Wiles and wanderings: immune-evasive maneuvers of skin-penetrating parasites

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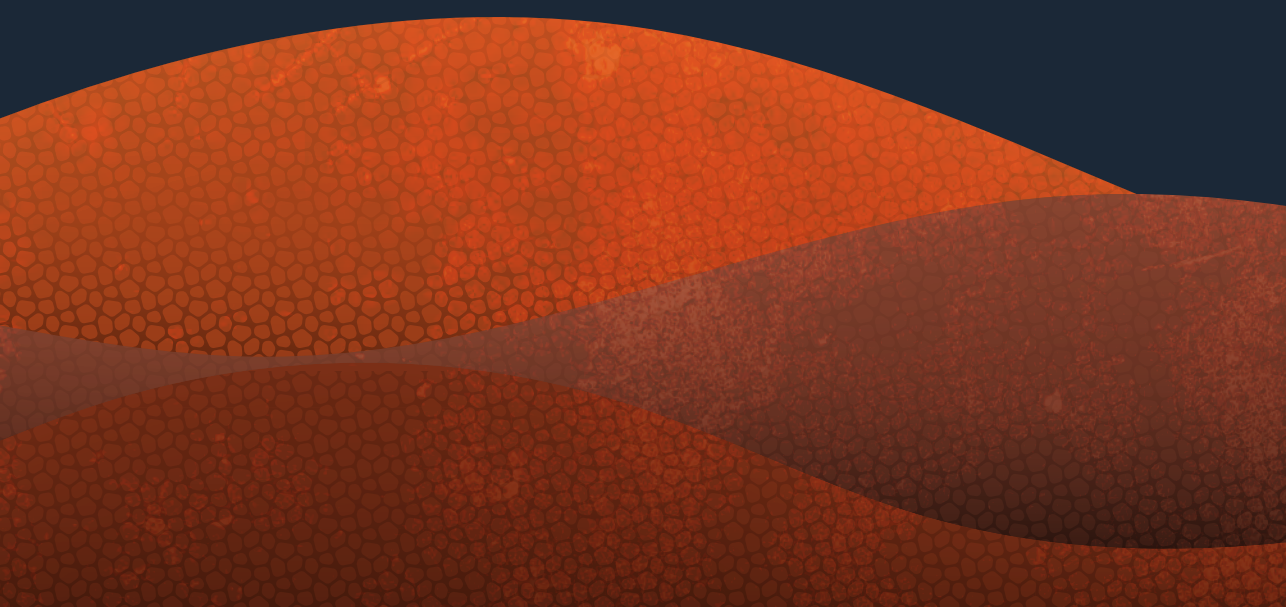

General introduction and thesis outline

GENERAL INTRODUCTION

Skin-penetrating parasites such as those causing malaria and schistosomiasis infect hundreds of millions of people annually. Over half of the world's population is at risk for malaria, and in 2017 alone, an estimated 216 million people were infected, eventually leading to the death of 435 thousand individuals, 61% of whom were children under the age of five[1]. Schistosomiasis is a less deadly disease yet causes substantial morbidity worldwide as patients suffer from chronic illness. Over 240 million people are infected worldwide and several million suffer from severe morbidity. It is estimated that around 200 thousand patients die yearly due to the disease and it is still thought that the burden of disease due to schistosomiasis is underestimated as it often goes unrecognized[2]. Given the global burden of these diseases, the need for an effective vaccine is evident[1,2]. Yet to date, no such vaccine exists for any parasitic disease. Naturally acquired protection following parasite exposure is a slow process that may take years or decades to develop and does not result in sterile immunity[3]. Currently, the most promising vaccine candidates are live-attenuated parasites, either yielding sterile immunity (in the case of malaria)[4,5] or decreasing parasite burden after subsequent infection (in the case of Schistosoma)[6-8].

Migration and kinetics

Malaria infection starts when the skin-penetrating form of the parasite, motile protozoans called sporozoites, are injected into the dermal stroma via the bite of infected mosquitoes while they search for blood[9]. Although the majority of parasites remain in the skin, within a matter of hours approximately 15% make their way through the dermis to reach the hosts vasculature which transports them to the liver, where they continue development intracellularly in hepatocytes[10-12]. Schistosoma cercariae, on the other hand, are released from freshwater snails and are aquatic multicellular organisms that bore their way into skin tissue upon contact. Subsequently, the tail detaches and the head of the cercaria continues as a schistosome; slowly making its way out of the skin in the span of days before gaining entry to the vascular space by entering venules or lymphatic vessels. Eventually, the schistosomes mature in the hepatic portal vein[13-15]. The dermal stage of both malaria and schistosomiasis is clinically silent, although the initial skin penetration is accompanied by a mosquito bite or a cercarial rash[16], showing that at least a mild form of immune activation at the site of parasite entry does exist.

Immunity to malaria and schistosomiasis

Immunity to malaria is complex and parasite-stage specific. When natural acquired immunity develops, it is primarily against blood-stage and does not appear to protect

against re-infection[17]. Vaccines against malaria are aimed to mediate protection at the pre-erythrocytic stage of disease, as up to this stage the disease is symptom free, parasites numbers are still low, parasites are located extracellularly making them vulnerable to antibody attack and blood-stage malaria does not yet activate immunoregulatory effects. RTS,S is currently the only licensed malaria vaccine and yields only approximately 30% protection over the course of the study period[18-20]. It is based on the circumsporozoite protein (CSP) which coats sporozoites and aims to increase anti-CSP antibodies in order to prevent migration of parasites from the skin to the liver. However, failure in blocking just one sporozoite from migrating into the vasculature would already render the vaccine inadequate as RTS,S initiates only modest T cell immunity.

The rationale for developing a live-attenuated parasite vaccine which confers sterile protection stems from findings by Nussenzweig and colleagues in the 1960's. They were the first to prove complete protection against pre-erythrocytic stages of malaria in mice vaccinated with radiation-attenuated sporozoites (RAS) that terminate development in the liver[21]. In the 70's it was then shown that exposure to over one thousand attenuated-parasite infected mosquitoes could indeed also protect human volunteers[22,23]. Currently, controlled human malaria infection studies using intravenously injected RAS show protective efficacy of up to 100%[24,25]. The immunological basis of protection by RAS has been characterized in murine malaria models. In mice, RAS vaccination results in antibody generation, which block migration of sporozoites during subsequent infections, and $CD8^+$ T cell activation, which eliminate infected hepatocytes[26-28]. Although antibodies significantly contribute to protection, $CD8^+$ T cells alone are capable of conferring sterile protection in mice[29,30]. In addition, more recently important roles for γδ-T cells as well as tissue resident memory (T(RM)) cells were suggested[5,31]. Overall, antibodies appear to play a role in sporozoite stage (anti-CSP antibodies) and blood-stage infection[32], and T cells have a dominant role in liver stage immunity, when parasites are localized intracellularly.

In the case of schistosomiasis, mouse studies have characterized immune responses to the various parasite stages. In the first 5 weeks of infection Th1 responses develop which are primarily associated with IFN-γ production. Subsequently starting at 6 weeks post infection, Th2 responses are initiated when parasites mature and start to generate eggs. Lastly, later stages of disease are characterized by immunomodulatory responses such as activation of alternatively-activated (M2) macrophages and regulatory T cells (Tregs), as well as IL-10 production as a regulatory feedback to proinflammatory immune activation[33-35]. Nonetheless, exposure to wild-type cercariae does not result in protective immunity. This has been suggested to result from the early increase in dermal

immunoregulatory mechanisms in the first few days post infection[36]. However, in the early 1960's mice were immunized using irradiated cercariae resulting in a reduction of worm burden post challenge[37]. Although immune responses to these diseases are slowly unraveled, none of the vaccination approaches have thus far resulted in long-term sterile protection to either parasitic disease.

Why the skin matters

The common ground for all skin-penetrating parasites is the route they must navigate from their site of entry through a densely packed immune organ: the skin[38]. The skin is the largest organ in the human body, and it is a specialized barrier to the outside world, both by forming a physiological barrier as well as functioning as an important immunological organ. Its most important function is to maintain the immunological balance between tolerance to commensal microbes and inflammation in response to pathogens. Within the skin reside a wide variety of immune cells such as dermal T cells, Natural Killer cells, innate lymphoid cells and mast cells. Importantly, the human dermis contains antigen presenting cells (APCs), dendric cells (DCs) and macrophages (MΦ), that sample the surroundings and present antigens to the adaptive immune system[39-42]. These cells coordinate the following adaptive immune responses by polarizing lymphocytes towards regulation or inflammation depending on the type of antigen encountered[42]. In addition, the dermis is rich in blood and lymphatic vessels which allow quick access to blood-derived immune cells such as neutrophils, monocytes, monocyte-derived macrophages and additional lymphocytes. Antigens are transferred continuously from the skin to the skin-draining lymph nodes either actively by migrating APCs, or passively through lymphatic vessels. It is here, in the skin draining lymph node, that an adaptive response is launched. For skin-penetrating parasites, the skin is the first site of interaction with the host's immune response.

In the case of malaria, the importance of the skin stage in vaccine development against parasitic infections is demonstrated by the importance of the route of administration of live-attenuated parasite vaccines on the protectivity of the response. In vaccination protocols against a variety of diseases dose reduction can be achieved by administering the vaccine not subcutaneously or intramuscularly, but directly into the skin[43-45]. In contrast, early clinical studies show that inoculation with malaria sporozoites results in a strong protective immune response when delivered by the bite of an infected mosquito or after intravenous administration of purified parasites[4]. However, the intradermal route of administration, often preferred due to practicalities, results in inferior protective immunity in both rodent and human models of malaria[46,47]. In addition to this, the skin may well be an important effector site for antibody responses against pre-erythrocytic

stages of malaria. Blocking dermal parasite migration retains sporozoites in the skin, preventing them from reaching the liver and initiating infection[48,49]. For schistosomiasis it was shown that irradiated cercariae could induce an APC-mediated IFN-γ response in the skin draining lymph node (sdLN) of mice. Additionally, non-attenuated larvae were demonstrated to induce the production of IL-10 in mouse skin[6]. These findings suggest that the skin stage of disease may be critical to the initiation of tolerance for both malaria and Schistosoma.

Could pathogens hijack existing immune pathways to avoid clearance?

Some pathogens, such as viruses and bacteria but also parasites, are capable of exploiting APC mechanisms for regulation in order to evade degradation by the immune system[50-55]. To convey a signal to the adaptive immune system, APCs present antigens in a context of co-stimulation and cytokine signals[42]. The human body contains a variety of mechanisms to down-modulate immune responses, in order to prevent continuous inflammation and subsequent tissue destruction. This can be achieved for example by secretion of regulatory cytokines such as interleukin 10 (IL-10)[56] or by co-stimulatory signaling though immune checkpoint molecules such as the PD-1/PD-L1 pathway[57,58]. Although immune modulation by parasites has widely been described during the blood stadia of these diseases[59-61], early immunoregulatory responses to skin stage parasites have not been investigated in the human host to date. Some murine models have begun to look into the skin-stage of skin-penetrating parasitic disease, nonetheless human responses have remained wholly uncharacterized. As murine skin differs drastically from human skin, both anatomically as well as functionally, looking into the human counterpart of the skin stage could prove critical in order to investigate some of the pitfalls in vaccine-induced immunity. In this thesis we aimed to test human responses to both malaria and Schistosoma, by characterizing responses of human monocyte-derived APCs as well as primary dermal APCs freshly isolated from human skin. In addition, we use a human skin explant model in order to expose skin to parasites in its natural three-dimensional state.

Parasite motility, prerequisite for immune responses

A critical feature in both parasite infectivity and subsequent responses is their motility. For malaria, parasites deficient in motility proteins do not establish an infection. In the case of Schistosoma parasite motility equally plays an important role; irradiated cercariae have been shown to persist in the skin much longer than their non-irradiated counterparts, increasing their time in the dermis from a few days up to a week[62,63]. In addition, over-irradiation of cercariae led to a much decreased number of parasites in the hepatic veins and a reduction of protective immunity[37]. Although the significance

of parasite movement has been widely accepted, motility analysis of live-attenuated sporozoite vaccines is routinely performed *in vitro*[64], and in-host motility of Schistosoma parasites has not been characterized at all. However, *in vitro* modelling passes over the consequential effect of the tissue environment on motility[65,66]. Therefore visualization and quantification of parasite motility in the three-dimensional setting may prove a crucial factor in the detailed comprehension of parasite-skin interactions. We dedicated the second part of this thesis to the development and implementation of (molecular) imaging techniques in the characterization of dermal parasite movement.

Understanding the details of dermal immune regulation by skin-penetrating parasites could significantly aid in the development and optimization of live-attenuated parasite vaccines. Bypassing or counteracting the regulatory effects of these parasites on dermal immune cells may optimize their protective effect. This thesis aims to unravel the pivotal role of the skin stage of skin-penetrating parasites in the potential polarization towards immune tolerance, both by investigating the kinetics of parasite migration, as well as the immune responses after exposure.

THESIS OUTLINE

The first part of this thesis describes the host immune-regulatory mechanisms that are exploited by two different skin-penetrating parasites immediately after their entry into the skin. In **chapter 2** we investigated APC responses to whole *Plasmodium falciparum* (*Pf*) sporozoite stimulation and show that malaria sporozoites induce regulatory MΦs that can suppress subsequent adaptive T cell responses. **Chapter 3** investigates the effect of the route of administration of whole sporozoites on their ability to skew towards dermal immune regulation. In **chapter 4** we show that a different skin-penetrating parasite, *Schistosoma mansoni*, is similarly capable of inducing regulatory immune responses in human skin and that its ability to do so is decreased upon radiation attenuation, the most commonly used mode of attenuation in parasite vaccines.

The second part of this thesis focusses on the motility behavior of malaria parasites in the human dermis. In **chapter 5**, we show that radiation attenuation impairs sporozoite movement in the human skin and reverts sporozoite motility back to "default", non-directional movement. **Chapter 6** describes a novel method for targeted molecular imaging of genetically wild-type *Pf* sporozoites, allowing for imaging and subsequent motility analysis of non-GMO *Pf* sporozoites in human skin tissue.

Finally, in **chapter 7** the results are summarized and discussed in the broader context of the current literature and regarding potential new lines of research necessary for

refinement and development of novel vaccines. We draw parallels between the two species of skin-penetrating parasites investigated and discuss the effect of radiation attenuation of parasites.

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