–specific T cells react to *Plasmodium* sporozoites in the skin of immunized individuals has yet to be studied. It is possible that, once activated, T_{RM} cells recruit circulating effector memory cells for support in a secondary challenge.[8] Whether T_{RM} cells are present at higher rates and how the dermal population of $Foxp3^+$ T_{reg} cells, which was found responsive to *P. berghei*–infected mosquito bites in naive skin,[20] changes with exposure to infected mosquito bites or immunization of the host remains unknown and may be interesting in the context of new vaccine strategies.

Potential immune-evasion strategies of *Plasmodium* sporozoites

Long-term coevolution of host and parasite has likely led to sporozoites that are successful at bypassing the dermal host response, and it is crucial for future vaccine approaches to advance our knowledge of how sporozoites escape innate and adaptive immunity. To yield sterile protection, the antibody-based adaptive response against the sporozoite appears to require high titers of sporozoite-specific antibody,[28–30] which may point to the ability of the

parasite to escape the humoral host response. Sporozoites have been reported to release a trail of parasite material while gliding *in vitro*,[35] and it has been discussed that this shedding may remove antibodies bound to the sporozoite surface. Also, host cell traversal has previously been discussed as a potential mechanism relevant for the sporozoite to evade phagocytosis in the skin, and parasites that were cell-traversal deficient are less infectious after intradermal inoculation and were found to associate more with $CD11b^+$ cells, monocytes, and neutrophils.[17] Additionally, the remarkable speed of 1–2 μm/s at which sporozoites glide in the skin[36] may be a mechanism to escape the much slower-moving host cells, which migrate at approximately 0.1 μm/s. One could speculate that sporozoites have adapted to exit the skin quickly before the innate response is initiated, thus escaping before infiltration of neutrophils and monocytes. Other parasites have evolved a variety of mechanisms to overcome the host response; however, little is known about how *Plasmodium* sporozoites interact with the immune system and whether they secrete factors designed to influence the immune response. Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine involved in many innate and adaptive immune functions, including recruitment of neutrophils and activation of macrophages and T lymphocytes.[37] A *Plasmodium* homologue of MIF is expressed in *P. yoelii* salivary gland sporozoites ,and Py*MIF* knockout sporozoites showed a liver-stage growth defect upon intravenous inoculation of sporozoites.[38] However, sporozoite infectivity upon intradermal inoculation or mosquito bite was not assessed, and may show a more enhanced phenotype through the interaction of sporozoites with a wide range of immune cells in the skin.

Researchers have attempted to answer the question of what percentage of the sporozoite inoculum is successfully entering dermal blood vessels, both in humans and rodent models. Two studies using the rodent parasite *P. yoelii*, which in terms of sporozoite infectivity models that of *P. falciparum* sporozoites closely, reported that the infectious dose (ID_{50}) of *P. yoelii* sporozoites inoculated intravenously ranges between 3 and 10.6 sporozoites, depending on the study and the strain of mice used in the study.[39,40] The infectivity of *P. yoelii* sporozoites after intradermal inoculation was reported to be comparable to infectivity of those inoculated intravenously.[1] For both *P. falciparum*– and *P. yoelii*–infected mosquito bites, it was found that approximately two infectious bites are required to result in blood-stage infection of 50% of the human subjects or mice.[40–42] This surprising inefficiency can largely be explained by a study that examined the sporozoite inoculum of *P. yoelii*–infected mosquitoes into the ears of mice, which found that, while the mean of sporozoite inoculation was 123 sporozoites, ~ 22% of *P. yoelii*–infected mosquitoes inoculated no sporozoites and ~ 25% injected fewer than 10 sporozoites.[43] These studies suggest that less than 20% of sporozoites successfully exit the dermis and travel to the liver. It remains possible that the innate immune response in the skin has an impact on the sporozoite inoculum, but more studies clearly need to be performed. Importantly, all of these studies investigated the number and infectivity of the sporozoite inoculum in naive individuals, and, while it has previously been suggested that a decreased number of sporozoites is inoculated in mice passively immunized with a CSP-specific monoclonal antibody,[29] the percentage of sporozoites that successfully invade blood vessels in saliva- and sporozoite-immunized individuals has not been studied.

Conclusions and outstanding issues

Over the course of a long evolutionary history with their mammalian hosts, *Plasmodium* sporozoites have developed mechanisms to evade the host immune system in order to establish infection. In the absence of strong data of an efficient dermal response against the parasite, one can only speculate that the sporozoite is capable of leaving the skin rapidly, giving the host little time to interfere. Conversely, it may be in the interest of the host to prevent a strong immune response against mosquito saliva to avoid severe reactions. A problem in our understanding of host–parasite interactions in the skin is that the majority of studies were performed in mice without prior exposure to mosquito saliva or the parasite. As a result, it remains unclear how presensitization alters the dermal response of mast cells, neutrophils, monocytes, or lymphocytes against the sporozoite, and the current data may be of limited relevance, considering the exposure to uninfected and infected mosquito bites in the field. It is crucial to understand, on a molecular level, the means by which sporozoites achieve this in order to design interventions aimed at making them more susceptible to the host response.

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