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The Skin: Where Malaria Infection and the Host Immune Response Begin[1]

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Abstract

Infection by malaria parasites begins with the inoculation of sporozoites into the skin of the host. The early events following sporozoite deposition in the dermis are critical for both the establishment of malaria infection and for the induction of protective immune responses. The initial sporozoite inoculum is generally low and only a small percentage of these sporozoites successfully reach the liver and grow to the next life cycle stage, making this a significant bottleneck for the parasite. Recent studies highlight the importance of sporozoite motility and host cell traversal in dermal exit. Importantly, protective immune responses against sporozoites and liver stages of Plasmodium are induced by dendritic cells in the lymph node draining the skin inoculation site. The cellular, molecular and immunological events that occur in the skin and associated lymph nodes are the topic of this review.

Keywords

malaria; dermis; sporozoites; plasmodium; dendritic cells; CD8+ T cells

Introduction

Malaria, one of the most important infectious diseases worldwide, is caused by protozoan parasites of the genus *Plasmodium*. These parasites cycle between a vertebrate and mosquito host and experience a significant reduction in numbers during transmission. Sexual or asexual reproductive cycles follow transmission and restore parasite numbers in the mosquito or vertebrate host, respectively. Thus, infection in the vertebrate host has two phases: an asymptomatic pre-erythrocytic stage, when parasite numbers are low, and a symptomatic erythrocytic stage, composed of iterative cycles of replication in host red blood cells. The pre-erythrocytic stage is short-lived, yet critical for the establishment of malaria infection. It is comprised of sporozoites, which are inoculated by infected mosquitoes, and the liver stages (or exoerythrocytic stages) into which they develop. In this review we will focus on the early stages of malaria infection, following the fate of inoculated sporozoites and outlining how the immune response to these stages is initiated and sustained. We will conclude with some thoughts as to how this knowledge can inform the generation of improved malaria vaccine candidates.

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The skin stage of malaria infection

Until recently, our knowledge of the molecular interactions between host and parasite during the early stage of malaria infection was limited due to the small numbers of sporozoites and liver stages present in the mammalian host. Indeed, a review of the literature between 1970 to 1995 indicates that malariologists labored under the assumption that sporozoites rapidly left the inoculation site and significant interactions between host and parasite did not begin until sporozoites invaded hepatocytes and began to develop. Recent studies have now shown that sporozoites spend several hours at the inoculation site [1] and initiate an immune response in the lymph nodes which drain this site [2], thus bringing this early stage of infection into the limelight.

Sporozoites reside in mosquito salivary glands and exit with the mosquito's saliva into the skin of the vertebrate host as the mosquito probes for blood. Salivation stops when the mosquito locates and begins to imbibe blood, thus sporozoites are primarily deposited into the skin and are not inoculated directly into the blood circulation [3, 4]. In most cases, the skin compartment into which sporozoites are inoculated is the dermis since this is the depth to which the mosquito's proboscis reaches. However, skin thickness varies from one location to another and a minority of sporozoites likely find themselves in the epidermis or sub-cutaneous tissue. The average number of sporozoites inoculated by a single infected mosquito varies enormously and is, to some extent, a function of the salivary gland load [5]. Studies using mosquitoes infected with rodent malaria parasites showed that a single infected mosquito injects between 0 and 1,300 sporozoites with the average inoculum being approximately 125 sporozoites [5]. These studies, however, used laboratory-raised mosquitoes where infection is optimized and salivary gland sporozoite numbers tend to be high. In the field, mosquitoes harbor lower numbers of parasites and it is likely that the inoculum is generally under 100 sporozoites [6, 7].

After their deposition in the skin, sporozoites must locate and penetrate blood vessels in order to reach the liver. Intra-vital microscopy studies show that sporozoites move randomly in the skin until they contact either endothelial cells of the blood or lymphatic system [8, 9]. Sporozoites glide around and along these vessels, enter by an as yet unknown mechanism and are carried away, either rapidly by the blood circulation, or slowly by the lymphatic system [8]. Although some sporozoites rapidly leave the injection site, many take hours to exit and enter the bloodstream. That transit to the bloodstream could take hours was initially suggested by experiments in monkeys with the primate malaria parasite *Plasmodium cynomolgi*, in which it was demonstrated that transplantation of skin containing the inoculation site, up to 2 hours after sporozoite injection, resulted in infection of naïve recipients [10]. More recently, using the rodent malaria parasite *Plasmodium yoelii*, which enables a more quantitative and comprehensive analysis, it was demonstrated that sporozoites exit the dermis and enter the blood circulation in a slow trickle extending for 2 to 3 hours after their inoculation [1]. Some sporozoites do not enter the bloodstream and instead enter the lymphatic circulation and go to the draining lymph node. Studies have shown that approximately 15–20% of the inoculum ends up in the draining lymph node [1, 2, 8]. These sporozoites, though at least initially alive, ultimately do not continue further and likely become fodder for the immune response [2, 8].

It is likely that the remainder of the inoculum is destroyed at the site of deposition, likely by the innate immune response of the host, although this has, to date, not been well-studied. Recently, however, it was shown that a small proportion, between 0.5 and 5%, of the inoculated sporozoites remain and begin to develop into exoerythrocytic stages at the inoculation site [11, 12]. Thus far this has only been studied using rodent malaria parasites so it is possible that the development of exoerythrocytic stages in an aberrant location may

result from a non-optimal host-parasite combination since the natural hosts of rodent malaria parasites are African thicket rats. Equally possible is that this may be an evolutionary relic since the avian malaria parasites, with whom the rodent and primate parasites share a common ancestor, develop into exoerythrocytic stages in mesodermal tissue including the skin. Importantly, these aberrantly developing parasites are not able to initiate a blood stage infection [12]. This is possibly because the merozoites within the exoerythrocytic forms do not fully mature although imaging data argues that this is not the case [11]. More likely it is because these parasites cannot easily access the blood circulation from this location: the liver sinusoids with their fenestrated endothelia provide a more direct route to the blood circulation than the closed endothelia of the blood. Nonetheless, it will be important to determine whether these skin exoerythrocytic stages contribute to the adaptive immune response that targets infected hepatocytes and whether this is a feature of malaria infection that is shared by all *Plasmodium* species.

Sporozoite exit from the dermis

Two sporozoite behaviors are required for dermal exit: motility and an ability to traverse cells. Sporozoites move by gliding motility, which is powered by an actin-myosin motor beneath their plasma membrane (reviewed in [13]. This motor is connected to the sporozoite surface via the cytoplasmic domain of a transmembrane surface protein called TRAP, (thrombospondin related anonymous protein) which has extracellular adhesive domains that bind to matrix such that the force of the motor translocates TRAP posteriorly, propelling the sporozoite forward. Previous studies have demonstrated that sporozoites actively invade hepatocytes and gliding motility is required for cell invasion [14]. More recently it has been shown that robust gliding is critical for sporozoite exit from the skin: sporozoites with mutations in TRAP which result in slow staccato movement have a much more pronounced effect on infectivity after intradermal inoculation than after intravenous injection [15]. Another critical property for dermal exit is the ability of sporozoites to traverse host cells, wounding these cells as they enter and exit [16, 17]. Mutants deficient in proteins required for cell traversal have normal infectivity when placed directly on hepatocytes in vitro yet are substantially less infective in vivo where they must exit the dermis and traverse the liver sinusoid to reach their target cell [16, 18–20]. In vivo imaging of fluorescent cell traversal mutants demonstrates that they are not able to efficiently move through the skin, becoming immobilized after contacting cells [16]. These data raise the possibility that cell traversal may also be a mechanism by which sporozoites escape phagocytic cells that arrive at the site in response to the mosquito's saliva [16]. Importantly migrating sporozoites must switch to an invasive phenotype once they reach the liver. Recent studies have shown that the major surface protein of sporozoites, the circumsporozoite protein or CSP, is critical for this switch [21]. CSP has a cell adhesive domain in its carboxy-terminus which is masked in salivary gland sporozoites. This domain remains masked as sporozoites migrate through the skin and then upon contact with hepatocytes, CSP is proteolytically processed by a parasite protease, revealing this domain and changing a migratory sporozoite into an invasive one. Although the signal for CSP cleavage and the switch to an invasive phenotype are incompletely understood, the highly sulfated heparan sulfate proteoglycans specific to hepatocytes likely play a role [17]. Thus, shortly after their arrival in the liver, cell traversal activity is stopped and invasion, with development into the next life cycle stage, proceeds.

Induction of protective anti-Plasmodium CD8+ T cell responses

Early studies using experimental models clearly demonstrated that protective immunity against sporozoite-induced infection requires antigen-specific $CD8^+$ T cells [22, 23]. Some of these $CD8^+$ T cells were specific for defined epitopes in CSP, and these T cells strongly inhibited the development of liver stage parasites [24]. Subsequent studies using T-cell

receptor transgenic CD8+ T cells specific for a CSP epitope, demonstrated that these T cells were primed primarily in lymph nodes draining the skin where sporozoites were deposited [2]. Forty-eight hours after immunization, either by the bites of irradiated infected mosquitoes or via intradermal inoculation of irradiated sporozoites, epitope-specific $CD8^+$ T cells producing IFN-γ were first detected only in the lymph nodes draining the inoculation site. Once CD8+ T cells are activated in lymph nodes, they migrate to other lymphoid and non-lymphoid organs including the liver. The importance of T cell priming in skin draining lymph nodes was demonstrated in experiments in which these lymph nodes were surgically ablated or through pharmacological inhibition of T-cell egress from lymph nodes. Under these experimental conditions the number of T cells reaching the liver was drastically reduced and the protective capacity of the anti-parasite CD8+ T cell-mediated protection was diminished.

An intriguing observation made in early studies indicated that protective immunity could be induced with irradiated yet live sporozoites [25]. Consistent with this observation, it was later shown that the induction of effector CD8+ T cell responses also requires immunization with live sporozoites [2, 26]. The strict requirement of viable sporozoites was previously interpreted as evidence that sporozoite invasion of hepatocytes was required for the induction of protective immune responses and this led to the idea that liver stage antigens were critical to induce protective immunity. However, the demonstration that protective CD8+ T cells are induced in the skin draining lymph node suggests that these responses are induced after complex interactions between professional antigen presenting cells and sporozoites.

Antigen presentation by dendritic cells (DCs)

It is well established that CD11c+ DCs play a critical role in the priming of *Plasmodium* specific $CD8^+$ T cells. Studies have shown that DCs incubated in vitro with sporozoites or obtained from lymph nodes of mice previously injected with sporozoites present parasite epitopes to T cells [2, 27, 28]. Moreover, *in vivo* depletion of the CD11c+ DCs abolishes the induction of parasite specific CD8+ T cell responses [29]. The skin is a tissue that harbors large numbers of DCs belonging to phenotypically and functionally distinct groups which are likely to interact with parasites. In addition, there are large numbers of lymph node-resident DCs which may also play a role in inducing T cell responses, particularly considering that a significant number of parasites migrate to lymph nodes draining the inoculation site.

The mechanisms by which DCs acquire antigen from live parasites is an intriguing yet poorly understood process. It is well known that the CSP and other parasite molecules are shed as sporozoites move and conceivably, these secreted molecules may be endocytosed and processed by DCs, and peptides within these antigens eventually presented to T cells. Alternatively, as was outlined earlier, the critical role of cell traversal for exit from the dermis means that sporozoites may also directly deposit antigen in the cytosol of DCs.

While the precise mechanisms of DC antigen uptake are unknown, experimental evidence indicates that DC priming of $CD8^+$ T cells occurs by antigen cross-presentation. This notion is supported by studies in which Toll-like receptor ligands administered prior to sporozoite immunization, inhibit the induction of CD8+ T cell responses [2]. It is known that TLR ligands hasten the maturation of DCs, a process that is accompanied by an inhibition of their endocytic activity [30, 31]. Recently, these studies were expanded using new methodological approaches and novel transgenic parasites. *P. berghei* parasites expressing mutant CSP containing the $H\text{-}2K^b$ SIINFEKL epitope have enabled studies in genetically modified C57Bl/6 ($H\text{-}2^b$) mice deficient in molecules involved in antigen processing and

presentation [32]. These studies demonstrated that priming of CD8+ T cells required intact endocytic function as experiments performed in mice lacking the endosomal protein Unc93B1 failed to develop robust CD8+ T cell responses. Unc93B1 is believed to mediate translocation of endocytosed molecules to the ER which are subsequently transported to the cytosol where they are processed to generate peptide epitopes [33]. A critical role for cross-presentation is further supported by in vivo experiments in which treatment of mice with cytochrome-c resulted in a severely reduced CD8+ T cell response [32]. Studies in other systems show that after cytochrome-c is internalized by endocytosis, it is translocated to the cytosol where it induces apoptosis, thus depleting antigen cross-presenting cells [34–36]. Finally, no CD8+ T cell responses were observed in mice lacking the TAP1 molecule which mediates the transport of the proteosome-processed CSP peptides from the cytosol to the ER. This clearly indicates that CSP must reach the cytosol where it is processed, generating epitope-containing peptides that are transported by TAP from the ER to where they bind to class l MHC molecules.

The precise tissue compartment where the capture of parasite antigen occurs and the identity of the DC subpopulation involved in this process, are critical matters that remain to be defined. As discussed in the preceding sections, intradermally inoculated sporozoites remain in the skin for over one hour and exit the skin in a slow trickle [1]. In addition a significant proportion of sporozoites migrate to the draining lymph nodes [1, 2, 8]. These findings raise an important question: where do DCs acquire sporozoite antigen? An obvious possibility is that sporozoite antigen is acquired in the dermis by skin-resident DCs that then migrate to the lymph nodes where they present antigen directly to naive $CD8^+$ T cells. At least three distinct subsets of skin-resident migratory DCs have been characterized: Langerhans cells, dermal DCs, and $langerin^+CD103^+$ dermal DCs [37]. $Langerin^+CD103^+$ dermal DCs are a subset of migratory DCs that play a key role in cross-presenting viral and self antigens [37–40] and their possible involvement in cross-presentation of sporozoite antigens requires further investigation. Alternatively, dermal DCs could transfer skin-derived antigen to lymph node resident DCs for $CD8^+$ T cell priming as shown in studies using herpes simplex virus [41]. Finally, it is also possible that $CD8^+$ T cell priming does not require skin-derived DCs, but instead occurs via direct acquisition of sporozoite antigen by lymph-node resident DCs. In this regard it is important that skin-inoculated sporozoites can be found associated with DCs in the lymph nodes [8].

Antigen persistence and maintenance of memory responses: another role for skin draining lymph nodes

Prolonged antigen presentation is crucial for maximal expansion of effector CD8+ T cell responses and recently it was demonstrated that continuous antigen presentation occurs for up to two months after immunization with irradiated sporozoites [42]. This observation is quite striking considering that irradiated sporozoites are not able to undergo proliferation and do not differentiate beyond early liver stages [43, 44]. Apparently, the parasite antigen does not persist as a dormant form of the parasite because treatment with primaquine to eliminate early liver stage parasites has no effect on continuous antigen presentation [42]. Antigen-presenting cells are responsible for trapping antigens, although the precise identity of the cell types involved in presenting persisting antigens is unclear and remains an area of further investigation. Persistent antigen is detected mostly in skin draining lymph nodes although it can also be found in spleen and liver. Continuous antigen presentation to CD8+ T cells is required for renewing and maintaining the memory $CD8^+$ T cell population and in fact naive cells such as recent thymic emigrants, are primed by persisting antigens. It is important that this prolonged antigen presentation does not induce $CD8^+$ T cell exhaustion as described in some chronic viral infection models [45, 46], on the contrary, persistent

antigen induces effector T cell differentiation and is necessary to develop or maintain optimal memory responses.

Relevance of the skin stage to the malaria vaccine effort

Several decades ago it was demonstrated that immunization with attenuated sporozoites could protective rodents, primates and humans from challenge with infected mosquito bites. Initially studies in rodents utilized irradiated sporozoites inoculated intravenously. In humans, immunization has always relied upon the bites of irradiated infected mosquitoes with ≥ 950 infected bites required for protection [47]. Calculations based upon the demonstration that individual mosquitoes inoculate on average 125 sporozoites [5], indicate that the dose required for protection in humans is not significantly different from rodent models. Thus, studies in humans clearly demonstrate that sporozoites inoculated into the skin can induce protective immune responses. More recently it has also been shown in the rodent models that sporozoites delivered by mosquito bite or intradermal injection can induce protection equivalent to that observed after intravenous immunization [2, 48, 49].

That attenuated sporozoites inoculated into the dermis induce protection should not be surprising given what we now know of sporozoite biology and priming of the immune response in the dermis and in the lymph nodes that drain this site. Moreover, a significant number of sporozoites, perhaps as high as 50%, may remain in the skin as non-viable parasites or undergoing further development to exoerythrocytic stages [1, 8, 11, 12]. Of the parasites that reach the bloodstream, most do not productively infect hepatocytes; studies with the rodent malaria parasites *P. yoelii* and *P. berghei* suggest that at most 25% and 10% of the inoculum, respectively, develops into liver stages [50, 51]. The skin, therefore, is the site where sporozoites may have their greatest exposure to the host immune system and it is not unexpected that a strong immune response is induced first in lymphoid organs associated with this tissue compartment.

Conclusions

Research conducted in several laboratories over the past 10 years has demonstrated that the skin, a compartment originally thought to be irrelevant to malaria infection, is a critical barrier for the sporozoite. Both robust gliding motility as well as the ability to traverse cells are required for dermal exit, yet even under optimal conditions, well-over 50% of sporozoites do not leave the inoculation site. Thus, this is a vulnerable time for the parasite suggesting that antibodies targeting critical processes such as gliding and cell traversal, could have a dramatic impact on infection. The recent explosion in our understanding of sporozoite biology at the inoculation site sets the stage for discovering and testing these new antibody targets.

This initial stage of malaria infection is also critical for the induction of T cell responses that ultimately target infected hepatocytes as priming occurs in the lymph node draining the inoculation site. Ultimately, the immunogenic properties of sporozoites combined with the rich array of immunologically active cells in the skin, confers upon give the dermis a critical role in determining the magnitude and quality of the anti-parasite immune response. As we move forward with candidate malaria vaccines, the biological and immunological importance of this early stage of infection is likely to play a role in vaccine design.

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