The bright and DARC side of detection

Role of membrane proteins during erythroid maturation

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Publication date
2020

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Chapter 7

General Discussion

Introduction

The research presented in this thesis aims at elucidating the role of membrane proteins during erythroid maturation. Understanding the environmental factors involved in the process of maturation of erythroblast to reticulocytes and to erythrocytes is required for the optimization of a culture system that can successfully produce transfusion-ready red blood cells. We show how and when these membrane proteins trigger signaling cascades during erythropoiesis, that have effects as diverse as differentiation induction, regulation of erythroblast adhesive properties, or tethering of cytokines involved in bone marrow hematopoietic homeostasis (retention and mobilization). Besides possible effects *in vivo*, these newly found regulatory axis are also important to optimize and develop turbulent bioreactor large scale cultures. In addition, the specific structure of membrane proteins in reticulocyte maturation has its implication in reticulocyte invasion by specific malaria species, generation of specific invasion model system and responses to inflammatory cytokines (in the case of DARC).

Role of Ca^{2+} during erythropoiesis

Ca^{2+} is one of the most important cellular second messenger. Ca^{2+} can be released from intracellular Ca^{2+} stores by inositol(1,4,5)P3 (IP3) following PLC-γ activation, which, for instance, occurs after EPOR activation [1-3]. Ca^{2+} can also enter cells from extracellular pools through actions of specific Ca^{2+} importers and exporters resulting in Ca^{2+} fluxes. In this thesis we have shown the effects of Ca^{2+} influx on erythroid differentiation, adhesion and signal transduction by hormones and growth factors such as EPO and SCF. Ca^{2+} levels need to be tightly balanced during erythropoiesis and inappropriate high or low concentrations lead to loss of cell viability, differentiation and/or proliferation defects (chapter 2).

PIEZO1 is the most prominent mechanosensitive protein expressed in erythroblasts. Therefore we assume that shear stress, as sensed by erythroblasts in a bioreactor, would activate PIEZO1 and result in Ca^{2+} influx. We investigated PIEZO1 induced signal transduction in erythroblasts using the synthetic PIEZO1 agonist Yoda1 and by culturing erythroblasts of HX patients that harbour an activating mutation in PIEZO1. We compared the activation of signaling pathways to erythroblasts submitted to orbital shaking. These three approaches have yielded similar result in terms of reduced cell proliferation, as well as pathway activation albeit with different kinetics. This is easily explained by the fact that addition of Yoda1 causes an acute effect, and only with a delay the signal becomes constitutive when Yoda1 and feedback signals are balanced. In contrast, the signal is constitutive in HX-derived erythroblasts and during orbital shaking. Orbital shaking resulted in prolonged ERK phosphorylation, while with Yoda1 the phosphorylation peaked after 10 minutes. Orbital shaking and Yoda1 treatment both resulted in activation of STAT5 and the MAPkinases ERK and p38. JNK was not activated during orbital shaking, but only after Yoda1 treatment; By

modulating Ca^{2+} entry, PIEZO1 contributes in balancing the signaling between proliferation and differentiation/apoptosis. Activation of PIEZO1 by Yoda1 and HX phenotypes is mainly caused by a prolonged opening of PIEZO1 following stimulation and this causes peaks in MAPkinases activation with additional activation of JNK. We assume that orbital shaking causes the mechanical activation of PIEZO1, which would be a more physiological PIEZO1 activation with distinct kinetics of opening and closing and therefore also different kinetics of downstream pathways. To proof that mechanical stress activates these pathways via PIEZO1 we would have to repeat these studies upon deletion of PIEZO1 for instance by CRISPR/Cas9.

Both PIEZO1 activation and mechanical stress also caused activation of NFATc2; the target genes activated by the NFAT pathway as well as their roles within erythropoiesis are partially elucidated. The NFAT pathway decreases KLF1 expression and therefore inhibits erythropoiesis [4]. NFAT is activated upon dephosphorylation by calcineurin. The fact that Ca^{2+} mediated NFAT activation can be abolished by the use of Tacrolimus, open possibilities to use this compound to modulate erythroblast proliferation.

The concurrent activation of STAT5 and NFAT is interesting, because STAT5 has been shown to cooperate with NFATc1 to modulate erythropoiesis [5]. STAT5 is a transcription factor important in EPOR signaling, by regulating transcription of multiple target genes (e.g. BcL-xL) that are essential for terminal erythroid differentiation [6, 7]. By showing the activation of STAT5 by phosphorylation, we proved involvement of a PIEZO1-mediated signalling acts upstream of EPOR receptor and may 'sensitise' the receptor for activation of STAT5, and potentially also for MAPKinases. The increased EPOR signalling strength in response to Ca^{2+}-dependent PKC activation is reminiscent of inside-out activation of integrin receptors.

In chapter 3 we have further investigated the role of Ca^{2+}, in particular the association between Ca^{2+} influx and integrin activation. Integrins (specifically VLA4 (α4β1) and VLA5 (α5β1)) play an important part in the interaction between erythroid cells and their environment. The importance of integrins for *in vivo* erythropoiesis is more argued below.

Having established that PIEZO1 mediated Ca^{2+} influx activates PKC and subsequently enhances EPOR signalling (chapter 2), we found that integrin activation in erythroblasts is also mediated by PKC and calpain. Blocking the activity of these proteins results in decreasing affinity for the ligand. It is important to make a distinction in the integrin ligands that play a role in erythroid development: VCAM1 is mainly mediating interactions between erythroblasts and central macrophage, while fibronectin is mediating the interaction between the erythroblasts and the surrounding niche. Our research focusses mainly on proerythroblasts, but it would be interesting to assess integrin activation throughout *in vitro* erythroid differentiation.

If PIEZO1 affects Ca^{2+} signalling leading to integrin activation, this raises the question which upstream stimuli cause PIEZO1 activation in the erythroblastic island. As PIEZO1 is a mechanosensitive channel, it can be assumed that forces like bone pressure and shear stress

caused by blood flow through bone marrow sinusoids may activate PIEZO1. For example, hydrostatic pressure improves the clonogenic potential and $CD34^+$ cell number in ex-vivo cultures [8]. However also crawling over the central macrophage within the bone marrow may cause mechanosensing within the microenvironment. Cell-cell contacts are a known generator of mechanical forces [9] and let us suppose that such forces exist also among erythroblasts and macrophages. This mechanosensing thus result in a Ca^{2+} influx which is vital for erythroid island development (as described in chapter 1). As PIEZO1 is sensitive to chemical activation by the agonist Yoda1, we could also assume that in the bone marrow one or more endogenous PIEZO1 agonists may exist.

Understanding the downstream pathways of PIEZO1 is of particular interest for the production of cultured RBC for transfusion purposes as we already showed for STAT5, MAPkinases and NFATc1. The transition from a static culture to a dynamic culture (e.g. bioreactor) where a strong mechanical force like shear stress can also have an effect on integrin adhesion, causing integrin mediated homophilic interactions, that may lead to premature erythroblast differentiation. While the strong integrin adhesive power makes erythroblasts adhere even to the culture dish (a phenomenon that has been observed in the context of integrin mediated osteoblast adhesion to implant materials [10]), erythroblast-erythroblast interaction are mediated by binding of α4 integrin to ICAM4 [11]. An interesting study that used G1E-ER2 cells has shown how in presence of SCF, adhesion of α5 integrin to fibronectin enhances SCF-induced ERK phosphorylation, while adhesion of α4 integrin to fibronectin leads to reduced SCF-induced Akt activation and apoptosis [12]. Such a selective integrin dependent signal transduction could occur in the case of erythroblast-erythroblast interactions, leading to premature differentiation and apoptosis. In conclusion, PIEZO1 activation is associated with activation of Ca^{2+} dependent signaling pathways, that have an effect on erythroblast proliferation, differentiation and cell death and changes in the context of the erythroblastic island. Moreover, PIEZO1 can modulate the function of multiple receptor, such as EPOR, integrins, and subsequently KIT.

Interaction of erythroid cells in the niche

In vivo, the interaction between the maturing erythroblast and the niche surrounding is essential. These interactions involve both extracellular matrix and cell-cell interactions. The interaction of erythroblasts in the niche is controlled by the binding of integrins to their ligands (VCAM expressed on macrophages and fibronectin present on the extracellular matrix). We found these integrins to be activated upon PIEZO1-dependent Ca^{2+} influx (chapter 3). The connection between the erythroid cell and the macrophage is maintained by integrins, in particular β1 integrins comprising VLA4 (α4β1) and VLA5 (α5β1). In the earliest stages these are stromal cells expressing membrane bound SCF, subsequently erythroblasts associate with macrophages, in the context of the erythroblastic island. Mice lacking β1 integrin are anemic and experience ineffective erythropoiesis (mainly caused by Reactive

Oxygen Species (ROS) accumulation), suggesting a crucial role for these adhesion molecules. Similarly, lack of α4 (which typically binds to VCAM1) but not α5 affects terminal erythroid differentiation [13]. This may be due to a different function, or because α4 is expressed on more differentiated erythroblasts that lack α5. Blocking of β1 integrin or VCAM-1 results in disruption of the erythroblastic island formation *in vitro* [14]. PIEZO1-induced Ca^{2+} influx leads to VLA4 and VLA5 activation, increasing their adhesion capacity to VCAM1 and fibronectin, which are respectively expressed on macrophages and present on the extracellular matrix in the niche. The downregulation of VLA4, VLA5 during differentiation and thus the interaction of erythrocyte precursors with their ligands represents one of the first events leading to enucleation. It is important to discuss the effect of PIEZO1-mediated integrin activation on both erythroblast and the formation and development of reticulocytes. Concerning the erythroblast, PIEZO1 could control integrin affinity for VCAM1 or fibronectin, contributing in keeping erythroblasts in the niche. Consequently, by mediating Ca^{2+} influx during the event of enucleation, PIEZO1 could be one of the sensor that regulates release of the newly forming reticulocytes from the erythroblastic island. In this line, a pathological overactivation of PIEZO1 could prematurely disrupt the binding between erythroid cells and macrophage, explaining the reticulocytosis in HX patients [15]. However, this hypothesis does not justify the reticulocytes levels of HX patients, that in some cases reach up to 20% (given a normal value between 0.8 and 3%). Thus, the reason behind the exorbitant reticulocytes values (which may lie in defective reticulocyte maturation or ineffective erythropoiesis) still has to be elucidated. The endoplasmic reticulum is already lost at this stage, so even if a spike of Ca^{2+} leads to enucleation, targeting PLC and therefore the pathway that mobilises intracellular Ca^{2+}, will not inhibit enucleation. On the contrary, chelating Ca^{2+} with EDTA did inhibit enucleation, proving that this event requires extracellular Ca^{2+} [15]. Moreover, Ca^{2+} ions contribute to chromatin condensation, one of the major event preceding enucleation, by activating calcium/calmodulin-stimulated protein kinase II (CaMKII) and increasing chromosome compaction [16]. It is unknown if PIEZO1 plays a role in these processes but as PIEZO1 is expressed throughout differentiation of erythroblasts to reticulocytes (chapter 3) it beckons to be investigated.

The connection between a mechanosensor like PIEZO1 and integrins on the erythroblastic island, raises the question which stimuli are important for the maintenance of the island. It is interesting to underline that the erythroblastic islands are not located close to the sinusoidal capillary lumen, as one would expect since reticulocyte are promptly released into the circulation, but they are spread across the bone marrow [17]. Moreover, islands far from the sinusoids contain more pro-erythroblasts, while islands close to the sinusoids contain more mature cells, suggesting that either the islands or either the precursors are able to migrate as they mature ([18] reviewed in [19]). In this light, PIEZO1 could, together with other players like EMP, CD163, CD169 (chapter 1), control the migration of erythroid cells from one island to another, as the PIEZO1-mediated integrin affinity for the ligand differs from a more mature erythroblast to a less mature (data not shown). In this light it is

also interesting to remark that during mouse stress erythropoiesis central macrophages are not a homogeneous cell population but change morphology and phenotype depending on the stage of erythroblasts attached [20].

The importance of erythroid island macrophages emerges in the context of stress erythropoiesis (a condition where a higher erythroid output is needed) in situations such as anemia. Induction of anemia caused slow recovery of erythropoiesis in mice were BM macrophages were ablated (in terms of limited erythropoiesis and decreased reticulocytosis). In a review from our group [19], we have hypothesized an increase in BM macrophages during stress erythropoiesis. Yokoyama et al. showed that erythroblastic islands can crawl towards the sinuses as differentiation of erythroid cells progresses, albeit through unknown mechanisms [18]. With this knowledge in mind, we can hypothesize that, under conditions of stress, this sort of mechanical force could cause PIEZO1 activation, followed by integrin activation. The binding of integrins to VCAM1 on macrophages is a trigger for signalling. Mice deficient for VCAM1 experience no erythroid proliferation defects but a decrease in macrophages [21]. Moreover, in response to EPO, erythroblasts release Gas6, that boosts EPO receptor signalling and enhances adhesion of VLA5 to fibronectin [22, 23]. Such a paracrine response could also be observed in our studies. Infact, in a similar way PIEZO1 could synergize with EPOR and initiate a response that triggers VCAM1 signalling and results in increase in macrophages in thus an increase in erythroblastic islands. Recently it was shown that EPOR is present not only on the surface of erythroblasts but also on the surface of erythroblastic island macrophages, which would add another level of complexity [24]. It is important to underlie that many data on integrins and VCAM1 expression on erythroblastic island are performed on mice, so one must be careful to apply this knowledge to the human setting. In conclusion, our findings enforce the concept of crosstalk between the erythroid cell and the macrophage in the context of the erythroblastic island.

In chapter 6 we explored the role of an important homing chemokine in the bone marrow niche, SDF-1 (Stromal Derived Factor 1). Signal transduction induced by this chemokine is known to retain neutrophils and hematopoietic stem and progenitor cells in the bone marrow [25, 26]. The results in this chapter show that this chemokine is able to bind DARC expressed on erythrocyte precursors, in contrast to DARC-expressing erythrocytes, [27] (chapter 6). A recent study showed that absence of DARC on nucleated erythroid cells induces altered haematopoietic transcriptional programming and neutropenia in mice [28]. In particular the composition of the HSPC compartment was altered in the BM of DARC-/- mice. LSK (lineage-negative (Lin^-) Sca-1^+c-kit^+) cells were reduced, and CD34 expression was increased. Despite these results, the peripheral blood count of the leukocyte population remained normal. Moreover, parabiosis experiments in which bone-marrow of DARC -/- mice is transplanted into DARC wild mice indicated that neutrophils migrate from DARC-/- bone marrow into $DARC^{+}/^{+}$ spleen, resulting in neutropenia [28]. It is important to underline that the neutropenia occurred only when DARC was absent in BM precursors and expressed in the venular endothelial cells. This suggests that absence of DARC could induce migration

of neutrophils to the circulation and ultimately to the spleen where endothelial cells are able to associate through DARC expression that is independent of GATA1. The authors excluded that endothelial cells could sequester SDF1 through DARC [29] as SDF-1 was described not to bind DARC. According to our results, SDF-1 indeed does not bind mature erythrocytes but erythrocyte precursors due to a change in the structure of DARC. Therefore, we propose that DARC through SDF-1 could be a potential candidate in regulating neutrophils retention and development in the bone marrow. At the same time neutrophil migration to the spleen could be driven by this DARC interacting chemokine. It is well established that individuals of African ancestry are neutropenic [30]. This neutropenia has been associated with the allelic variant rs2814778(G) of the ACKR1 gene (DARC) that is prevalent in Africa, which abolishes DARC expression on erythrocytes but not on endothelial cells and provides protection against Plasmodium infection [31, 32]. It would be interesting to assess whether splenectomized individuals from African ancestry present neutropenia or not, although no literature reports are present to our knowledge. In conclusion, the finding that SDF-1 interacts with DARC mainly on nucleated progenitors may be of importance in understanding the underlying cause of neutropenia in DARC-/- individuals.

Reticulocyte maturation

In the bone marrow, erythropoiesis progresses until the enucleated reticulocyte stage, after which the reticulocytes are released into the circulation where final maturation occurs. Is there an evolutionary benefit for this maturation to occur in the circulation and not within the bone marrow? Early reticulocytes seem non-optimally adapted to the shear stress within the circulation and display lower deformability [33]. Therefore, it is important to mature cRBC *in vitro* beyond the early reticulocyte stage before the cells are released in large numbers into the circulation. It becomes increasingly clear that reticulocyte maturation is an interplay between intrinsic processes and extrinsic processes, including exosome formation, interactions with splenic and liver cells (e.g. macrophages) and circulation-induced shear forces. During maturation in the peripheral blood the reticulocyte loses 20% of the membrane, which requires conformational changes in the membrane structure. We characterized *in vivo* peripheral blood reticulocytes which appears to be an heterogeneous population of reticulocytes in different stages of progression to biconcave erythrocytes (chapter 4). We confirmed the progressive loss of RNA and transferrin receptor (CD71), and proposed an updated reticulocytes classification based on the expression of CD71 and residual RNA (R1, R2, R3, R4, E). Even in the short time span in which reticulocytes mature into erythrocytes in the circulation (about 24h [34]) an array of changes occurs, one example being: an intense membrane remodeling, for example loss of exposure of specific epitopes within DARC, the same exposure being crucial for *P.vivax* binding and eventual invasion (chapter 4). Interestingly, in a co-authored publication (Nicolet et al., 2018; Appendix: list of

publications), we have shown that despite progressive loss of RNA in maturing reticulocytes, circular RNA is still maintained. The presence of circRNA in reticulocytes but also within vesicles shed by erythrocytes could lead to signalling in cells that phagocyte either the complete erythrocytes or vesicles. In addition the crRNA may be templates of specific translation [35].

One of the major events comprised in reticulocyte maturation is volume control, of which one of the major regulators is the non selective cation channel PIEZO1. In chapter 2 we show how PIEZO1 modulates signaling pathways during erythropoiesis. This channel is also implicated in reticulocytes volume control: the volume loss is regulated by two processes, i) through loss of membranes and intracellular remnants due to exosome/vesicle formation/ shedding and ii) through actions of transporters (such as PIEZO1 and the potassium chloride cotransported (KCC) that regulate the cation/anion and water content and thereby cell volume. PIEZO1 mediated Ca^{2+} influx may have a role in intracellular vesicle formation and release. For instance, influx of Ca^{2+} caused phosphatidylserine (PS) exposure and increased vesiculation in erythrocytes [36]. Moreover, deformability is decreased, and maturation is delayed in reticulocytes from splenectomized patients affected by Hereditary Xerocytosis (characterized by a gain-of-function PIEZO1 mutation and therefor an overactive channel). In particular, however, reticulocytes from HX patients experience an increased intracellular vesicle content [37]. This suggests how aberrant influx of Ca^{2+} can cause adverse events during reticulocytes maturation, including aberrant vesicle formation. Moreover, splenectomy may cause a more detrimental outcome in HX patients, as there is less turnover of deformed cells.

Another major event is membrane remodeling [38]. As described in chapter 1, reticulocyte lose 20% of their membrane during maturation. It is not always easy to assess the expression of specific membrane proteins, as detection differs among different techniques; in chapter 4, chapter 5 and chapter 6 we compared the surface expression of proteins with flow cytometry versus total protein expression with western blot. During reticulocytes maturation, the expression of some proteins (e.g. glycophorin A) remains constant even considering the 20% loss, while surface expression other proteins, such as DARC, seem to decrease because of a differential epitope exposure while the protein content remains constant when detected by Western blot. It is therefore a challenge to determine whether a certain protein is lost, or its expression on the surface of the membrane decreases because of a conformational change. Techniques like mass spectrometry are helpful to assess the absolute amount of protein in cell subsets, but they require an higher amount of material compared to flow cytometry and western blot. The scarcity of the starting material to explore the erythroid proteome is amplified by the fact that reticulocytes are composed by 97% of haemoglobin [39].

The insight that protein remodeling during reticulocyte maturation enabled us to overcome a well-known challenge in *P.vivax* field: establishment of an *in vitro Plasmodium vivax* invasion model using our *in vitro* cultured reticulocytes (chapter 5). We have shown that these cells express all the different membrane proteins associated with invasion. Importantly, the

host cell and the malaria parasite probably need to mature side by side in order to achieve an optimal invasion and propagation [40]. To achieve this co-development, the host cell (reticulocyte) acquired a unique membrane conformation (e.g. correct epitope exposure, correct membrane lipid composition) to avoid infection of old reticulocytes (negative for CD71) by *P.vivax* [41].

The conformation shift of DARC is exploited by a different cellular process. The ability of DARC to bind SDF-1 can be modulated by the inflammatory cytokine IL-8 and reflects whether DARC conformation is open or not. This conformation can be detected by antibodies against specific DARC epitopes. In reticulocytes, treatment with anti-Fy6 decreases the ability of binding SDF-1, while treatment with anti-Fya and anti-FyB increases this ability. Surprisingly, even in erythrocytes, SDF-1 binding is increased, proving that the ability of DARC to bind SDF-1 and therefore to change its conformation is not indefinitely lost and can be induced. Even more surprisingly, treatment with IL-8 in both reticulocytes and erythrocytes increases SDF-1 binding, indicating opening of DARC conformation. Given that *P.vivax* does not invade erythrocytes because of a closed DARC conformation, treatment of IL-8 could open this conformation and allow the parasite to enter. Moreover, it opens the possibilities to use certain chemokines (after assessing whether they could modulate and close DARC conformation) as therapeutic approaches.

Pathophysiological implications

The topics covered in this thesis have different pathophysiological implications, which make a fascinating connection especially between two major topics investigated throughout this thesis, malaria and PIEZO1. Erythropoiesis is defected in patients affected by Hereditary Xerocytosis, an hemolytic anemia characterized by a gain-of-function mutation of *PIEZO1* (chapter 2). Interestingly, the infection rate of *P.falciparum* is decreased in RBC of HX patients[42]. In a mouse model of HX, a *PIEZO1* gain-of-function mutation (E756del) is responsible for the protection of the mice from cerebral malaria [43]. The same variant is associated with protection from severe malaria in Gabonese children [44]. Such a mechanism of protection is also known for sickle cell disease (SCD). It has been shown that carrying one allele of HbS or HbC protects from *P.Falciparum* infection [45, 46]. A recent study that associated the variant E756del in PIEZO1 with clinical parameters in SCD showed that having this variant was associated with increased dehydration. This suggests an increase in severity of SCD form in individuals with PIEZO1 mutations, although a recent report excluded this possibility [47]. Sickle cells are already extremely dehydrated due to activation of 3 ion-transport pathways: KCl cotransport (KCC), the Gardos channel, and Psickle [48], Psickle is a non selective cation channel which mediates Ca^{2+} entry into the sickle erythrocyte [49]. Of note, since the molecular identity of Psickle is unknown, PIEZO1 has been suggested as candidate, since its activation is caused by the sickling membrane deformation [50]. In

chapter 5 we have showed how *in vitro* erythroid culture can be used as source for *P.vivax* invasion. Such an *in vitro* system could be useful to further understand *P.vivax* invasion and biology, as well as therapy possibilities and vaccine development [51]. In the context of *P.vivax* malaria, a potential vaccine could be a molecule that blocks the connection between the parasite membrane and the host cell (a blocking agent). Based on the studies above mentioned, drug-induced overactivation of PIEZO1 could also represent a potential candidate for targeted therapy. While most potential candidate are still at the preclinical stage, *P.vivax* Duffy Binding Protein (PvDBP) represents a good candidate [52]. PvDBP is the essential protein that will bind to DARC (specifically to the DBP binding site, corresponding to the Fy6 epitope which is exposed in reticulocytes as we have shown in chapter 4 and 6). A clinical trial for a vaccine candidate targeting DBP has been already completed (https://clinicaltrials.gov; trial NCT01816113). This vaccine represents a combination of two viral vectors encoding PvDBP: chimpanzee adenovirus and modified vaccinia virus Ankara (MVA). Preclinical studies in mice and clinical studies in human showed that these vectors elicited an immune response, with production of antibodies against PvDBP, as well as T cell response [53, 54].

It is clear from this thesis, that an optimized *in vitro* erythroid culture becomes essential to allow the expression of membrane proteins that could become immune targets. This could serve as a new vaccine that may trigger a long-term immune response, as in the case of the DBP binding site. Maintaining *P.vivax* in culture would allow more functional assays to study the immune reaction caused by potential antigen candidates. Interestingly, malaria disease is associated with an increase in inflammatory cytokines, among which IL-8 [55, 56], with *P.vivax* eliciting a bigger inflammatory response than *P.falciparum* [57]. However, the role of IL-8 has been mostly investigated in the context of *P.falciparum* malaria. IL-8 promotes production of ROS (reactive oxygen species) in myeloid cells, to induce endothelial cell activation and to promote T cell response [58]. In chapter 6 we show how we can increase or decrease DARC exposure in erythrocytes and reticulocytes by treating with antibodies recognizing the different epitopes. We also show that well known DARC-binding chemokines like IL-8 are able to open DARC epitope even in erythrocytes, where *P.vivax* is not expected to invade because such epitope is closed [59, 60]. DARC is considered a scavenger receptor, meaning that it removes inflammatory chemokines (like IL-8) and peptides from the circulation. It could be tempting to add IL-8 to *P.vivax* cultures, because it would allow DARC opening and invasion in erythrocytes; however, considering the knowledge that the parasite co-matures with the reticulocyte, culturing *P.vivax* in erythrocytes would not be feasible, as these cells have terminally rearranged their membrane. It would also not be recommended for *in vivo* studies, as it would cause an additional state of inflammation. The question arises whether the other way around is also true: does a state of inflammation increase susceptibility to malaria? Inflammatory responses have been shown to influence clinical severity [61]. But whether this is caused by cytokines altering the epitope morphology of DARC in erythroid cells, remains unclear.

Future perspectives

Erythroid culture protocols are mainly driven by the intrinsic ability of erythroblasts to differentiate into reticulocytes but fail to produce fully biconcave erythrocytes, stressing that specific maturation cues are missing. Although these missing signals may partly originate from the bone marrow niche, it is surprising that pure >95% enucleated reticulocytes can be cultured without this support [33, 62-64]. Failure to progress from reticulocytes to fully biconcave erythrocytes thus suggests a lack of signals that would otherwise come from the bone marrow niche, or from the circulation. A membrane protein like DARC, may not only be essential to *P.vivax* infection, but also for the homeostasis of the bone marrow niche. Of note, also stromal co-cultures (MS5 or OP9) do not completely result in reticulocyte maturation to erythrocytes [65, 66]. Nevertheless, a recent study published a characterization of the secretory proteins from OP9, and such studies may eventually point to specific factors to will further facilitating erythropoiesis and possibly reticulocyte maturation [67]. Extrinsic signals, such as circulation-induced shear stress, may induce transport/exchange of ions to regulate volume control, and to facilitate reticulocyte maturation [37]. The link between integrins and their ligands induces a signal that may crucially influence proper maturation, a link that is missing when culturing cells without macrophages. However, more research must be performed to dissect the contribution of, and the connections between, intrinsic processes and extrinsic factors to reticulocyte maturation. Even prior to this, the field is currently still assessing the identity/nature of these external factors and their concomitant erythrocyte "signal transducer" counterparts before characterization and optimization can begin. For instance, the connection between exosome formation, cargo selection (e.g. ubiquitination) and ion transport mediated by ion channels is interesting to pursue. The importance of such cation/anion channels is given in this thesis by indicating the strong regulatory role that the cation (and thus Ca^{2+}) mechanosensitive channel PIEZO1 is playing on erythropoiesis. Such knowledge will become essential in the setup of a non-static shear stress producing bioreactor to upscale erythroid culture, which will overcome the challenge of producing large quantities of cells for transfusion product, in a cost-effective manner. A second challenge to overcome is to deliver a quality product. The aid of novel techniques to separate and discriminate the various reticulocyte maturation stages and the progressive use of lower cell numbers in -omic approaches as outlined in this thesis will facilitate this research. Future erythroid culture protocols will need to incorporate the regulation (activation) of these processes to ensure complete differentiation to fully biconcave erythrocytes. The promise of *in vitro* cultured erythrocytes that could on one side be personalized and loaded with specific cargo and on the other side be used as model system to understand erythropoiesis in health and disease (e.g. vaccine development in *P.vivax*), justifies research into this last elusive step of erythropoiesis.

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