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

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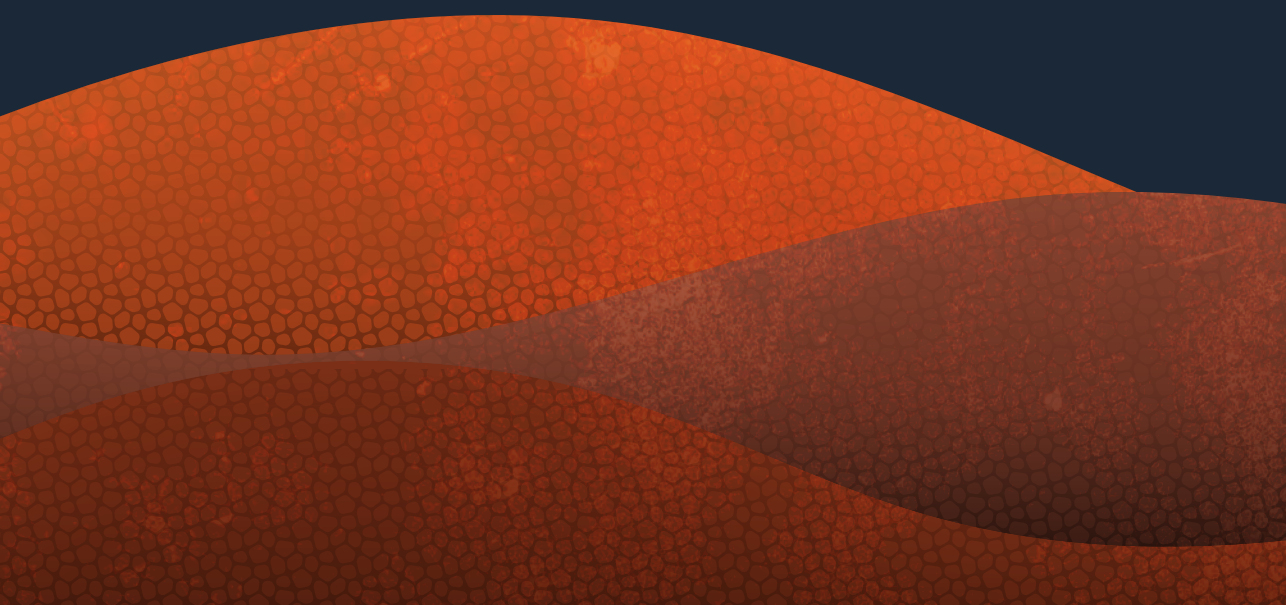

Summarizing discussion

With this thesis we attempted to shed light on the thus far uncharacterized human skin stage of skin-penetrating parasites. We present three novel key concepts to the field.

1. By analyzing immune responses to both malaria and schistosomiasis in the skin of their natural host, we have demonstrated that parasite exposure initiates immune regulation at the dermal site, by exploiting host immune-regulatory mechanisms. A characteristic relating them to tumor cells, which have been described to recruit immune cells to their aid in a similar fashion (**Chapter 2 and 4**).

2. We applied imaging techniques which allowed us to quantitatively image parasite movement within skin tissue. Our efforts yielded the first video-microscopic images of *Plasmodium falciparum* migrating through human skin, as well as of invading *Schistosoma mansoni* cercariae and allowed us to quantitatively compare the *in-skin* motility of radiation-attenuated and non-attenuated parasites. We describe how sporozoite migration is altered after radiation-attenuation (**Chapter 5 and 6**).

3. Importantly, our studies are performed directly in human skin, using either *Schistosoma mansoni* or the deadliest *Plasmodium* species to man; *Plasmodium falciparum*. Thus far, investigation into the skin-stage of both schistosomiasis and malaria was performed solely on rodents and non-human primates. Using human skin explants allows for a more direct translation of our findings to the *in vivo* setting in the human host (**all chapters**).

In this final chapter our findings will be discussed in the context of the known pathophysiology of disease and with regards to future prospects in parasite vaccine development. Additionally, we will draw parallels between the two very different skin-penetrating parasites investigated in this thesis.

Dermal immune responses to malaria and schistosomiasis; of mice and men

The discovery that the majority of *Plasmodium* sporozoites remain in the skin and that a large quantity of injected parasites drains to the skin draining lymph node (sdLN)[1,2], combined with the fact that protective immune responses can be primed in the sdLN[3,4], has sparked new interest in the skin-stage of malaria. In mice, intradermal delivery of sporozoites results in a local inflammatory response consisting of sustained neutrophil and monocyte recruitment as well as mast cell activation[5-7]. 2-4 hours after injection, neutrophil and monocyte recruitment can also be seen into the sdLN. Additionally, both in the skin as well as in the sdLN, sporozoites were seen to co-localize with $CD11b^+$ myeloid cells[2,5]. Within the sdLN, both a Th1 response as well as an immune regulatory response,

indicated by an increase in IFNγ producing T cells and IL10 producing, CTLA-4 expressing T cells respectively, has been shown previously[3,5,8]. Although research has focused solely on murine dermal responses, the importance of dermal mechanisms in the human host is demonstrated by the crucial role of the route of administration of whole-sporozoite vaccines to their protective effect. Both in controlled mouse and human models of malaria infection, intradermal vaccine delivery greatly reduces their efficacy compared to both mosquito bite delivery as well as intravenous injection[8,9]. We aimed to elucidate the immunological mechanisms underlying these differences by examining the effects of sporozoites on APCs, and their subsequent effect on T cell responses (**Chapter 2**) as well as comparing the dermal immune responses after needle or mosquito bite delivery of parasites (**Chapter 3**). Our findings show that immune regulation by parasites is not limited to the blood stage of infection but initiates at the first site of interaction with the host immune system, the skin site. We show clear upregulation of immunoregulatory mechanisms on monocyte-derived macrophages with a concomitant similar response by primary dermal APCs after sporozoite stimulation in vitro. However, despite our ex vivo method indicating a role for the $CD14^+$ APC subset (proposed to be macrophages; **Chapter 3**), it did not shed light on whether specific DDC subsets are involved. Whether skin-resident DCs have no place in the response to skin-stage malaria, or it reflects the limitations of the *ex vivo* protocol (described in **chapter 3**) remains to be determined.

Skin-penetration by *Schistosoma* cercariae results in dermal inflammation maximal at day 4 post invasion in mice[10,11]. Dermal APCs show increased activation and increase their migration to the skin draining lymph node. However, already at day 2 post invasion a regulatory response mounts in the skin characterized by IL-10 and IL-1ra production eventually resulting in a quick abrogation of the inflammatory responses and failure to induce a protection against reinfection[11]. In humans, cercarial dermatitis has been described after exposure showing that at least some form of immune activation exists[12-14], however the human skin-stage of schistosomiasis remained uncharacterized to date. We show that the initiation of the regulatory response in humans occurs in the skin by IL-10 producing DDCs and that attenuated cercariae are less capable of inducing this response. These findings corroborate with murine skin responses[15].

For both parasite species lasting sterile natural and vaccine-induced immunity fails to develop, although vaccination efforts using live attenuated parasites show promising results[15-18]. However, parasites do not go entirely undetected by the host immune system, as residents in endemic areas do show increased levels of circulating antibodies and altered cytokine responses[19]. So why does it seem impossible to initiate a lasting sterile immune response? We hypothesized that either, 1) parasites divert immune responses by inactivation of host immune cells through their (secreted) products or

direct interactions with host immune cells, or 2) the response mounted by the host is abrogated by activation of host regulatory mechanisms. In **chapter 2** we tested whether the regulatory propensity of sporozoites was the result of responses to the immunodominant circumsporozoite protein (CSP), secreted by sporozoites upon migration. We did not find any effect of CSP on both dendritic cells and macrophages, indicating CSP plays no role in immune-modulation through APCs. CSP has been proposed to be a 'decoy' protein. A protein shed in large quantities in order to generate 'smokescreen' for antibody development, leading away from the migrating sporozoite[20]. In vaccination studies using RTS,S, antibodies against CSP do correlate with protection[21]. However, recently it was found that RTS,S also increases vaccine-unrelated antibody responses against *Plasmodium*[22]. In the case of Schistosoma, although early studies have demonstrated a role for schistosome excretory/secretory (ES) products in the suppression of (dermal) immune responses by cercariae, our results in chapter 4 did not show cercarial ES products to have an effect on immune responses *in vitro*. We show that direct contact of cercariae is necessary for modulation of the response. However, as our results suggest that the in vitro setting (used to determine the effect of ES products) is not affected by changes in migration or motility, an effect of ES on the motility of APCs, and thereby altering their function cannot be ruled out. Whether other (secreted) proteins or compounds such as parasite exosomes[23] play a role in the activation of immune regulation on APCs remains to be determined.

Surviving in a hostile host: where tumor cells and parasites intersect.

For microbes residing in an immune competent host, exploitation of immune regulatory pathways is a powerful tool to steer clear of elimination. The same can be said for tumor cells. Tumor cells use a variety of mechanisms in order to escape the immune system, including the expansion of regulatory T cells (Tregs), increased production of regulatory cytokines such as IL-10 and TGFβ[24] as well as affecting the phenotype and function of APCs leading to decreased T cell activation. These mechanisms are also utilized by some parasites[25,26].

Our findings implicate a central role for the regulatory macrophage (Mreg) in malaria dermal immunology (**Chapter 2**). Macrophages are terminally differentiated skin resident cells that have antigen presenting abilities and display high levels of plasticity. Historically, macrophages were subdivided into categories based on their response to either TLR ligands or Th2 cytokines: (classically activated) M1 macrophages secreting high levels of pro-inflammatory Th1 cytokines and (alternatively activated) M2 macrophages involved in tissue repair respectively. Further investigation led to a subdivision of M2 macrophages in Mregs and tissue repair macrophages based on

their cytokine production and role in wound healing. It is currently thought that the classification of macrophages in M1, M2 and Mreg might be an over-simplification of a continuous spectrum of macrophage phenotypes[27,28]. Primarily described in the context of tumor immunology, the regulatory macrophage is characterized as a macrophage producing large quantities of IL-10 and expressing PD-L1 on its surface despite its retained ability to produce proinflammatory cytokines and express activation markers such as CD80[27,29]. They are described to have a local effect on the tumor environment by production of cytokines and chemokines as well as on the recruitment of circulating immune cells homing to the tumor and induction of T cell anergy[30]. This is distinct from parasite infection, where parasites actively migrate away from the site of injection and do not wait for immune cells to arrive. However, as the majority of parasites remain in the skin, and a large proportion is found in the skin draining lymph node where immune responses to malaria can be primed, this local effect may still have widespread consequences to the overall induction of immunity. In addition, antigen uptake by macrophages does not only influence them directly, but also shapes their behavior and responses to subsequent antigen-sensing, therefore containing an integrated adaptive component[31]. This indicates that the first exposure to (malaria) parasites in the right context may be crucial in order to launch a protective response. Therefore, it could have implications for the translation of vaccine studies on malaria-naïve individuals to the endemic field.

We established a role for DDCs in the immune-regulation by *Schistosoma* parasites. However, we did not examine dermal macrophages in our skin explant setup due to difficulties in the distinction between DDCs and skin-residing macrophages in human tissues lacking clear macrophage markers such as F4/80 in mice. Interestingly, alternatively activated macrophages have been implicated to prevent helminth and protozoan killing in mice[32]. In addition, PD-L1/2 expression on macrophages has been demonstrated in splenic macrophages of mice infected with *S. mansoni* and was found to induce T cell anergy[33]. These findings could indicate that macrophages might also be involved in dermal immune regulation by *S. mansoni* and should be the topic of future investigation.

Although PD-L1 is generally described as a regulatory marker, its upregulation does not inherently mean a cell has immunosuppressive capabilities. We see PD-L1 also upregulated after exposure to LPS, a finding previously described as a negative feedback loop preventing excessive inflammation in the context of endotoxin tolerance. We use salivary gland extract of similarly handled uninfected mosquitoes as a control in all our experiments, ruling out that the effect on phenotype and function is due to endotoxin contamination of the sporozoites. However, although the macrophages with regulatory

phenotype investigated in this thesis did result in a suppression of T cell responses, where LPS stimulated macrophages were not, the induction of this phenotype as a feedback response cannot be completely ruled out. Indeed, blocking of the PD-1/PD-L1 pathway in bystander macrophages did not influence $CD8^+$ T cell responses to DCs, however, blocking of the IL-10 pathway partially restored $CD8^+$ T cell IFNγ production, indicating that IL-10 production by these macrophages may be critical to their suppressive effect.

The proposed model of immune-modulation

We propose a model where regulatory macrophages impair immune priming against parasite antigens from skin-penetrating parasites, starting in the dermis (Figure 1). In the case of *Plasmodium* sporozoites, the majority of injected parasites are sacrificed for the greater cause and are phagocytosed by macrophages, be it directly at the injection site or after passive transfer to the skin draining lymph node. These macrophages decrease their migration (**Chapter 2 and 3**) in order to remain at the hot spot of parasite entry and gain a regulatory phenotype which hinders the activation of $CD8^+$ T cells by dendritic cells (**Chapter 2**). The site of this interaction might well be extended to the sdLN[3,8]. $CD8^+$ T cells that DDCs did manage to activate and prime enter the skin and find themselves in a regulatory environment containing high concentrations of IL-10 which may directly impair their function[34] (Figure 1a).

Schistosoma mansoni cercariae utilize similar mechanisms in order to suppress dermal immune responses. Direct interaction with this parasite pushes dermal dendritic cells towards a regulatory phenotype and results in a decreased Th1 response by $CD4^+$ T cells. A role for dermal macrophages is possible but has not been studied to date (**Chapter 4**). Additionally, a role for liver-resident macrophages, Kupffer cells, in the modulation of immune responses is possible, and should be studied in the future. (Figure 1c). Sporozoites have been shown to primarily utilize Kupffer cells in order to enter the liver. Contact with sporozoites could result in a similar response of these cells thereby initiating an equally regulatory environment at the site of hepatocyte infection[35-37].

Establishing immunity to malaria and schistosomiasis is a complex and multi parasite-stage endeavor that is not limited to the importance of one cell type during one parasite-stage, but most likely hinges on the combined efforts of both innate and adaptive immune cells during all stages of infection. Although the exact molecular mechanisms and cellular interactions underlying the regulatory responses shown still remain unclear, our findings are a first step in the characterization of the human skin-stage of skin-penetrating parasites and may help to explain the inferiority of the intradermal route of vaccination, why natural immunity fails to develop in endemic areas and why no effective vaccine to either disease exists to date.

Common ground for all skin-penetrating parasites? The 'fallen heroes' hypothesis.

Whether exploitation of the IL-10 and/or the PD-1 pathway is conserved across different parasites remains an interesting question. In **chapter 3 and 4** we show upregulation of PD-L1 in *Plasmodium* sporozoite as well as *S. mansoni* cercariae stimulation. In addition, a role for PD-1 has recently been demonstrated for yet another skin-penetrating parasite species, *Brugia malayi*, one of the causative agents of lymphatic filariasis[38,39]. If immune suppression via conserved pathways is characteristic for skin-penetrating parasite species, this pathway would be a promising target for vaccine development, potentially in the form of adjuvants or adaptations to existing parasite proteins. Evolutionarily, initiation of immune regulation as early as possible has advantages for the parasite life cycle. As for all skin-penetrating parasites described, only a minority of invading parasites will make it to adult-hood. Regulation at early stages in this 'funnel-effect' is primarily important for parasites such as *S. mansoni*, which do not replicate inside the human host. We propose that parasites stranding in the skin and/or skin draining lymph node, initiating a regulatory response, is an important mechanism to overall parasite survival. We named this the 'fallen heroes' hypothesis. For malaria, where the sporozoite stage is relatively short-lived, and where after the initial pre-erythrocytic stage the subsequent expansion leads to high levels of parasite antigen during blood stage, this is potentially less crucial. Having immune regulatory mechanisms in play during the blood stage of malaria might aid parasite invasion during subsequent infection[40].

Future skin focus

Although the use of ex vivo skin circumvents the pitfalls regarding the use of murine models for human dermal immunology questions (**Chapter 2-5**), the limitations of explant models (described in **chapter 3 and 4**) highlight the additional need for alternatives. Given the advances in the field of controlled human infections[41-44], future research should focus on *in vivo* application of both immunological assays as well imaging. Controlled human infections have the capacity to elucidate these mechanisms directly within the host, without facing the challenges of either *ex vivo* or murine models of disease. Taking dermal biopsies from volunteers would allow for cytological, histological as well as functional read-outs directly *in vivo*. However, murine models should be used additionally, in order to systematically examine possible mechanisms for dermal immune regulation. Furthermore, mouse models and non-human primate models are not limited to skin and blood samples but allow for determination of responses in both the sdLN as well as the liver. Non-human primate skin has the advantage of being more comparable to human skin, and although more costly, should be considered as a valuable alternative. Knockout models can be used to investigate the role of dermal

dendritic cells and macrophages. The effect of neutralizing the PD-1 pathway or other checkpoint molecules is worth investigating using neutralizing antibodies, siRNA or specific genetic knockouts. In addition, targeting dermal dendritic cells and/or macrophages specifically by administration of liposomes containing parasite proteins, or coupling of antigens and adjuvants to dendritic cell-specific antibodies should be studied in order to gain insight in the regulatory mechanisms in play[45-47].

Implications of dermal immune regulation to vaccine refinement

In order to generate an effective live attenuated parasite vaccine, parasites should initiate sustained adaptive immune responses without the interference of modulatory mechanisms. One obvious possibility to circumvent dermal immune regulation is to bypass the skin altogether. Intravenous injection of cryopreserved sporozoites can indeed induce sterile protection in volunteers[16,48,49]. However, also after IV vaccine injection protection wanes over time[17], indicating an additional role for regulatory mechanisms outside of the skin such as in the liver. Furthermore, intravenous injection of attenuated parasite vaccines is currently possible for malaria sporozoites, but not for the multicellular, and much larger, Schistosoma parasites.

In order to skew immunity to pro-inflammatory responses, adjuvants are often used. RTS,S for example is delivered in a hepatitis B envelope and is co-administered with adjuvant AS01B to increase antibody responses[50]. Adjuvants used for dermal applications such as imiquimod (a TLR7 agonist) and resiquimod (a TLR 7 and 8 agonist) can be tested in order to stimulate dermal macrophages and DDCs and to increase the pro-inflammatory response to intradermal delivered vaccines[51-53]. In addition, if the molecular pathways underlying immune regulation are elucidated, parasites could hypothetically be genetically modified to circumvent these pathways.

Lastly, our findings on dermal immunity could potentially have great implications for the immunization protocols used in vaccine efficacy studies. In **chapter 2**, we show that antibody opsonization of sporozoites greatly increases their uptake by macrophages, without affecting the subsequent regulatory response. This could imply that a prime-boost protocol using high numbers of cryopreserved sporozoites may not be optimal for vaccination. Firstly, increased phagocytosis results in fewer parasites able to initiate a protective response in the skin draining lymph node or after reaching the liver. In addition, the increased numbers of phagocytosed parasites result in larger numbers of immune-suppressive macrophages further tempering immune responses with each boost. It can be argued that a selection of only the most potent and viable sporozoites should be used as a vaccine in order to decrease the amount of skin-residing (and

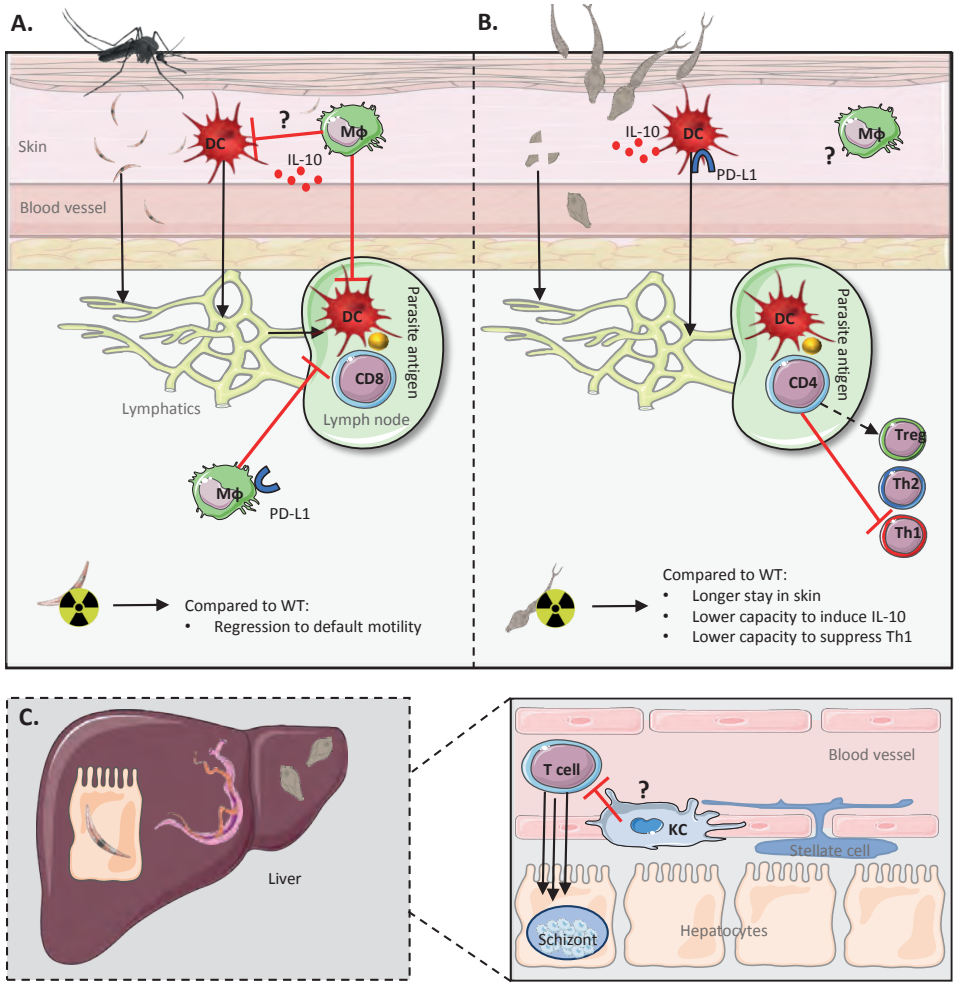

Figure 1. Proposed model of immune regulation by sporozoites. **A.** An infected anopheles mosquito injects SPZ into the dermis of the host. SPZ migrate through the dermis to reach a blood or lymphatic vessel. The majority stay within the skin. Skin resident macrophages (MΦ, green) phagocytose SPZ and gain a regulatory phenotype and are able to modulate CD8 responses to antigen loaded dendritic cells (DCs, red). Additionally (sdLN resident?) macrophages can interfere with CD8 T cell priming directly though cell-cell interactions. **B.** *S. mansoni* cercariae bore their way into the skin, shed their tails and migrate as schistosomula to the vasculature. Direct contact with skin resident dendritic cells (DC, red) leads to upregulation of PD-L1 and the production of IL-10. This eventually results in a decrease in Th1 skewing of naïve T cells. The role for skin resident macrophages remains to be determined. Irradiated cercariae are not as efficient as wild-type cercariae to induce this phenotype. **C.** Whether Liver resident macrophages, Kupffer cells (KC), play a similar role in immune regulation remains to be determined.

thereby immunoregulatory) parasites. The same can be argued for vaccine application in the endemic setting, where residents already have circulating anti-CSP antibodies ready to immobilize parasites upon entry.

Overall, our findings may have widespread implications in the field of live-attenuated parasite vaccine development. In order to refine current vaccine formulations, these important questions should be addressed before costly, grand-scale clinical trials take place in the field.

The effect of radiation attenuation on live parasite vaccines

We characterized the effect of radiation-attenuation of parasites on both their immune-suppressive function (Schistosoma, **Chapter 4**) as well as their motility (malaria, **Chapter 5**). Although radiation-attenuation is a powerful method to inhibit parasite maturation, our findings indicate that its effect is much more extensive and has previously uncharacterized repercussions.

Using *S. mansoni* parasites, we demonstrated decreased immune regulatory propensity after radiation attenuation. Irradiation of cercariae has been demonstrated to reduce migration through the skin[11,54] and it was proposed that prolonged antigen exposure underlies the improved immunogenicity of radiation-attenuated cercariae. We show that direct interaction of radiation-attenuated cercariae leads to decreased PD-L1 expression on and decreased IL-10 production by DDCs, and their increased ability to skew towards Th1 responses (Figure 1b). Whether radiation-attenuation alters cercarial surface antigens or whether molecular mechanisms used to inactivate host immune responses are hampered by radiation is not yet clear.

In line with motility alteration previously described after radiation-attenuation of cercariae, *Plasmodium falciparum* sporozoites were shown to display impaired motility after irradiation (**Chapter 5**, Figure 1a). Potentially RAS will remain longer in the skin than their non-attenuated counterparts, as movement was shown to revert to default patterns including circulatory and reversal patterns. Whether these patterns also increase their chances of passive transfer to skin draining lymph nodes has not been investigated due to the limitations of the explant model.

In addition, whether radiation attenuation has a similar effect on the sporozoites ability to modulate APC responses is the subject of further research. Both radiation effects have the potential to work in synergy: the slower migration indeed does prolong

antigen exposure at the immunocompetent skin site, and priming is now potentially able to occur since the immune-regulatory propensity of parasites is diminished upon attenuation.

A role for (molecular) imaging in vaccine advancement

Molecular imaging is a powerful tool to directly analyze the behavior of (live-attenuated) parasites. In **chapter 5 and 6** we used novel imaging strategies in order to visualize and characterize dermal migration of malaria sporozoites. Motility and migration are important features of parasite infection especially during the skin stage where their most important objective is to exit the skin and enter the vasculature. Indeed, genetically modified parasites deficient of proteins crucial for migration fail to establish an infection and, in attenuated form, fail to confer protection[55-58]. Therefore, a detailed understanding of this movement may be crucial to design and optimize attenuated-parasite vaccines.

Next to mapping of parasite motility alone, (molecular) imaging has the potential to go deeper into dermal immune interactions. In example, it can be used to answer the question whether irradiation of sporozoites results in prolonged exposure to dermal regulatory cells. Optimization of our protocols can potentially chart cellular interactions of sporozoites with immune cells. The altered movement patterns within our dataset could signify these interactions, however, the complexity of the interactions warrant visualization to a level of detail not yet feasible in our current setup. Immunofluorescent staining of cell types by camelid single-domain antibodies could overcome the limitations faced in *ex vivo* staining of dermal structures[59,60]. Additionally, the use of spinning-disk confocal systems or the optimization of tracer penetration by emission in the far-red spectrum could potentially increase the depth of analysis.

Not only would molecular imaging advance our understanding of dermal immune interactions, it could facilitate testing of novel anti-sporozoite antibodies as well as the characterization, optimization and comparison of different parasite-attenuation techniques. Antibody binding of attenuated sporozoites likely contributes to protective immunity by altering sporozoite motility[61-63]. Yet the role of these antibodies in the overall immune response against malaria remains controversial, despite anti-CSP antibody titers correlating with protection. To date, the relative contribution of anti-CSP antibodies in sporozoite migration or invasion blocking remains uninvestigated. Molecular-imaging based techniques that allow for sporozoite tracking can be used as a novel tool to study the effect of antibodies on dermal sporozoite migration.

Additionally, molecular imaging could be extended to imaging beyond the skin stage with a nuclear medicine-based approach. Hybrid (both fluorescent and nuclear) tracer development would facilitate its use in controlled human infections where it could help to elucidate the role of lymph node trafficking and extra-hepatic parasite development in parasite immunity[64]. Lastly, dermal imaging of pathogens is not limited to host-parasite interactions but could be used in the visualization and movement quantification of all manner of pathogens.

Concluding remarks

By studying the combined immune response to parasites as well as parasite motility, this thesis offers a new scope to enhance our understanding of parasite skin stages. Although the molecular mechanisms and cellular interactions that play a role in the dermal immune-regulatory propensity of parasites require further investigation, this thesis highlights the importance of the skin stage of different skin-penetrating parasites and offers novel (imaging) strategies in order to further characterize and subsequently optimize attenuated-parasite vaccine design. These insights are a pivotal first step in broadening our understanding of pre-erythrocytic natural immunity and the pitfalls of intradermal vaccination-induced immunity.

Outstanding questions

- Where does immune modulation take place? Solely in the dermis, or in addition in the sdLN and/or liver?
- What is the role of liver-resident macrophages (Kupffer cells) in pre-erythrocytic immune modulation?
- Do macrophages themselves also present sporozoite antigen to T cells or are their effects modulated via dermal- or lymph node-resident dendritic cells?
- Does the PD-1/PD-L1 pathway indeed play a role *in vivo*?
- Is there a role for neutrophils and/or monocyte-derived macrophages in dermal immune modulation?
- How do sporozoites exert their effects on macrophages? Which molecular pathways are involved in the upregulation of PD-L1 and IL-10?
- Can we use adjuvants, delivered directly to dermal macrophages in order to circumvent the modulatory response?
- What is the duration of the APC mediated immune-suppressive effect? Does this impact prime-boost vaccination strategies?
- Does radiation attenuation alter sporozoite-dermal APC interaction?
- What is the role of sporozoite motility on their interaction with dermal immune cells?

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