Journal of Circulating Biomarkers

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Uncovering the Role of Erythrocyte-derived Extracellular Vesicles in Malaria: From Immune Regulation to Cell Communication

Review Article

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Received 18 Feb 2014; Accepted 12 Mar 2014

DOI: 10.5772/58596

Abstract Investigation of the involvement of extracellular vesicles (EVs) in parasite biology has burgeoned in recent years. Human infecting protozoan parasites, such as *Trypanosoma cruzi*, *Lesihmania sp.* and *Trichomonas vaginalis*, have all demonstrated the utilization of EVs as virulence factors in order to activate or hamper host immunity. Novel findings have provided evidence that the deployment of EVs by *Plasmodium sp*. has a major impact in disease outcomes and serves as an integral part in controlling stage switching in its life cycle. Clinical studies have highlighted elevated levels of EVs in patients with severe malaria disease and EVs have been linked to increased sequestration of infected red blood cells to the endothelium, causing obstruction of blood flow. It has also been found that EVs produced during malaria disease activate innate immunity. Intriguingly, recent discoveries indicate that *Plasmodium sp*. "highjack" the erythrocyte microvesiculation system in order to cross-communicate. Both the transfer of DNA and parasite density regulation has been suggested as key mechanisms of EVs in malaria biology.

Keywords Extracellular Vesicles, Malaria, Cell Communication, Inflammation, Plasmodium falciparum

1. Introduction

'Quorum sensing' refers to the regulation of gene expression in an organism as a response to variations in cell density in a population of cells. This phenomenon has been well studied in bacteria where certain species produce and secrete molecules called auto-inducers. The concentration of these molecules increases with increased cell density, while differential gene expression in a cell occurs once a minimal threshold stimulatory concentration has been reached [1]. More recently, it has been found that similar phenomena occur among disease-

causing protozoan parasites such as *Leishmania sp.*, which causes leishmaniasis, *Trypanosoma cruzi*, the causative agent of Chagas disease, *Trichomonas vaginalis*, which induces vaginitis and *Plasmodium sp.*, the malefactor of malaria disease. Malaria currently affects upwards of half a billion people each year and mortality rates have been estimated to exceed 600,000. The majority of deaths occur in the sub-Saharan regions of Africa, where young children, pregnant women and the elderly are at elevated risk of morbidity and mortality. Several species of Plasmodium can infect humans, namely *P. ovale*, *P. malariae*, *P. knowlesi*, *P. vivax* and *P. falciparum*, where the latter accounts for the majority of deaths. Malaria disease includes symptoms such as fever and headaches, which in approximately 1% of *P. falciparum* infections can progress to severe cases and ultimately result in coma and death. The most common form of severe malaria is anaemia, but cerebral malaria, which comprises neurological disorder, is among the deadliest [2].

Plasmodium species are obligate intracellular protozoan parasites that belong to the phylum Apicomplexa. The complex life cycle of the human infecting *Plasmodium* species entails switching between a mosquito vector and a primate host. In the host, the majority of parasites reproduce asexually; this stage gives rise to the severe symptoms and the mortality of the disease. However, differentiation into gametocytes (the sexual stage) also occurs inside the human host (Figure 1). Gametocytogenesis is the destiny of a minority of the total parasite population and the mechanisms underlying sexual differentiation are largely unknown. The gametocyte is the sole causative agent of transmission from the human to the mosquito, as this is the life cycle stage that is actively transmitted during a mosquito blood meal. In the mosquito midgut, male and female gametocytes reproduce sexually through meiosis, giving rise to millions of sporozoites ready to infect new human hosts upon subsequent mosquito feedings. Several factors are hypothesized for controlling the rate of malarial sexual stage commitment. These include host immunity, chemotherapeutical agents and anaemia [3], however, the exact mechanisms remain largely unknown [4]. It has been proposed that unidentified factors secreted or released by parasites trigger differentiation to gametocytes [5]. Recently however, extracellular vesicles (EVs) derived from iRBCs have been proposed to be a key factor in the regulation of sexual stage commitment.

EVs are small vesicles derived from intact cells. Different types of vesicles have been described and are named according to their origin and size, such as exosomes, microvesicles, apoptotic bodies and oncosomes [6]. Exosomes, which are a type of EVs, originate from late endosomes and are formed in multi-vesicular bodies (MVB). Once the MVB fuse with the cell membrane, the exosomes are released in the extracellular space. Although exosomes were first described in reticulocytes in 1987 by Johnstone and colleagues, they can be secreted by a large variety of cells, in particular, upon cell activation [7]. There are currently several markers used to identify exosomes, such as Alix, TSG101, heat shock proteins and the tetraspanins (CD9, CD63 and CD81) [8,9]. Exosomes mediate cell to cell transfer of information [10,11] and regulate the immune response during infections and tumourogenesis [12]. Microvesicles (MVs) (syn. Ectosomes, microparticles) are secreted after budding or shedding from the plasma membrane [6] and they display similar physiological features as the cells from which they originate. For example, MVs express receptors from the cell of origin on their surface, allowing their identification by antibody labelling in complex biological samples [13,14]. Although it is not always possible to distinguish between exosomes and MVs, MVs are generally larger in size (0.1 - 1 μM), whereas exosomes range from 50-150 nm. In several published works, it is impossible to clearly distinguish between exosomes and MVs; thus, we will use the abbreviation EV as a general term for both types of vesicles. In this review, we discuss the involvement of EVs in malaria pathology and disease, as well as the recent discoveries of EVs as quorum sensing-like auto-inducers of parasite density regulation, including their suggested functional mechanisms.

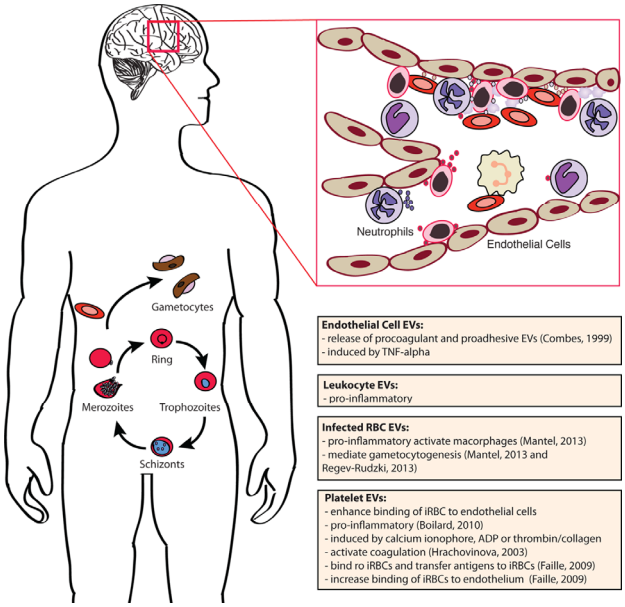

Figure 1. Parasite life-cycle and pathogenesis: the blood stage is responsible for the pathology and symptoms observed in malaria infection. Within the 48 hours of the life cycle, the parasite develops into the RBC from a ring form to trophozoites and finally schizonts; after rupture of the iRBCs, the merozoites invade uninfected RBCs to repeat a new cycle. During the asexual cycle iRBC produce EVs that interact with immune cells and mediate stage conversion to gametocytes. Mature stages of parasites sequester in tissues to avoid clearance by the spleen, triggering local inflammation and activation of the endothelium and accumulation of immune cells, particularly in the brain. Vascular cells release EVs, contributing to the pathology.

2. Extracellular vesicles are elevated in the plasma of malaria patients

EVs can be detected in the different body fluids of healthy donors such as urine [15], plasma [16], breast milk [17] and saliva [18]. Circulating EVs are mostly derived from platelets, but RBCs, blood leukocytes and endothelial cells are also an important source of EVs. EV production is elevated in several diseases, such as cancer [19], inflammation [20], cardiovascular disease [21], idiopathic thrombocytopenic purpura [22], thalassaemia [23] [14] in human immunodeficiency virus (HIV) patients [24] and Ebola Virus-infected macaques [25]. Thus, EVs are promising biomarkers for the diagnosis of pathologies, as well as in monitoring treatment response in patients. Furthermore, EVs are important mediators and regulators of physiological responses. They contribute to coagulation, inflammation, vascular homeostasis and the development of tumours and metastases [26]. Several studies have highlighted elevated levels of EVs during malaria episodes, especially in patients experiencing a severe form of the disease. In a study performed in Malawi with children aged six months to 12 years infected with *P. falciparum*, the plasmatic concentration of EVs-derived from endothelial cells was increased at the time of admission in patients with fatal malaria, as measured with the endothelial cell-specific marker CD51 by flow cytometry [27], whereas in uncomplicated cases, the level of EVs remained at the same level as in healthy controls. The EV release may be the result of endothelium activation or a direct mechanical result of cytoadherence of iRBC to the endothelium [28]. It has been shown that activation of the endothelial cells by TNF-alpha in lupus leads to the production of EVs [29]. Similarly to lupus, plasma TNF concentration is increased in patients with malaria [30] and TNF can induce the release of procoagulant and proadhesive EVs from cultured cells in vitro [29]. Therefore, TNF is likely to play a role in local inflammation and the release of endothelial EVs. While the endothelium in the capillaries is activated, it interacts with leukocytes, platelets and iRBCs; therefore, other cell types are likely to produce EVs during malaria. A study performed in Douala, Cameroon, found that platelet, RBC, endothelial and leukocytic EV levels were elevated and to a lesser extent lymphocyte EVs in malaria infected patients with cerebral dysfunctions; however, these had returned to normal at the time of discharge. Interestingly, EV levels were found to correlate with a number of clinical and biological parameters. In CM patients, the platelet-EV increase was associated with the depth and duration of the coma, as indicated by the negative correlations between EV levels and Coma Resolution Time (CRT). Thus, platelet-EVs have the potential to be implemented as candidate markers for follow up diagnosis of patients post treatment, a topic of tremendous clinical importance.

While the microvascular sequestration primarily involves iRBCs, there was no correlation between parasitaemia and RBC-EVs CD235a [31]. Another study composed of 36 patients suffering from acute *P. falciparum*, *P. vivax* or *P. malariae* infection based in Thailand, found that patients with *P. falciparum* had the highest concentration of RBC-derived EVs compared to healthy controls. In this study, a specific marker for phospholipid PS (annexin V) and anti-glycophorin A were used for the identification of RBC EVs. In vitro, the EVs were released mostly by mature stage parasites. Infected RBCs release 10-15 times more EVs than uninfected. About 40% of the EVs were positive for RESA, a parasite protein, which is exported by the parasite to the RBC cell membrane [32]. The concentration of RBC-derived EVs quickly decreases after treatment with standard courses of artemisinin derivatives. EVs are likely to be removed by the spleen, since splenectomised malaria patients have increased levels and prolonged circulation of EVs [23]. The splenic reticular-endothelial system removes cells and particles that express PS on their surface. The liver and the lungs can also contribute to the clearance of EVs, as has been shown in a murine model [33]. Furthermore, it has been found that the concentration of EVs was elevated in *P. vivax* and *P. malariae* infection, although to a lesser extent than in *P. falciparum* infection [34]. In a fourth study based in Brazil, the EVs derived from RBC (CD235a), CD45 (leukocytes), CD41a (platelets), CD144 (endothelial cells) and CD41 (monocytes) [35] were analysed. The study was composed of 37 *P. vivax* malaria patients between 15-66 years of age, 15 healthy donors and 12 ovarian cancer patients (an EV-inducing disease) [36]. The study concluded that most EVs found in plasma during *P. vivax* infections are derived from platelets, RBCs and leukocytes. Platelet-derived EVs increased in a linear fashion, with the presence of fever at the time of blood collection [35]. The conclusion of these studies are somewhat varied and it is important to note that the number of patients was limited and the clinical profile, as well as the geographic location, may in part have contributed to the heterogeneity observed in the results. In addition, the method of isolation, storage and measurement of EVs were not optimal. Yet several laboratories have reported that the minimal size cut-off for measuring EV by flow cytometry is between 200-500nm [37]. Since EVs range from 100 nm-1 um in size, it is likely that the smaller vesicles were not detectable by flow cytometry. Additionally, antigen expression might have been too low in certain subsets of the samples, which would have prevented accurate measurements.

Technological advances are likely to offer better instruments for more accurate measurements in the future. In general, protocols used to prepare samples for EV analysis are relatively facile and based on differential centrifugations [38].

The above mentioned studies have demonstrated a direct correlation between EV levels and disease severity; it was not, however, possible to assess the direct role of EVs in malaria pathology based on the above described sets of data and further studies are mandatory to fully elucidate the role of EVs in malaria.

2.1 The role of EVs in malaria disease

Autopsy studies of brain tissue from patients who died of cerebral malaria revealed multifocal capillary obstruction by iRBCs, platelets and leukocytes [39]. Malaria is a complex disease in which the outcome is determined by a combination of several factors. Despite intense research, there is still no clear understanding of the mechanisms leading to severe disease or death. However, obstruction of capillaries and a reduction in perfusion of essential organs, as well as leukocyte activation and inflammation, are thought to contribute to the disease [40]. An exaggerated immune response compromises the integrity of the BBB [41] and CM has a high mortality rate of about 20%, despite treatment [42].

Infected RBCs sequester in capillaries and cause a considerable obstruction in blood flow, thereby compromising the perfusion of organs. The sequestration is mediated by the interactions of the plasmodial family of receptors, i.e., *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) with host receptors expressed on the host endothelial cells, such as ICAM1, CD36 and VCAM1 [43]. During infection, the endothelium is further activated and as such, the expression of host receptors favouring sequestration of iRBCs is increased. Only mature parasites sequester in the capillaries, whereas the immature ring stages are found in circulation. Adherence protects the parasite from destruction and clearance by the spleen as non-adherent mature stages are rapidly cleared [44]. Parasites sequester in various organs including the heart, lung, brain, liver, kidney, subcutaneous tissues and placenta. In addition to the accumulation of iRBCs, several histopathological studies have demonstrated excessive accumulation of leukocytes [45] and platelets [46] in the brain of deceased patients. All the vascular cells produce EVs, including monocytes [47], RBCs [48], leukocytes [24], platelets and endothelial cells [29]. It is therefore highly likely that EVs contribute to the pathology by triggering inflammation and promoting sequestration of iRBCs to the endothelium.

2.2 The role of platelets as EV producers

In vitro, platelets produce EVs when stimulated by calcium ionophores, ADP or thrombin/collagen. The platelet-EVs can activate coagulation in vitro and participate in thrombus formation in vivo in a model of vessel injury through their surface expression of tissue factor and phosphatidylserine (PS) [49]. Depending on the agonist used to stimulate vesiculation, the amount and composition of the EVs will vary and therefore their effect on the recipient cells may vary as well, depending on the stimulus; for example, after thrombin stimulation platelets release EVs enriched in miRNAs [50]. Platelets not only contribute to wound healing but also play a role in fighting infections, platelet-deficient or aspirin-treated mice are more susceptible to death when infected with *P. chabaudi* [51]. Platelets have been found to bind to the iRBCs and kill the parasites within post release of PF4 [52]. Under some circumstances, platelets contribute to inflammation, for example, platelet-EVs accumulate in the joint fluid of patients with rheumatoid arthritis. There, the EVs are pro-inflammatory and can induce the secretion of IL-1 from synovial fibroblasts [53].

Furthermore, platelets play an important role in the induction of the clumping of iRBCs [54] and *in vitro* induce the cytoadherence of iRBCs to endothelial cells [55,56]. Interestingly, platelet-EVs bind to iRBCs and transfer platelet antigens into the infected cells; binding occurs only to iRBCs in a PfEMP1 dependent-manner, whereas platelet-EVs do not bind to uninfected RBCs. The platelet-EVs dramatically increase the binding of iRBCs to the endothelial cells [57]. Therefore, platelet-EVs contribute to sequestration and the avoidance of clearance of iRBCs by the spleen.

3. Activation of endothelial cells during malaria

Endothelial cells, when stimulated with tumour necrosis factor (TNF) or LPS, are able to produce increased numbers of EVs, thereby showing the role played by TNF in endothelial activation and the capability of cells to vesiculate in response to infectious stimuli. Interestingly, *ex vivo*, the endothelial cells from patients with complicated malaria are more responsive to TNF-alpha stimulation and produce an elevated amount of EVs [58], suggesting that some people may be genetically more prone to developing severe disease.

3.1 Malaria in vivo models and the discovery of EVs in severe disease

The first direct evidence for a role of EVs in the pathogenesis of severe malaria came from the mouse model of CM with *P. berghei*, using the ATP-binding-cassette transporter 1 (ABC1) knock-out mice. ABC1 is a transporter involved in membrane-lipid turnover and promotes Ca2+-induced exposure of PS at the membrane surface, an early step in microvesiculation. The ABCA1 gene is mutated in Tangier disease, a disorder of free cholesterol efflux to high density lipoprotein [59]. Hamon et al. discovered that the shedding of RBC EVs is reduced by about 70% upon ionophores stimulation in the

ABC1-/- mice [60]. The ABC1 -/- mice were completely resistant to cerebral malaria upon infection with the lethal strain *P. berghei* ANKA. All the ABC1 deficient mice survived the neurological phase, whereas 90% of the wild-type mice died within the first seven days following infection. The immune response was impaired in the absence of ABC1 and the mice had a lower plasma level of TNF-alpha. These mice also had a weaker up-regulation of endothelial adhesion molecules in the brain micro-vessels, reduced leukocyte sequestration, as well as an ablated platelet accumulation. Moreover, the number of EVs was found to be dramatically reduced [61]. In addition to RBC, platelets and macrophages harbour a vesiculation defect upon stimulation by an agonist *in vitro*. Interestingly, the WT and the KO showed comparable levels of EVs prior to infection. However, the level of EVs increased significantly in the WT at the time of the cerebral syndrome, whereas it remained low in the ABCA1 KO. After isolation, the EVs from wild-type mice were more efficient at inducing clotting and TNF production from macrophages than the EVs derived from the ABCA1 KO [61]. Similarly, the administration of the low-molecular-weight thiol pantethine prevented the cerebral syndrome in *P. berghei* ANKA-infected mice. The protection was associated with an impairment on the part of the host's response to the infection, in particular, with a decrease of circulating EVs and preservation of the blood-brain barrier integrity without affecting parasite development [62].

3.2 EV activation of innate immunity

The interaction of malaria parasite-derived moieties with cells of the immune system is regarded as the initial step in the induction of the inflammatory response that determines the severity of the disease's condition. Several parasite factors are believed to be released during egress and to induce a potent pro-inflammatory response. Although the exact mechanisms and nature of these factors remain unknown, several "malaria toxins" have been identified. The Plasmodium glycosylphosphatidylinositol (GPI) triggers a potent immune response by macrophages and the vascular endothelium [63,64]. In addition, the parasite digests haemoglobin and the resulting products, the hemozoin crystals, are coated with plasmodial DNA that trigger TLR9 [65,66]. The AT-rich DNA can induce type I IFNs in a TLR-independent fashion [67]. Additional factors are likely to contribute to the immune modulation; in fact, another study demonstrated that EVs, purified from the blood plasma of mice infected by malaria induced potent activation of macrophages in a Toll-like receptor dependent manner. Immunofluorescence staining revealed that EVs contained significant amounts of parasite material, indicating that they were derived primarily from iRBCs rather than platelets or endothelial cells [68]. Furthermore, EVs more potently induced macrophage activation than intact iRBCs. Macrophage activation by EVs was mediated by a TLR-4- and MyD88-dependent pathway [68]. A role for iRBC-EVs was further confirmed with the isolation of EVs from in vitro cultured *P. falciparum* iRBCs. These studies directly demonstrated that EVs originate from iRBCs and infection of the iRBCs with *P. falciparum* increases the secretion of EVs from iRBCs by 15 fold when compared with uninfected RBCs [69,34]. Proteomic analyses further demonstrated that EVs contain malaria specific proteins; most of the plasmodial proteins are known to be exported and derived mainly from the Maurer's cleft, which are small organelles connected by actin filaments to the RBC membrane [70,69]. Interestingly, small vesicles were found by electron tomography to be bound to the actin filaments. The Maurer's clefts serve as a platform for the export of parasite proteins to the RBC cell surface [71]. Interestingly, EVs interact with the innate immune system and are rapidly phagocytosed by macrophages, inducing their activation and secretion of TNF-alpha [69]. The TNF production due to macrophage activation following infection is a major source of TNF *in vivo* [72]. There is a positive correlation between TNF level and EVs found in the plasma of patients with malaria [73]. In conclusion, these data indicate that iRBC EVs are highly likely to contribute to the inflammation observed in severe malaria and might therefore be a novel target for the development of therapies to prevent severe disease (Figure 1).

4. RBC-derived EVs in cell-to-cell communication

In addition to the contribution of EVs from different cell sources to malaria pathology, recent evidence indicates that EVs derived from *P. falciparum* iRBCs can regulate parasite density and mediate horizontal transfer of nucleic acids to regulate the rate of conversion into gametocytes, i.e., the transmission stage. To determine whether iRBCs communicate and transfer information, Regev-Rudzki and colleagues used two strains expressing different drug resistance cassettes, which were linked to different fluorescent markers expressing red or green fluorescence. When the two transgenic strains (one containing a blasticidin (BSD) resistance marker and the other a WR resistance marker) were co-cultured in vitro, the parasites initially died. However, five days post initial drug treatment, recrudescence occurred and the surviving parasites contained both the green and red fluorescent markers, demonstrating the transfer of plasmids between the two transgenic strains. Using a trans-well membrane system it was subsequently shown that the transfer of drug resistance is not dependent on cell to cell contact, but instead depends on soluble factors from iRBCs smaller than 400 nm [74]. The transfer is inhibited by cytochalasin D, an inhibitor of actin polymerization and oryzalin [75]. Finally, the transfer of

DNA was demonstrated by using in vitro purified EVs. Vesicles could be observed budding at the surface of the iRBCs by atomic force microscopy (AFM) [74]. Quantification of vesicles by Nanosight in supernatants of synchronized parasite culture indicated that most EVs were released during the late stages and the size of the vesicles varied from 50-250 nm [69]. The vesicular shape, size and lipid bilayer were further confirmed by electron microscopy and cryo-electron microscopy. Fluorescent-labelled EVs were internalized by iRBCs and targeted towards the nucleus, and accordingly, the transferred plasmid DNA was localized in the nuclear periphery, thereby suggesting that EV cargo might target gene regulation in the recipient cell. Furthermore, in vitro analyses have shown that iRBC EVs induce sexual stage conversion in cultured parasites in a dose dependent manner. Highly elevated levels of gametocytes were found in cultures where EVs had been introduced [69,74].

The Maurer's clefts are important for the production of EVs and the PTP2 knock-out parasites produce less EVs as measured by AFM. PTP2 is expressed on budding vesicles from the Maurer's clefts and is essential for PfEMP1 export to the host cell membrane [76]. Interestingly, the PTP2 KO parasites were also defective in the uptake of MVs, since transfer of the BSD gene was not possible using this strain. The localization of PTP2 on vesicles budding from the Maurer's clefts suggests that EVs may rather be derived from the Maurer's clefts rather than from the host cell membrane, illuminating the possibility that different kinds of vesicles with diverse functions are released by iRBCs (Figure 2).

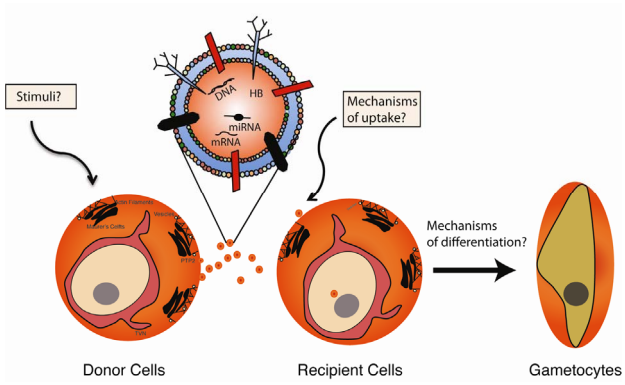

Figure 2. *P falciparum* infected RBCs (donor cells) secrete EVs that are subsequently taken up by another iRBC (recipient cell). EVs act as a quorum sensing-like mechanism by inducing differentiation towards gametocytes. The stimuli that trigger EV release from the donor cell, as well as the mechanisms of uptake by the recipient cells are still unknown.. However, the Maurer's clefts seem to play a role particularly in the protein PTP2. The molecular mechanisms of differentiation are unknown; however, EVs contain several factors such as DNA, mRNA, miRNA, proteins and lipids that can potentially be involved.

5. Quorum sensing-like mechanisms in *Plasmodium* infections

Since iRBC-derived EVs are able to transfer nucleic acids from parasite to parasite and to induce differentiation to gametocytes, they have been proposed to exhibit quorum sensing-like mechanisms. Previous investigations into bacteria have shown that prokaryotes use signalling molecules to control traits such as antibiotic production, nutrient storage, sporulation, biofilm formation and virulence factor secretion. This phenomenon, which was initially discovered in bacteria, has been termed 'quorum sensing' and in general refers to activities using signalling molecules to synchronize activities among large populations of cells.

Some basic principles can be distinguished in QS according to Rutherford et al.; in the first stage, the bacteria produce the signalling quorum-sensing molecules. While at low cell density, the molecules diffuse without reaching the threshold required for detection. As the cell density increases, the cumulative production of molecules leads to a local high concentration sufficient for reaching the threshold necessary for detection and response. Finally, after detection by specific receptors in the cells and activation of the expression of genes necessary for the cooperative behaviours, the QS molecules induce the release of more QS molecules. This positive feedback mechanism of induction further increases bacterial synchronicity [77]. The success of infection depends on the ability of the parasite to survive in its mammalian host and to ensure transmission to future hosts. Overgrowth can damage the host and thereby limit the parasites transmission potential [78]. The quorum-sensing mechanism has been observed in vector-borne parasites such as *Trypanosoma brucei*, which is the causative agent of African sleeping sickness. The parasites differentiate from replicating slender bloodstream forms to non-dividing stumpy forms, thereby limiting the parasite's population size and allowing survival of the mammalian host and establishment of a stable host-parasite relationship [79]. In the protozoan parasite *T. brucei*, a density-sensing mechanism activates the differentiation of proliferative slender cells to stumpy forms through the release of stumpy induction factor (STI) [80,81,82]. It would be a major advantage for parasites such as *P. falciparum* to communicate during blood-stage infection in order to enable populations to react to changing conditions in the host and to regulate cell density [83].

In conclusion, EVs constitute a potential target for the control and management of severe *P. falciparum* infections; it also poses as a target for novel transmission blocking strategies of this important human pathogen. It is a major advantage for *P. falciparum* to communicate during the blood-stage infection in order to enable parasites at the population level to react to changing conditions in the host.

6. The involvement of EVs in gametocyte induction

Although EVs can serve as vehicles for transferring plasmid DNA from parasite to parasite, the real factor responsible for cell-cell communication and the induction of gametocytes is yet to be identified. Several studies in other systems have pointed out a role for lipids, proteins, DNA and RNA in transfer of information mediated by EVs. For example, EVs may contain different forms of DNA, genomic DNA fragments [84] [85], mtDNA [86] ssDNA and cDNA retrotransposon [87]. It has been shown that gDNA can be transported by EVs and increase the gDNA-coding mRNA and protein expression in the recipient cells [84]. For example, the BCR/ABL hybrid gene was transferred from K562 EVs to normal neutrophils and localized close to the nucleus, as demonstrated by DNA-FISH, thereby suggesting that tumours can transfer genetic mutations from cell to cell as a new avenue for expanding tumours [84].

EVs can mediate the transfer of functional transmembrane proteins and proteases from the donor to the target cell. In the context of a tumour, the glioblastoma cells can share the oncogenic receptor EGFRvIII through EVs, thereby favouring tumour growth [88] [89] by providing a functional receptor on the target cells. The EV-derived from a tumour can transfer the oncogenic receptor tyrosine kinase MET to bone marrow-derived cells to promote their education, mobilization and pro-invasive behaviour [90]. Active Wnt is secreted in exosomes during Drosophila development and in human cells [91] also Wnt11 secreted in fibroblast EVs drive breast cancer cell invasive behaviour [92]. Some pathogens use EVs secretion to their own advantage to favour their growth and evade the immune system.

GP63, a Zn-metallo-protease secreted in EVs by *Leishmania donovani*, targets pre-miRNA processor Dicer1 to prevent miRNP formation in *L. donovani* interacting hepatic cells, thereby shutting down the lipid metabolism and promoting parasite growth [93]. The CCR5 chemokine receptors are transported on EVs and transfer of CCR5+ EVs by PBMCs to CCR5- PBMCs render the CCR5- cells susceptible to HIV infection [94].

Additionally, EVs contain mRNA and miRNA that can be transferred to recipient cells and regulate gene transcription in the recipient cell [10]. The RNA profile of EVs is different than the RNA profile of the source cells and EVs seem to lack ribosomal RNA, while being enriched in small RNA [95]. The role of miRNA has been studied in a different context. The EVs can transfer miRNA between T cells and dendritic cells (DC) at the immunological synapse [96]. DCs release EVs that contains miRNA. Upon maturation of the DCs, the miRNA profile varies [97], regulating the transcription of genes in the target DCs; therefore, immature DC-EVs have a different function than mature DC-EVs. Epstein Barr viruses infect B cells and functional mature EBV-encoded miRNAs are secreted in EVs by EBV-infected B cells and mediate repression of CXCL11/ITAC, an immunoregulatory gene down-regulated in primary EBV-associated lymphomas in recipient immature monocyte-derived dendritic cells [98].

Platelets have been shown to contain an abundant and diverse array of microRNAs and platelet-derived MVs are the most abundant EVs in the blood circulation. Upon activation, platelets release EVs containing functional Ago2:miR223 complexes [99], which can induce apoptosis after internalization by recipient cells (HUVEC) by down-regulating the expression of the insulin-like growth factor 1 receptor [50]. EVs secreted by myotubes play a role in myogenesis by transferring miRNA and down-regulating Sirtuin1 in myoblasts [100]. Interestingly the proteomics analysis of iRBC-EVs revealed the presence of human Argonaute 2 as a component of iRBC EVs. Furthermore, RNA can be isolated from EVs and is mainly composed of small RNA, as shown by Bioanalyzer analyses, thus raising the possibility that EVs contain functional RISC complexes that could be transferred to the recipient cell in order to regulate gene transcription. Interestingly, it has recently been shown that RBC miRNA can regulate that gametocytaemia. La Monte and colleagues provided evidence for the presence of miRNA in HbSS and HbSA in patients with sickle cell anaemia, regulates gametocyte differentiation [101]. Intriguingly, at a similar level as that seen by Mantel et al., where EVs from *P falciparum* iRBCs were shown to have an important role in parasite density regulation [69]. It has been shown that EVs from iRBCs contain small RNAs, suggesting a similar function as that seen in patients with sickle cell traits. The presence of functional miRNAs in EVs has also been found in other systems [98,99]. Although plasmodium is not thought to possess the machinery for RNAi pathway [102], it has been demonstrated that host miRNAs regulate gametocytaemia.

A strong link between the transcription factor APapi2 and sexual stage conversion has been indicated [103]. It would be highly interesting to try and identify the links between EVs and epigenetic factors that influence gametocytogenesis. Investigating the content of EVs in terms of lipids, DNA, RNA and proteins will likely reveal the molecular pathways involved in the quorum sensing-like mechanisms in parasite communication and density regulation.

7. Conclusion

With escalating evidence that the role of EVs are highly influential in malaria biology, it has become clear that investigations concerning synthesis, mechanisms of

uptake, content, as well as their direct role in transmission are highly important as part of an incentive for understanding malaria biology. The recently discovered link between EVs and malaria sexual stage switching holds promising potential for solving the long-standing problem of hindering malaria transmission and ultimately eradicating malaria disease.

8. Acknowledgements

Johan Ankarklev is sponsored by a grant from the Swedish Research Council (VR).

9. Compliance with ethical research standards

The authors declare no conflict of interest.

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