PORTLAND PRESS

Review Article

The enemy within: lipid asymmetry in intracellular parasite–host interactions

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Eukaryotic pathogens with an intracellular parasitic lifestyle are shielded from extracellular threats during replication and growth. In addition to many nutrients, parasites scavenge host cell lipids to establish complex membrane structures inside their host cells. To counteract the disturbance of the host cell plasma membrane they have evolved strategies to regulate phospholipid asymmetry. In this review, the function and importance of lipid asymmetry in the interactions of intracellular protozoan parasites with the target and immune cells of the host are highlighted. The malaria parasite *Plasmodium* infects red blood cells and extensively refurbishes these terminally differentiated cells. Cholesterol depletion and an altered intracellular calcium ion homeostasis can lead to disruption in erythrocyte membrane asymmetry and increased exposure of phosphatidylserine (PS). Binding to the PS receptor on monocytes and macrophages results in phagocytosis and destruction of infected erythrocytes. *Leishmania* parasites display apoptotic mimicry by actively enhancing PS exposure on their surface to trigger increased infection of macrophages. In extracellular *Toxoplasma gondii* a P4-type ATPase/CDC50 co-chaperone pair functions as a flippase important for exocytosis of specialised secretory organelles. Identification and functional analysis of parasite lipid-translocating proteins, i.e. flippases, floppases, and scramblases, will be central for the recognition of the molecular mechanisms of parasite/host interactions. Ultimately, a better understanding of parasitic diseases, host immunity, and immune escape by parasites require more research on the dynamics of phospholipid bilayers of parasites and the infected host cell.

Introduction

Many important pathogens, such as *Plasmodium spp.*, the causative agent of malaria, or *Leishmania spp.*, which cause a spectrum of skin, muco-cutaneous, and visceral diseases, are single-cell eukaryotes, which adopted a parasitic lifestyle. These protozoan parasites share all hallmarks of eukarya, including large genomes, cellular organelles, and complex phospholipid bilayers in their plasma membranes (PMs). Beyond barrier functions and nutrient uptake, the parasite PM facilitates pathogen/host interactions through modulation of immune cells and, for intracellular parasites, by driving host cell invasion. Accordingly, analysing the dynamics of parasite and parasitised phospholipid bilayers is central for a better understanding of disease, host immunity, and immune escape by the parasite.

Red blood cells (RBCs) maintain an asymmetry between the lipids present in each layer of their membrane bilayer (Figure 1). Anionic phospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) are sequestered to the inner (cytoplasmic) leaflet of the membrane, while other lipids, such as phosphatidylcholine (PC) are more abundant in the outer (extracellular) leaflet [5]. Indeed, the concept of membrane asymmetry was first described in RBCs nearly half a century ago [6,7]. Technological advancements and concentrated research efforts have vastly improved our knowledge and understanding of membrane asymmetry since these early studies. We now know that membrane asymmetry is not restricted to RBCs, but a feature of all mammalian cells and across the different kingdoms of life, and of organelle membranes, too [8–13]. Membrane asymmetry is
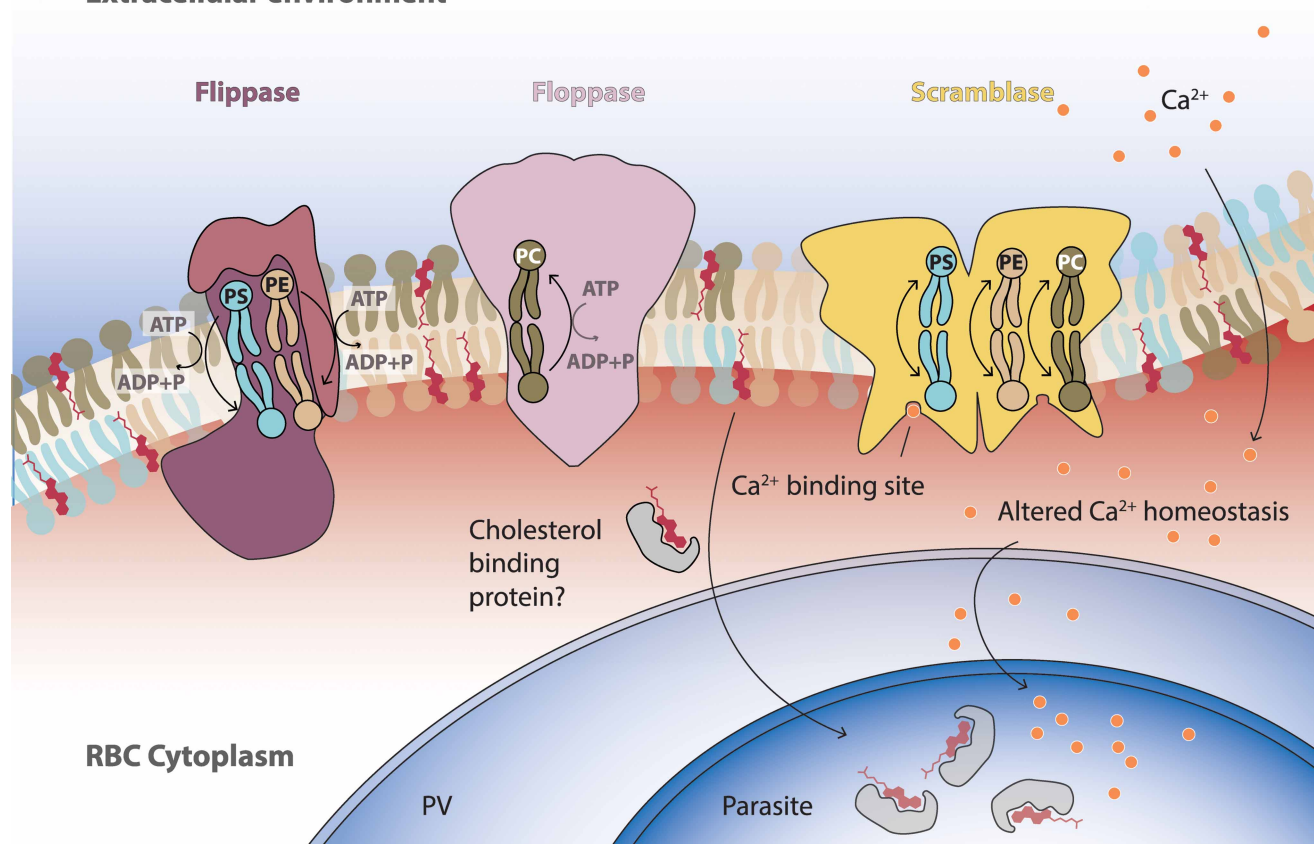
Figure 1. Dynamics of phospholipid asymmetry and cholesterol uptake upon parasite infection exemplified by a lipid bilayer of a *Plasmodium*-infected erythrocyte. Shown are major phospholipid species, the sterol cholesterol, Ca^{2+}, and candidate membrane-bound enzymes that catalyse phospholipid shuttling between the outer and inner lipid leaflet. Enrichment of phosphatidylserine (PS; blue) and phosphatidylethanolamine (PE; cream) in the interior lipid sheet is catalysed by a flippase (purple) under ATP hydrolysis. In contrast, the exterior layer facing the blood stream, endothelial cells and phagocytes is enriched in phosphatidylcholine (PC; brown) by an ATP-hydrolysing floppase (pink). Cholesterol (red) is sequestered by the growing parasite, whereas Ca^{2+} homeostasis is modulated in infected red blood cells. Activation of scramblase (yellow) by an increase in intracellular Ca^{2+} results in the random shuttling of all three phospholipids across the two layers. Enrichment of host-derived cholesterol might be mediated by a cholesterol-binding protein. ADP, adenosine diphosphate; ATP, adenosine triphosphate. Shape of proteins is based on structures of representative flippase (P4-ATPase Drs2p-CDC50p [1]), floppase (ABCG2 [2]), and scramblase molecules (TMEM16F [3]) deposited in the protein data bank (PDB [4]).

important for a whole range of cellular functions — from membrane biophysical properties, protein–membrane interactions, and cell-to-cell interactions, including as a principal signal to initiate apoptosis acting as an 'eat me' label to a neighbouring cell, which will initiate phagocytic uptake [14,15]. This review will concentrate on the function and importance of lipid asymmetry in the interactions of intracellular protozoan parasites with the target and immune cells of the host, with a particular emphasis on the malaria parasite *Plasmodium*, which infects RBCs.

Lipid asymmetry regulation in red blood cells

Phospholipid asymmetry is established, regulated, and ultimately collapsed by three classes of lipid-translocating enzymes, termed flippases, floppases, and scramblases (Figure 1) [5,16,17]. These terms are used as broad descriptors of multiple proteins that perform the respective functions. Flippases move anionic phospholipids, particularly PS and with lower affinity PE, from the outer layer to the inner layer in an

ATP-dependent manner [5,18]. This unidirectional movement of these negatively charged lipids creates and maintains the unequal distribution between the bilayers (Figure 1). The most studied flippases consist of members of the P4-type ATPase protein family and an accessory CDC50 subunit, which is located at the outer layer and likely regulates the binding of the lipid substrate by acting both as a chaperone and integral part of the lipid-translocating machinery [18]. P4-ATPases localise to the PM and intracellular membranes and display different substrate selectivity for the aminophospholipids PS, PE, or PC, but also a broader range of lipid substrates, such as lysophospholipids. Thus, identifying bona fide flippases among the range of P4-ATPase members necessitates biochemical assays and experimental genetics. In human RBCs ATP11C is the most abundant P4-type ATPase, followed by ATP11A and ATP11B [19–21]. Forward and reverse genetic approaches in humans and mice, respectively, have revealed the functional importance of ATP11C for maintaining membrane asymmetry in RBCs and other cell types, and its mutation can lead to altered cell development and lifespan [22–24]. Floppases similarly function in an ATP-dependent manner, but instead shuttle lipids such as PC to the outer layer [5] (Figure 1). Several ATP-binding cassette (ABC) proteins have been ascribed floppase function, such as ABCC1, ABCA7, and ABCG2, which are present in RBCs [19,21,25,26].

The third class of these enzymes, scramblases, are responsible for the disruption of lipid asymmetry [5] (Figure 1). They do not require ATP to function, but instead indiscriminately scramble lipids in both directions, allowing the membrane to reach a symmetric equilibrium [5]. Scramblase activity is generally repressed in cell membranes to ensure that asymmetry is maintained, and only activated under specific circumstances — by increased intracellular calcium, cholesterol depletion, or by caspase-mediated cleavage; these circumstances can occur in response to cell damage, signalling cascades, or during apoptosis [5,21,27–29]. PS exposure can act as an 'eat me' signal to phagocytes, such as monocytes and macrophages, which express PS receptors on their surfaces, in order to engulf and clear away these damaged or apoptotic cells [30,31]. Scramblase activation and subsequent PS exposure are also a crucial part of platelet activation [5,27]. Several proteins and their family members are suggested to act as scramblases, such as TMEM16F (involved in platelet activation), XKr8 (involved in caspase-mediated apoptotic PS exposure), and PLSCR1–4 [21,32–34]. The annotation of the latter has received scrutiny over whether it truly acts as a scramblase under physiological conditions, but there are still suggestions that it could play a role [21,28,35]. In RBCs, PLSCR1 and PLSCR4 are highly abundant, TMEM16F levels are low, and XKr8 appears to be absent [19,21].

The regulation of programmed cell death (or apoptosis) is an important mechanism for multicellular organisms to remove unwanted cells during development or damaged cells during infection or senescence, with phagocytic cells such as macrophages and monocytes participating in this process [31,36]. Since RBCs do not contain a nucleus, mitochondria, or protein synthesis machinery, they constitute elementary cells and have been a popular model system for membrane research. However, this also means that RBCs are not able to perform the same apoptotic processes as nucleated cells, such as the fragmentation of DNA and mitochondrial membranes [36,37]. Nonetheless, RBCs can enter an apoptotic-like state, termed 'eryptosis,' which shares other common markers of apoptosis — like altered ion balance, particularly of calcium and potassium, cell shrinkage, membrane blebbing, and a loss of membrane asymmetry, resulting in PS exposure [38,39]. Indeed, RBCs may be particularly dependent on PS exposure as a signal for cell death, since they lack the other apoptotic signals available to nucleated cells. These eryptotic markers generally begin to appear as the RBC ages, when factors such as ATP depletion hamper the cells' ability to actively maintain ion homeostasis, water balance, and deformability [40,41]. These changes signal that the aging RBC is ready to be removed from circulation, and it is generally filtered out and phagocytosed in the spleen or liver [42].

The case of intracellular parasites: refurbishing host cells

Several parasites have chosen RBCs as their home, since they provide abundant nutrients, shelter from the host's immune system, and transportation through the human body. These parasites are members of the phylum apicomplexa, which are all obligate intracellular pathogens and infect a wide variety of host cells [43]. This phylum encompasses parasites such as *Plasmodium*, the causative agent of malaria, *Babesia* and *Theileria*, tick-borne pathogens of veterinary importance [44–46]. As the parasite grows inside, it modifies the RBC to support its survival — scavenging lipids, proteins, and other molecules from the host cell and extracellular environment. Parasites introduce new channels and virulence factors in the RBC membrane to ensure adequate nutrient uptake and aid in the avoidance of the immune system [47,48]. Since the parasite seemingly places a heavy burden on the RBC — even before it completes its replication and ultimately destroys the cell to seek out

new host cells — many researchers have sought to investigate whether these consequences include changes to membrane asymmetry.

Asymmetry at the *Plasmodium*-infected RBC membrane: cell culture artefacts and clinical relevance

Many studies have attempted to determine whether lipid asymmetry in the RBC membrane is disrupted when parasitised with *Plasmodium*, often reaching contradictory results (for example, [49–54]). A recent meta-analysis of these studies looked at the contradictory body of evidence for whether or not PS is exposed on the surface of infected RBCs [22]. A main conclusion was that much of the controversy is likely attributable to the variety of methods to assay PS exposure — lipid hydrolysis, anti-PS antibodies, thrombin-activation, and eventually annexin V staining — and the shortcomings associated with each [22]. In addition, several of the experimental conditions might have artificially exacerbated PS exposure — glucose deprivation, high potassium concentrations, extreme parasite burdens — suggesting that observed disruptions to lipid asymmetry may not necessarily reflect physiological conditions [22]. More recent techniques, such as combined annexin V and DNA staining to distinguish infected and uninfected RBCs without artificial separation, have addressed many of these arguments [22].

On the balance of evidence, PS is likely exposed in a portion of infected RBCs under *in vitro* culture conditions, and this disruption to membrane asymmetry can be exacerbated by a number of factors such as heat stress (mimicking malarial fevers), hyperparasitaemia (leading to an increase in parasite burden), and treatment with chemical agents, which directly or indirectly lead to infected RBCs entering eryptosis [52,54–56].

Two mechanisms have been identified as the source for this disruption in membrane asymmetry (Figure 1): an increase in calcium levels in the otherwise low-calcium environment of the RBC cytoplasm, and cholesterol depletion from the RBC membrane — both of which can activate scramblase proteins and lead to PS exposure [28,29,57]. The infected RBC population has a higher level of scramblase activity compared to uninfected RBCs, although a concurrent increase in flippase activity has also been observed [54]. It has been hypothesised that cholesterol depletion likely plays a role in both phenomena, since this lipid affects membrane fluidity and can, therefore, affect the turnover rate of these proteins [28,29,58,59].

Asymmetry of internal membranes: more questions than answers

While mature human RBCs lack internal membranous structures, parasitic invasion creates a complex structure of membranes, where asymmetry could play a role in parasite survival (Figure 2). *Plasmodium* parasites invade RBCs using a variety of receptors and ligands, with signalling cascades in both parasite and host cell culminating in the invagination of the RBC membrane around the parasite [60,61]. The parasite remains in this resulting membrane-bound organelle, termed the parasitophorous vacuole (PV), adding newly synthesised lipids and proteins as it grows and multiplies, until the daughter cells (merozoites) are ready to egress both the PV and the host cell before invading new RBCs [47,62–64]. As a consequence, molecules that are exchanged between the host serum and the parasite have to transverse three membranes: RBC plasma membrane, PV membrane (PVM), and parasite plasma membrane (PPM).

One notable phenomenon is that the PVM membrane is created from the 'outside in' host cell membrane, and, accordingly, the proteins may also be orientated in the opposite direction [65,66]. This has been proposed as a mechanism for retaining high calcium concentrations in the vacuolar compartment, since the human calcium extrusion pump, PMCA, may now be orientated to pump calcium in this direction [65,66]. Similarly, erythrocyte flippases and floppases may be orientated and functioning in the new direction, keeping PS and PE on what is still technically the cytoplasmic side. However, not all host proteins are translocated into the PVM [67], and so the localisation, orientation, and function of lipid-translocating proteins in this membrane are yet to be confirmed.

In addition to the PVM, the parasite also establishes membranous structures within the host cell cytoplasm, such as the Maurer's clefts and tubovesicular network (TVN), which are involved in the trafficking of lipids and proteins between the host and parasite [68] (Figure 2). The parasite contains membrane-bound organelles originally derived from evolutionary endocytosis — the double-membraned mitochondria, and the quadruple-layered apicoplast [69,70]. This non-photosynthetic plastid likely resulted from the secondary endocytosis of algae, and compartmentalises processes, such as isoprenoid biosynthesis via methyl erythritol phosphate
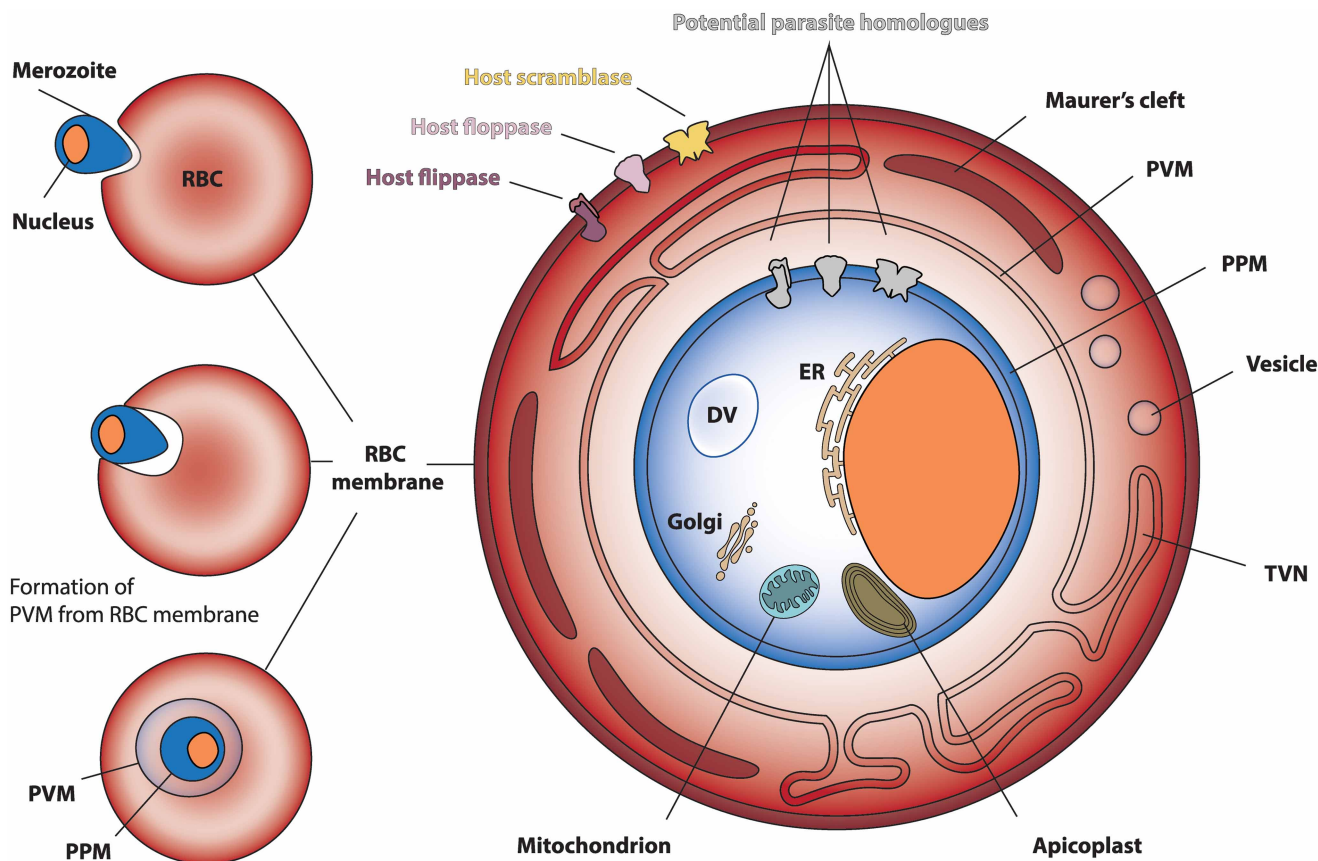
Figure 2. Establishment of multiple membrane structures in a *Plasmodium falciparum*-infected erythrocyte.
Shown are the invagination of the host cell plasma membrane during parasite invasion resulting in a parasite-derived organelle, the parasitophorous vacuole, and the intracellular growth stage of *P. falciparum* in an infected erythrocyte. Host-encoded flippases (purple), floppases (pink), and scramblase (yellow) are highlighted and potential parasite homologues are shown in grey. Note multiple parasite organelles, which include the classic eukaryotic single membrane organelles, ER and Golgi cistern, the double membrane organelles, nucleus (orange) and mitochondrion, a specialised lysosome (termed digestive vacuole), and a relict plastid organelle. The latter, termed the apicoplast, is non-photosynthetic, and contains four lipid membranes, which originate from the secondary endosymbiosis of a protist algae. In addition, the growing parasite induces membranous structures in the host cell, including vesicles, a tubovesicular network, and Maurer's clefts. DV, digestive vacuole; ER, endoplasmic reticulum; PPM, parasite plasma membrane; PVM, parasitophorous vacuole membrane; RBC, red blood cell/erythrocyte; TVN, tubovesicular network.

(MEP), fatty acid chain elongation, and parts of the haem biosynthetic pathway [71]. This multitude of membranes raises many potential questions about the existence, role, and maintenance of membrane asymmetry between these different compartments.

The overall lipid composition of the RBCs changes significantly upon infection with *Plasmodium*. As the parasite develops, it synthesises and scavenges lipids, increasing the amounts in total lipids, phospholipids, neutral lipids, sphingolipids, and cholesterol [71–74]. Changes to individual structures are much harder to measure, as protocols for separating the parasite from its host cell generally leave segments of the latter attached [75]. It is well established that the parasite PM normally contains a relatively low abundance of cholesterol, since it shows resistance to saponin lysis, while both the host PM and PVM can be lysed (though not fully removed) [75,76]. It has also been demonstrated that the PVM initially matches the cholesterol composition of the host cell membrane it was derived from, but this cholesterol is diluted as the PVM grows with the incorporation of parasite-synthesised phospholipids [64]. A lipidomic study of isolated apicoplasts also reveals an atypical lipid composition compared to the overall composition found in infected RBCs [77]. Apicoplasts are particularly rich in saturated phospholipids (in particular phosphatidylinositol) and contain also lipids that are rather unusual for plastids (like sphingomyelins, ceramides, and cholesterol). Collectively, these studies

highlight clear differences between host and parasite membrane composition, however, none have probed the asymmetric state of any of these membranes. While the need to sequester certain lipids on either side of these internally hidden membranes may not be as important for intracellular parasites in the absence of phagocyte recognition, membrane asymmetry may have other functional roles at these sites, such as protein binding. Whether there are functional roles of asymmetry or differences between different compartments and organelles remains to be investigated.

In the absence of experimental evidence, we can only speculate about the asymmetrical nature of these membranes based on the existence of putative lipid-translocating proteins. The parasite genome encodes several proteins that show homology to human flippases, floppases, and scramblases. In the case of flippases, this includes P4-ATPases PF3D7_1219600 (*Pf*ATP2) and PF3D7_1223400 (*Pf*ATP8). These are likely essential to the parasite's survival, and orthologs in another *Plasmodium* species localise to the PPM [78–80]. *Plasmodiun falciparum* encodes three CDC50 orthologues, PF3D7_0719500 (*Pf*CDC50A), PF3D7_1133300 (*Pf*CDC50B), and PF3D7_1029400 (*Pf*CDC50C), the latter being the chaperone partner of *Pf*ATP2 [81,82]. Other P4-ATPases, such as PF3D7_031900 (*Pf*ATP7), may localise to internal membranes [80]. The parasite encodes 11 ABC family members containing transmembrane domains, of which PF3D7_0112200 (*Pf*MRP1/ABCC1), PF3D7_1229100 (*Pf*MRP2/ABCC2), PF3D7_1447900 (*Pf*MDR2/ABCB2), PF3D7_1339900 (*Pf*MDR5/ABCB5) have been localised to the PPM [81,83,84]. These proteins might operate as floppases, though no information is currently available to assign functions to any of these candidate transport proteins. A parasite candidate scramblase protein, PF3D7_1022700 (*Pf*PLSCR), was identified based on homology to the human PLSCR proteins, and displayed scramblase-like activity when expressed in proteoliposomes [85]. It appears to localise to membranes within the parasite during the majority of intraerythrocytic asexual development, with the potential exception of merozoites.

The existence of these proteins could indicate that parasite membranes are asymmetrically arranged, though further evidence is clearly required to determine the localisation and function of these proteins, Of note, none of the parasite-encoded, candidate lipid-translocating proteins has yet been localised to the PVM. None of the proteins contains common export motifs indicative of export beyond the PPM, but this does not preclude the possibility that they could be exported in a different manner [86]. There is a need to ultimately determine the distribution of lipids within different parasite compartments through studies, which probe the asymmetrical nature of internal membrane structures, preferably without destructive separation techniques. Towards this goal, organelle enrichment by affinity purification of epitope-tagged signature membrane proteins, as established for the apicoplast [77], is expected to reveal the organellar lipid composition and permit conventional cell biological assays, e.g. annexin V stain. Symmetric membranes are less common than asymmetric membranes, likely because they are less selective for transiting molecules. The superior selectivity together with the 'scaffolding effect', where different lipid compositions on both bilayers can provide greater mechanical stability, hold the membrane in its proper shape, and prevent it from deformation, makes it likely that the dynamic intracellular environments are reflected by asymmetric internal membranes.

Consequences of altered asymmetry: phagocytosis of infected erythrocytes

Disruption to membrane asymmetry has several conceivable biological consequences, some of which have been explored experimentally. The essential nature of parasite proteins, such as *Pf*ATP2, suggests an important functional role during asexual growth. Due to lack of information about asymmetry in internal membranes, we will focus on the host cell membrane, where there is potential for interaction with immune cells, such as monocytes and macrophages, which recognise exposed PS as a signal for phagocytosis (Figure 3). There is *in vitro* evidence for a role of PS exposure in monocyte phagocytosis of infected RBCs, and these interactions can be blocked with either the PS-binding protein annexin V on iRBCs, or with PS liposomes on the phagocytes [54,87]. Similarly, blocking a phagocyte PS-receptor protein, CD36, decreases monocyte and macrophage phagocytosis of infected RBCs. However, since CD36 is a promiscuous 'scavenger' receptor, and also recognises other ligands on parasitised RBCs, this provides less direct evidence than inhibition of PS exposure on infected RBCs. In patient samples, monocytes with phagocytosed parasites are frequently observed (Figure 3). More broadly, monocytes and macrophages influence the course of malaria infection through their pro-inflammatory response after classical (M1) activation, which includes the release of IL-1, IL-12, IL-23, and chemokines [88,89]. Aside from phagocytosis, *in vitro* evidence has also suggested a role for PS exposure in parasite cytoadherence to
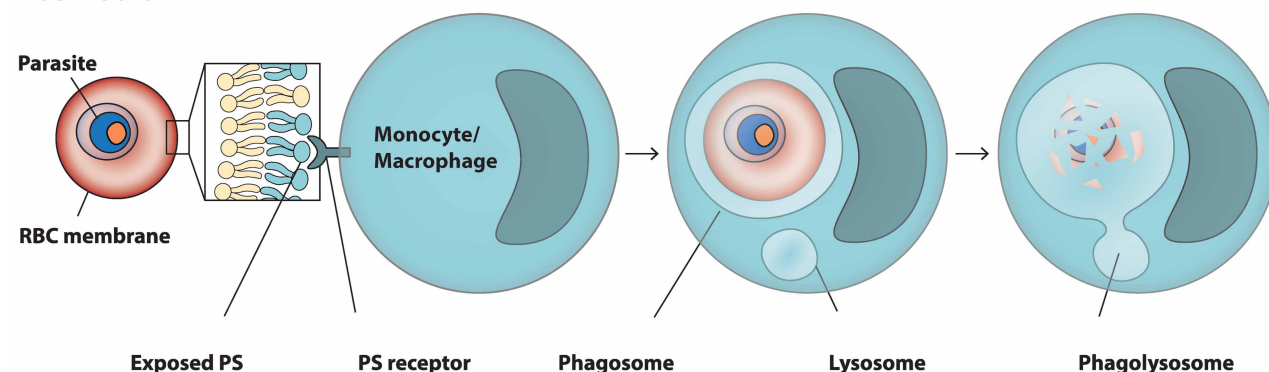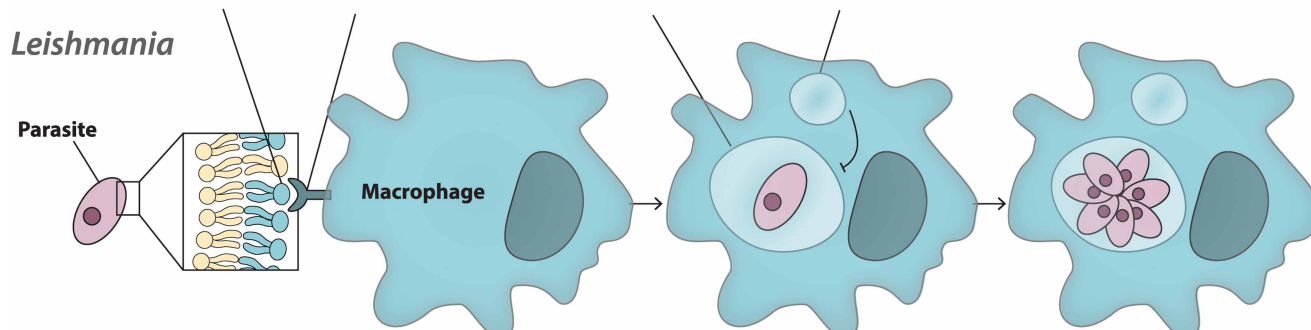
Figure 3. Alternative interference with phagocytosis by protozoan parasites.

Plasmodium-infected cells (top) are recognised through the exposure of phosphatidylserine (PS) on the erythrocyte plasma membrane (and additional parasite-encoded proteins; not shown). Binding to the PS-receptor on macrophages results in phagocytosis and lysosomal fusion with the endocytic compartment leading to the formation of a phagosome. Killing inside a phagosome occurs through classical activation (M1) and is accompanied by a pro-inflammatory response. *Leishmania* parasites (bottom) enhance PS exposure on their surface to increase phagocytic uptake by macrophages, which constitute their only host cell for growth and replication. *Leishmania* parasites have evolved mechanisms to inhibit lysosomal fusion and/or acidification of the phagosome, resulting in a replication-competent niche inside infected macrophages.

ligands present on the vascular endothelium — an important part of the parasites' defence against the host immune system, which sequesters them away from circulation and prevents splenic clearance [52,54,90]. While the importance of the avoidance strategy is well-accepted, a causal relationship between PS exposure and cytoadherence *in vivo* has not been established.

As outlined above, there are many gaps in our understanding of membrane asymmetry and its role in malaria infection. It is also clear that there is no simple answer for who benefits most from this process — a typical phenomenon of these close relationships between parasite and host, which have evolved together in a constant arms race [91]. Being able to recognise and phagocytose an infected cell may seem to be a simple benefit to the host, but if this comes at the cost of a hyperinflammatory response, then it is no longer advantageous [92,93]. Similarly, it may seem like immune evasion is the ultimate goal of a parasite, but perhaps the clearance of a subsection of parasites could be an advantage, since it lowers the chance of the host dying before the infection can be transmitted — an especially important consideration for *P. falciparum*, which requires 10–14 days to fully develop transmissible parasites [94]. It may, therefore, be beneficial for the parasites to reduce parasite burden, and recognition by exposed PS may trigger a less severe immune response than opsonisation by antibodies or complement [40,95]. Collapsing membrane asymmetry may also have other complex consequences in processes such as invasion, egress, and nutrient uptake. The threat of immune discovery could mean that the parasite is limited in which modifications it can induce in the RBC before collapsing asymmetry and hence impose some restrictions on the parasite. With this complicated relationship in mind, we will turn our attention to another intracellular parasite which appears to take advantage of PS exposure.

Apoptotic mimicry and the *Leishmania* parasite

Like the apicomplexans, *Leishmania* is also a protozoan parasite, belonging to the class Kinetoplastida. This parasite is the causative agent of leishmaniasis, which affects the epithelia of skin, mucosa and inner organs. During chronic infections, *Leishmania* parasites reside in macrophages [96–99]. Rather than avoiding PS exposure and the detection by the immune system, these parasites exploit this mechanism to end up in their host cells (Figure 3). To aid in its uptake, the parasite uses 'apoptotic mimicry' — where it exposes PS in the outer layer of its PM and hence increasing phagocyte recognition [98–102]. After subsequent engulfment, the parasites employ a number of strategies to prevent their degradation, allowing them to live and multiply in the macrophage without being targeted by the immune system [98,103]. The molecular basis for enhanced PS exposure implicated in *Leishmania*-macrophage interaction is under investigation, but one candidate protein, LABCG2, a *Leishmania* member of ABC half-transporters (ABCG subfamily), has been implicated in PS exposure and macrophage infectivity of *Leishmania major* metacyclic promastigotes, the parasite stage transmitted by infected sand-flies (Phlebotominae) [104]. We note that some studies have failed to detect PS in the PM of *Leishmania donovani* promastigotes by complementary lipid analysis techniques [105,106]. Parasites were still stained with annexin V [106], illustrating that annexin V, and perhaps also phagocytic PS receptors, may not be completely specific to PS, despite consistent detection of PS on early apoptotic cells by fluorescently labelled annexin V [107].

Drug-induced increase in intracellular Ca^{2+} levels of *Leishmania* promastigotes, the replicative stage in the sandfly vector, induced swift, bi-directional and non-specific trans-bilayer movement of phospholipids indicative of scramblase activity [108,109], but a candidate scramblase has not yet been identified in *Leishmania* genomes [110]. Accordingly, functional analysis of *Leishmania* lipid-translocating proteins and more cell biological assays are needed to clarify the molecular basis of this phenomenon. Apoptotic mimicry has also been described for other parasites which can infect macrophages, such as *Toxoplasma gondii*, a food-borne apicomplexan parasite that causes toxoplasmosis in immune-suppressed individuals and foetuses, and *Trypanosoma cruzi*, the causative agent of Chagas disease and transmitted by kissing bugs, where it seems to promote an anti-inflammatory response [111,112]. More research is warranted to resolve whether apoptotic mimicry of parasites is an active regulatory mechanism that contributes to host colonisation *in vivo* or whether it can be at least partially attributed to experimental conditions during cell culture.

Asymmetry and *Toxoplasma gondii*: flippase contributes to microneme secretion

Membrane asymmetry might also be important for infections by *T. gondii*, a universal parasite capable of infecting nucleated cells of a wide range of homeothermic hosts [113]. While data on potential interference with the dynamics of lipid asymmetry of *Toxoplasma*-infected cells are lacking, the import of PS and PE, but not PC, into extracellular parasites (so-called tachyzoites) has been reported [114]. *T. gondii* encodes six candidate P4-type ATPases and four CDC50 co-chaperomes, of which one pair, ATP2B and CDC50.4, mediates flipping of PS, which in turn acts as a lipid mediator for the last step in microneme exocytosis, i.e. fusion of micronemes with the PM [115]. Flippase activity was measured at the single-cell level employing a lactadherin C2 domain — GFP fusion protein as genetically encoded molecular probe [15]. These findings will inform corresponding work in malaria research, and, indeed, a recent experimental genetics analysis of the three *Plasmodium* CDC50 members uncovered roles in the maturation of blood stages and efficient parasite egress for CDC50C and CDC50B, respectively [82]. Candidate floppase and scramblase genes have not yet been assigned in the *T. gondii* genome [116], perhaps indicating that intracellular replication in nucleated cells might require less regulation of lipid asymmetry of host cell PMs than inside erythrocytes, which are refractory to *T. gondii* infection.

Conclusions

While many aspects of membrane asymmetry have been elucidated in eukaryotic cells, there are still many questions about what happens when one eukaryote parasitises another. The added complexity of having two organisms with fundamentally different objectives, along with the deeply nested structure of so many membranes within membranes, presents additional challenges for those attempting to tease apart the organisation of the bilayers. However, this system — although complicated — allows for the exploration of the functional role of membrane asymmetry and the process of its regulation. We have highlighted some intriguing gaps in our

collective understanding of membrane organisation in the context of host–parasite interactions, focusing mainly on the malaria parasite *Plasmodium*. Without a doubt, further investigations into this fascinating topic will reveal more ways how membrane asymmetry is used in the tug-of-war between the parasite and the host.

Summary

- Intracellular parasites establish complex membrane structures inside host cells.

- Protozoan parasites encode candidate lipid-translocating enzymes, but data on the dynamics of lipid asymmetry on the plasma and internal membranes are scarce.

- Erythrocyte infections by malarial parasites lead to changes in lipid asymmetry on the RBC membrane and phagocytosis.

- *Leishmania* parasites use disrupted phospholipid asymmetry on their surface to enhance infection of macrophages by phagocytic uptake.

- A *Toxoplasma gondii* flippase mediates the final step in membrane fusion during exocytosis.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Open Access

Open access for this article was enabled by the participation of Australian National University in an all-inclusive *Read & Publish* agreement with Portland Press and the Biochemical Society under a transformative agreement with CAUL.

Author Contributions

All authors wrote and edited the manuscript.

Abbreviations

ABC, ATP-binding cassette; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PM, plasma membranes; PPM, parasite plasma membrane; PS, phosphatidylserine; PV, parasitophorous vacuole; PVM, PV membrane; RBCs, red blood cells.

References

1 Timcenko, M., Lyons, J.A., Januliene, D., Ulstrup, J.J., Dieudonne, T., Montigny, C. et al. (2019) Structure and autoregulation of a P4-ATPase lipid flippase. *Nature* **571**, 366–370 https://doi.org/10.1038/s41586-019-1344-7112

2 Orlando, B.J. and Liao, M. (2020) ABCG2 transports anticancer drugs via a closed-to-open switch. *Nat. Commun.* **11**, 2264 https://doi.org/10.1038/s41467-020-16155-2

3   Feng, S., Dang, S., Han, T.W., Ye, W., Jin, P., Cheng, T. et al. (2019) Cryo-EM studies of TMEM16F calcium-activated ion channel suggest features important for lipid scrambling. *Cell Rep.* **28**, 567–579 https://doi.org/10.1016/j.celrep.2019.06.023

4   Berman, H., Henrick, K. and Nakamura, H. (2003) Announcing the worldwide Protein Data Bank. *Nat. Struct. Mol. Biol.* **10**, 980 https://doi.org/10.1038/nsb1203-980

5   Hankins, H.M., Baldridge, R.D., Xu, P. and Graham, T.R. (2015) Role of flippases, scramblases and transfer proteins in phosphatidylserine subcellular distribution. *Traffic* **16**, 35–47 https://doi.org/10.1111/tra.12233

6   Bretscher, M.S. (1972) Asymmetrical lipid bilayer structure for biological membranes. *Nat. New Biol.* **236**, 11–12 https://doi.org/10.1038/newbio236011a0

7   Gordesky, S.E. and Marinetti, G.V. (1973) The asymmetric arrangement of phospholipids in the human erythrocyte membrane. *Biochem. Biophys. Res. Commun.* **50**, 1027–1031 https://doi.org/10.1016/0006-291X(73)91509-X

8   Voeltz, G.K. and Prinz, W.A. (2007) Sheets, ribbons and tubules — how organelles get their shape. *Nat. Rev. Mol. Cell. Biol.* **8**, 258–264 https://doi.org/10.1038/nrm2119

9   Ikeda, M., Kihara, A. and Igarashi, Y. (2006) Lipid asymmetry of the eukaryotic plasma membrane: functions and related enzymes. *Biol. Pharm. Bull.* **29**, 1542–1546 https://doi.org/10.1248/bpb.29.1542

10 Van Meer, G., Voelker, D.R. and Feigenson, G.W. (2008) Membrane lipids: where they are and how they behave. *Nat. Rev. Mol. Cell. Biol.* **9**, 112–124 https://doi.org/10.1038/nrm2330

11 Tjellström, H., Hellgren, L.I., Wieslander, Å. and Sandelius, A.S. (2010) Lipid asymmetry in plant plasma membranes: phosphate deficiency-induced phospholipid replacement is restricted to the cytosolic leaflet. *FASEB J.* **24**, 1128–1138 https://doi.org/10.1096/fj.09-139410

12 Rizzo, J., Stanchev, L.D., da Silva, V.K.A., Nimrichter, L., Pomorski, T.G. and Rodrigues, M.L. (2019) Role of lipid transporters in fungal physiology and pathogenicity. *Comput. Struct. Biotechnol. J.* **17**, 1278–1289 https://doi.org/10.1016/j.csbj.2019.09.001

13 Low, W.Y. and Chng, S.S. (2021) Current mechanistic understanding of intermembrane lipid trafficking important for maintenance of bacterial outer membrane lipid asymmetry. *Curr. Opin. Chem. Biol.* **65**, 163–171 https://doi.org/10.1016/j.cbpa.2021.09.004

14 Leventis, P.A. and Grinstein, S. (2010) The distribution and function of phosphatidylserine in cellular membranes. *Annu. Rev. Biophys.* **39**, 407–427 https://doi.org/10.1146/annurev.biophys.093008.131234

15 Yeung, T., Gilbert, G.E., Shi, J., Silvius, J., Kapus, A. and Grinstein, S. (2008) Membrane phosphatidylserine regulates surface charge and protein localization. *Science* **319**, 210–213 https://doi.org/10.1126/science.1152066

16 Pomorski, T. and Menon, A.K. (2006) Lipid flippases and their biological functions. *Cell. Mol. Life Sci.* **63**, 2908–2921 https://doi.org/10.1007/s00018-006-6167-7

17 Daleke, D.L. (2007) Phospholipid flippases. *J. Biol. Chem.* **282**, 821–825 https://doi.org/10.1074/jbc.R600035200

18 López-Marqués, R.L., Gourdon, P., Pomorski, T.G. and Palmgren, M. (2020) The transport mechanism of P4 ATPase lipid flippases. *Biochem. J.* **477**, 3769–3790 https://doi.org/10.1042/BCJ20200249

19 Andersen, J.P., Vestergard, A.L., Mikkelsen, S.A., Mogensen, L.S., Chalat, M. and Molday, R.S. (2017) P4-ATPase as phospholipid flippases - structure, function, and enigmas. *Front. Physiol.* **7**, 275 https://doi.org/10.3389/fphys.2016.00275

20 Bryk, A.H. and Wiśniewski, J.R. (2017) Quantitative analysis of human red blood cell proteome. *J. Proteome Res.* **16**, 2752–2761 https://doi.org/10.1021/acs.jproteome.7b00025

21 Liou, A.Y., Molday, L.L., Wang, J., Andersen, J.P. and Molday, R.S. (2019) Identification and functional analyses of disease-associated P4-ATPase phospholipid flippase variants in red blood cells. *J. Biol. Chem.* **294**, 6809–6821 https://doi.org/10.1074/jbc.RA118.007270

22 Fraser, M., Matuschewski, K. and Maier, A.G. (2021) Of membranes and malaria: phospholipid asymmetry in *Plasmodium falciparum*-infected red blood cells. *Cell. Mol. Life Sci.* **78**, 4545–4561 https://doi.org/10.1007/s00018-021-03799-6

23 Yabas, M., Coupland, L.A., Cromer, D., Winterberg, M., Teoh, N.C., D'Rozario, J. et al. (2014) Mice deficient in the putative phospholipid flippase ATP11C exhibit altered erythrocyte shape, anemia, and reduced erythrocyte life span. *J. Biol. Chem.* **289**, 19531–19537 https://doi.org/10.1074/jbc.C114.570267

24 Arashiki, N., Takakuwa, Y., Mohandas, N., Hale, J., Yoshida, K., Ogura, H. et al. (2016) ATP11C is a major flippase in human erythrocytes and its defect causes congenital hemolytic anemia. *Haematologica* **101**, 559–565 https://doi.org/10.3324/haematol.2016.142273

25 Yabas, M., Jing, W., Shafik, S., Bröer, S. and Enders, A. (2016) ATP11C facilitates phospholipid translocation across the plasma membrane of all leukocytes. *PLoS One* **11**, e0146774 https://doi.org/10.1371/journal.pone.0146774

26 Quazi, F. and Molday, R.S. (2011) Lipid transport by mammalian ABC proteins. *Essays Biochem.* **50**, 265–290 https://doi.org/10.1042/bse0500265

27 Neumann, J., Rose-Sperling, D. and Hellmich, U.A. (2017) Diverse relations between ABC transporters and lipids: an overview. *Biochim. Biophys. Acta - Biomembr.* **1859**, 605–618 https://doi.org/10.1016/j.bbamem.2016.09.023

28 van Kruchten, R., Mattheij, N.J.A., Saunders, C., Feijge, M.A.H., Swieringa, F., Wolfs, J.L.N. et al. (2013) Both TMEM16F-dependent and TMEM16F-independent pathways contribute to phosphatidylserine exposure in platelet apoptosis and platelet activation. *Blood* **121**, 1850–1857 https://doi.org/10.1182/blood-2012-09-454314

29 Arashiki, N., Saito, M., Koshino, I., Kamata, K., Hale, J., Mohandas, N. et al. (2016) An unrecognized function of cholesterol: regulating the mechanism controlling membrane phospholipid asymmetry. *Biochemistry* **55**, 3504–3513 https://doi.org/10.1021/acs.biochem.6b00407

30 van Zwieten, R., Bochem, A.E., Hilarius, P.M., Van Bruggen, R., Kees Hovingh, G. and Verhoeven, A.J. (2012) The cholesterol content of the erythrocyte membrane is an important determinant of phosphatidylserine exposure. *Biochim. Biophys. Acta* **1821**, 1493–1500 https://doi.org/10.1016/j.bbalip.2012.08.008

31 Connor, J., Pak, C. and Schroit, A. (1994) Exposure of phosphatidylserine in the outer leaflet of human red blood cells. Relationship to cell density, cell age, and clearance by mononuclear cells. *J. Biol. Chem.* **269**, 2399–2404 https://doi.org/10.1016/S0021-9258(17)41959-4

32 Naeini, M.B., Bianconi, V., Pirro, M. and Sahebkar, A. (2020) The role of phosphatidylserine recognition receptors in multiple biological functions. *Cell. Mol. Biol. Lett.* **25**, 23 https://doi.org/10.1186/s11658-020-00214-z

33 Suzuki, J., Imanishi, E. and Nagata, S. (2014) Exposure of phosphatidylserine by Xk-related protein family members during apoptosis. *J. Biol. Chem.* **289**, 30257–30267 https://doi.org/10.1074/jbc.M114.583419

34 Suzuki, J., Fujii, T., Imao, T., Ishihara, K., Kuba, H. and Nagata, S. (2013) Calcium-dependent phospholipid scramblase activity of TMEM16 protein family members. *J. Biol. Chem.* **288**, 13305–13316 https://doi.org/10.1074/jbc.M113.457937

35   Wiedmer, T., Zhou, Q., Kwoh, D.Y. and Sims, P.J. (2000) Identification of three new members of the phospholipid scramblase gene family. *Biochim. Biophys. Acta - Biomembr.* **1467**, 244–253 https://doi.org/10.1016/S0005-2736(00)00236-4

36   Bevers, E.M. and Williamson, P.L. (2010) Phospholipid scramblase: an update. *FEBS Lett.* **584**, 2724–2730 https://doi.org/10.1016/j.febslet.2010.03.020

37   Elmore, S. (2007) Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* **35**, 495 https://doi.org/10.1080/01926230701320337

38   Hengartner, M.O. (2000) The biochemistry of apoptosis. *Nature* **407**, 770–776 https://doi.org/10.1038/35037710

39   Pretorius, E., du Plooy, J.N. and Bester, J. (2000) A comprehensive review on eryptosis. *Cell. Physiol. Biochem.* **39**, 1977–2000 https://doi.org/10.1159/000447895

40   Lang, E., Qadri, S.M. and Lang, F. (2012) Killing me softly - suicidal erythrocyte death. *Int. J. Biochem. Cell Biol.* **44**, 1236–1243 https://doi.org/10.1016/j.biocel.2012.04.019

41   Wesseling, M.C., Wagner-Britz, L., Huppert, H., Hanf, B., Hertz, L., Nguyen, D.B. et al. (2016) Phosphatidylserine exposure in human red blood cells depending on cell age. *Cell. Physiol. Biochem.* **38**, 1376–1390 https://doi.org/10.1159/000443081

42   Franco, R.S., Puchulu-Campanella, M.E., Barber, L.A., Palascak, M.B., Joiner, C.H., Low, P.S. et al. (2013) Changes in the properties of normal human red blood cells during in vivo aging. *Am. J. Hematol.* **88**, 44–51 https://doi.org/10.1002/ajh.23344

43   de Back, D.Z., Kostova, E.B., van Kraaij, M., van den Berg, T.K. and van Bruggen, R. (2014) Of macrophages and red blood cells; a complex love story. *Front. Physiol.* **5**, 9 https://doi.org/10.3389/fphys.2014.00009

44   Morrison, D.A. (2009) Evolution of the Apicomplexa: where are we now? *Trends Parasitol.* **25**, 375–382 https://doi.org/10.1016/j.pt.2009.05.010

45   Maier, A.G., Matuschewski, K., Zhang, M. and Rug, M. (2019) *Plasmodium falciparum. Trends Parasitol.* **35**, 481–482 https://doi.org/10.1016/j.pt.2018.11.010

46   Liu, M., Igarashi, I. and Xuan, X. (2022) *Babesia gibsoni. Trends Parasitol.* **38**, 815–816 https://doi.org/10.1016/j.pt.2022.03.001

47   Liu, J., Guan, G. and Yin, H. (2022) *Theileria annulata. Trends Parasitol.* **38**, 265–256 https://doi.org/10.1016/j.pt.2021.11.001

48   Maier, A.G., Cooke, B.M., Cowman, A.F. and Tilley, L. (2009) Malaria parasite proteins that remodel the host erythrocyte. *Nat. Rev. Microbiol.* **7**, 341–354 https://doi.org/10.1038/nrmicro2110

49   de Koning-Ward, T.F., Dixon, M.W.A., Tilley, L. and Gilson, P.R. (2016) *Plasmodium* species: master renovators of their host cells. *Nat. Rev. Microbiol.* **14**, 494–507 https://doi.org/10.1038/nrmicro.2016.79

50   Moll, G.N., Vial, H.J., Bevers, E.M., Ancelin, M.L., Roelofsen, B., Comfurius, P. et al. (1990) Phospholipid asymmetry in the plasma membrane of malaria infected erythrocytes. *Biochem. Cell Biol.* **68**, 579–585 https://doi.org/10.1139/o90-083

51   Sherman, I., Prudhomme, J. and Fair, J. (1997) Altered membrane phospholipid asymmetry in *Plasmodium falciparum*-infected erythrocytes. *Parasitol. Today* **13**, 242–243 https://doi.org/10.1016/S0169-4758(97)85284-2

52   Eda, S. and Sherman, I.W. (2002) Cytoadherence of malaria-infected red blood cells involves exposure of phosphatidylserine. *Cell. Physiol. Biochem.* **12**, 373–384 https://doi.org/10.1159/000067908

53   Engelbrecht, D. and Coetzer, T.L. (2016) *Plasmodium falciparum* exhibits markers of regulated cell death at high population density *in vitro. Parasitol. Int.* **65**, 715–727 https://doi.org/10.1016/j.parint.2016.07.007

54   Zhang, R., Chandramohanadas, R., Lim, C.T. and Dao, M. (2018) Febrile temperature elevates the expression of phosphatidylserine on *Plasmodium falciparum* (FCR3CSA) infected red blood cell surface leading to increased cytoadhesion. *Sci. Rep.* **8**, 15022 https://doi.org/10.1038/s41598-018-33358-2

55   Fraser, M., Jing, W., Bröer, S., Kurth, F., Sander, L.E., Matuschewski, K. et al. (2021) Breakdown in membrane asymmetry regulation leads to monocyte recognition of *P. falciparum*-infected red blood cells. *PLoS Pathog.* **17**, e1009259 https://doi.org/10.1371/journal.ppat.1009259

56   Boulet, C., Doerig, C.D. and Carvalho, T.G. (2018) Manipulating eryptosis of human red blood cells: a novel antimalarial strategy? *Front. Cell. Infect. Microbiol.* **8**, 419 https://doi.org/10.3389/fcimb.2018.00419

57   Pattanapanyasat, K., Sratongno, P., Chimma, P., Chitjamnongchai, S., Polsrila, K. and Chotivanich, K. (2010) Febrile temperature but not proinflammatory cytokines promotes phosphatidylserine expression on *Plasmodium falciparum* malaria-infected red blood cells during parasite maturation. *Cytometry A* **77A**, 515–523 https://doi.org/10.1002/cyto.a.20879

58   Bogdanova, A., Makhro, A., Wang, J., Lipp, P. and Kaestner, L. (2013) Calcium in red blood cells - a perilous balance. *Int. J. Mol. Sci.* **14**, 9848–9872 https://doi.org/10.3390/ijms14059848

59   Lee, A.G. (2004) How lipids affect the activities of integral membrane proteins. *Biochim. Biophys. Acta - Biomembr.* **1666**, 62–87 https://doi.org/10.1016/j.bbamem.2004.05.012

60   Morrot, G., Hervé, P., Zachowski, A., Fellmann, P. and Devaux, P.F. (1989) Aminophospholipid translocase of human erythrocytes: phospholipid substrate specificity and effect of cholesterol. *Biochemistry* **28**, 3456–3462 https://doi.org/10.1021/bi00434a046

61   Gao, X., Gunalan, K., Yap, S.S.L. and Preiser, P.R. (2013) Triggers of key calcium signals during erythrocyte invasion by *Plasmodium falciparum. Nat. Commun.* **4**, 2862 https://doi.org/10.1038/ncomms3862

62   Molina-Franky, J., Patarroyo, M.E., Kalkum, M. and Patarroyo, M.A. (2022) The cellular and molecular interaction between erythrocytes and *Plasmodium falciparum* merozoites. *Front. Cell. Infect. Microbiol.* **12**, 272 https://doi.org/10.3389/fcimb.2022.816574

63   Matz, J.M., Beck, J.R. and Blackman, M.J. (2020) The parasitophorous vacuole of the blood-stage malaria parasite. *Nat. Rev. Microbiol.* **18**, 379–391 https://doi.org/10.1038/s41579-019-0321-3

64   Kilian, N., Choi, J.-Y., Voelker, D.R. and Ben Mamoun, C. (2018) Role of phospholipid synthesis in the development and differentiation of malaria parasites in the blood. *J. Biol. Chem.* **293**, 17308–17316 https://doi.org/10.1074/jbc.R118.003213

65   Tokumasu, F., Crivat, G., Ackerman, H., Hwang, J. and Wellems, T.E. (2014) Inward cholesterol gradient of the membrane system in *P. falciparum*-infected erythrocytes involves a dilution effect from parasite-produced lipids. *Biol. Open* **3**, 529–541 https://doi.org/10.1242/bio.20147732

66   Gazarini, M.L., Thomas, A.P., Pozzan, T. and Garcia, C.R.S. (2003) Calcium signalling in a low calcium environment. *J. Cell Biol.* **161**, 103–110 https://doi.org/10.1083/jcb.200212130

67   Camacho, P. (2003) Malaria parasites solve the problem of a low calcium environment. *J. Cell Biol.* **161**, 17–19 https://doi.org/10.1083/jcb.200303116

68 Samuel, B.U., Mohandas, N., Harrison, T., McManus, H., Rosse, W., Reid, M. et al. (2001) The role of cholesterol and glycosylphosphatidylinositol-anchored proteins of erythrocyte rafts in regulating raft protein content and malarial infection. *J. Biol. Chem.* **276**, 29319–29329 https://doi.org/10.1074/jbc.M101268200

69 Beck, J.R. and Ho, C.M. (2021) Transport mechanisms at the malaria parasite-host cell interface. *PLoS Pathog.* **17**, e1009394 https://doi.org/10.1371/journal.ppat.1009394

70 Hayward, J.A. and van Dooren, G.G. (2019) Same same, but different: uncovering unique features of the mitochondrial respiratory chain of apicomplexans. *Mol. Biochem. Parasitol.* **232**, 111204 https://doi.org/10.1016/j.molbiopara.2019.111204

71 Buchanan, H.D., Goodman, C.D. and McFadden, G.I. (2022) Roles of the apicoplast across the life cycles of rodent and human malaria parasites. *J. Eukaryot. Microbiol.* **69**, e12947 https://doi.org/10.1111/jeu.12947

72 Tran, P.N., Brown, S.H.J., Rug, M., Ridgway, M.C., Mitchell, T.W. and Maier, A.G. (2016) Changes in lipid composition during sexual development of the malaria parasite *Plasmodium falciparum*. *Malar. J.* **15**, 73 https://doi.org/10.1186/s12936-016-1130-z

73 Ridgway, M.C., Cihalova, D., Brown, S.H.J., Tran, P., Mitchell, T.W. and Maier, A.G. (2022) Analysis of sex-specific lipid metabolism of *Plasmodium falciparum* points to the importance of sphingomyelin for gametocytogenesis. *J. Cell Sci.* **135**, jcs259592 https://doi.org/10.1242/jcs.259592

74 Gulati, S., Ekland, E.H., Ruggles, K.V., Chan, R.B., Jayabalasingham, B., Zhou, B. et al. (2015) Profiling the essential nature of lipid metabolism in asexual blood and gametocyte stages of *Plasmodium falciparum*. *Cell Host Microbe* **8**, 371–381 https://doi.org/10.1016/j.chom.2015.08.003

75 Wein, S., Ghezal, S., Buré, C., Maynadier, M., Périgaud, C., Vial, H.J. et al. (2018) Contribution of the precursors and interplay of the pathways in the phospholipid metabolism of the malaria parasite. *J. Lipid Res.* **59**, 1461–1471 https://doi.org/10.1194/jlr.M085589

76 Beaumelle, B.D., Vial, H.J. and Philippot, J.R. (1987) Reevaluation, using marker enzymes, of the ability of saponin and ammonium chloride to free *Plasmodium* from infected erythrocytes. *J. Parasitol.* **73**, 743–748 https://doi.org/10.2307/3282405

77 Das, S., Bhatanagar, S., Morrisey, J.M., Daly, T.M., Burns, J.M. et al. (2016) Na$^+$ influx induced by new antimalarials causes rapid alterations in the cholesterol content and morphology of *Plasmodium falciparum*. *PLoS Pathog.* **12**, e1005647 https://doi.org/10.1371/journal.ppat.1005647

78 Botté, C.Y., Yamaryo-Botté, Y., Rupasinghe, T.W.T., Mullin, K.A., MacRae, J.I., Spurck, T.P. et al. (2013) Atypical lipid composition in the purified relict plastid (apicoplast) of malaria parasites. *Proc. Natl Acad. Sci. U.S.A.* **110**, 7506–7511 https://doi.org/10.1073/pnas.130125111

79 Lamy, A., Macarini-Bruzaferro, E., Dieudonné, T., Perálvarez-Marín, A., Lenoir, G., Montigny, C. et al. (2021) ATP2, the essential P4-ATPase of malaria parasites, catalyzes lipid-stimulated ATP hydrolysis in complex with a Cdc50 β-subunit. *Emerg. Microbes Infect.* **10**, 132–147 https://doi.org/10.1080/22221751.2020.1870413

80 Zhang, M., Wang, C., Otto, T.D., Oberstaller, J., Liao, X., Adapa, S.R. et al. (2018) Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. *Science* **360**, eaap7847 https://doi.org/10.1126/science.aap784

81 Kenthirapalan, S., Waters, A.P., Matuschewski, K. and Kooij, T.W.A. (2016) Functional profiles of orphan membrane transporters in the life cycle of the malaria parasite. *Nat. Commun.* **7**, 10519 https://doi.org/10.1038/ncomms10519

82 Patel, A., Nofal, S.D., Blackman, M.J. and Baker, D.A. (2022) CDC50 orthologues in *Plasmodium falciparum* have distinct roles in merozoite egress and trophozoite maturation. *mBio* **13**, e0163522 https://doi.org/10.1128/mbio.01635-22

83 Kavishe, R.A., Van Den Heuvel, J.M., Van De Vegte-Bolmer, M., Luty, A.J., Russel, F.G. and Koenderink, J.B. (2009) Localization of the ATP-binding cassette (ABC) transport proteins PfMRP1, PfMRP2, and PfMDR5 at the *Plasmodium falciparum* plasma membrane. *Malar. J.* **8**, 205 https://doi.org/10.1186/1475-2875-8-205

84 Rubio, J.P. and Cowman, A.F. (1994) *Plasmodium falciparum*: the pfmdr2 protein is not overexpressed in chloroquine-resistant isolates of the malaria parasite. *Exp. Parasitol.* **79**, 137–147 https://doi.org/10.1006/expr.1994.1073

85 Haase, S., Condron, M., Miller, D., Cherkaoui, D., Jordan, S., Gulbis, J.M. et al. (2021) Identification and characterisation of a phospholipid scramblase in the malaria parasite *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **243**, 111374 https://doi.org/10.1016/j.molbiopara.2021.111374

86 Spielmann, T., Hawthorne, P.L., Bixon, M.W.A., Hannemann, M., Klotz, K., Kemp, D.J. et al. (2006) A cluster of ring stage-specific genes linked to a locus implicated in cytoadherence in *Plasmodium falciparum* codes for PEXEL-negative and PEXEL-positive proteins exported into the host cell. *Mol. Biol Cell* **17**, 3613–3624 https://doi.org/10.1091/mbc.e06-04-0291

87 Turrini, F., Ginsburg, H., Bussolino, F., Pescarmona, G.P., Serra M, V. and Arese, P. (1992) Phagocytosis of *Plasmodium falciparum*-infected human red blood cells by human monocytes: involvement of immune and nonimmune determinants and dependence on parasite developmental stage. *Blood* **80**, 801–808 https://doi.org/10.1182/blood.V80.3.801.801

88 Royo, J., Rahabi, M., Kamaliddin, C., Ezinmegnon, S., Olagnier, D., Authier, H. et al. (2019) Changes in monocyte subsets are associated with clinical outcomes in severe malarial anaemia and cerebral malaria. *Sci. Rep.* **9**, 17545 https://doi.org/10.1038/s41598-019-52579-7

89 Taliaferro, W.H. and Mulligan, H. (1937) The histopathology of malaria with special reference to the function and origin of macrophages in defense. *Indian Med. Res. Mem.* **29**, 1–138

90 Lee, W.C., Russell, B. and Rénia, L. (2019) Sticking for a cause: the *falciparum* malaria parasites cytoadherence paradigm. *Front. Immunol.* **10**, 1444 https://doi.org/10.3389/fimmu.2019.01444

91 Ebert, D. and Fields, P.D. (2020) Host–parasite co-evolution and its genomic signature. *Nat. Rev. Genet.* **21**, 754–768 https://doi.org/10.1038/s41576-020-0269-1

92 Chua, C.L.L., Brown, G., Hamilton, J.A., Rogerson, S. and Boeuf, P. (2013) Monocytes and macrophages in malaria: protection or pathology? *Trends Parasitol.* **29**, 26–34 https://doi.org/10.1016/j.pt.2012.10.002

93 Schwarzer, E., Turrini, F., Ulliers, D., Giribaldi, G.-, Ginsburg, H. and Arese, P. (1992) Impairment of macrophage functions after ingestion of *Plasmodium falciparum*-infected erythrocytes or isolated malarial pigment. *J. Exp. Med.* **176**, 1033–1041 https://doi.org/10.1084/jem.176.4.1033

94 Baker, D.A. (2010) Malaria gametocytogenesis. *Mol. Biochem. Parasitol.* **172**, 57–65 https://doi.org/10.1016/j.molbiopara.2010.03.019

95 Birge, R.B., Boeltz, S., Kumar, S., Carlson, J., Wanderley, J., Calianese, D. et al. (2016) Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer. *Cell Death Differ.* **23**, 962–978 https://doi.org/10.1038/cdd.2016.11

96 Alexander, J., Satoskaar, A.R. and Russell, D.G. (1999) Leishmania species: models of intracellular parasitism. *J. Cell Sci.* **112**, 2993–3002 https://doi.org/10.1242/jcs.112.18.2993

97 Späth, G., Lye, L.-F., Segawa, H., Sacks, D.L., Turco, S.J. and Beverley, S.M. (2003) Persistence without pathology in phosphoglycan-deficient *Leishmania major*. *Science* **301**, 1241–1243 https://doi.org/10.1126/science.1087499

98   Bogdan, C. (2020) Macrophages as host, effector and immunoregulatory cells in leishmaniasis: impact of tissue micro-environment and metabolism.
      Cytokine X **2**, 100041 https://doi.org/10.1016/j.cytox.2020.10004193

99   Serafim, T.D., Iniguez, E. and Oliveira, F. (2020) *Leishmania infantum*. *Trends Parasitol.* **36**, 80–81 https://doi.org/10.1016/j.pt.2019.10.006

100   Wanderley, J.L.M., Damatta, R.A. and Barcinski, M.A. (2020) Apoptotic mimicry as a strategy for the establishment of parasitic infections: parasite- and
      host-derived phosphatidylserine as key molecule. *Cell Commun. Signal.* **18**, 10 https://doi.org/10.1186/s12964-019-0482-8

101   Wanderley, J.L.M., Moreira, M.E.C., Benjamin, A., Bonomo, A.C. and Barcinski, M.A. (2006) Mimicry of apoptotic cells by exposing phosphatidylserine
      participates in the establishment of amastigotes of *Leishmania (L) amazonensis* in mammalian hosts. *J. Immunol.* **176**, 1834–1839 https://doi.org/10.
      4049/jimmunol.176.3.1834

102   Balanco, J.M.D.F., Costa Moreira, M.E., Bonomo, A., Bozza, P.T., Amarante-Mendes, G., Pirmez, C. et al. (2001) Apoptotic mimicry by an obligate
      intracellular parasite downregulates macrophage microbicidal activity. *Curr. Biol.* **11**, 1870–1873 https://doi.org/10.1016/S0960-9822(01)00563-2

103   Arango Duque, G. and Descoteaux, A. (2015) *Leishmania* survival in the macrophage: where the ends justify the means. *Curr. Opin. Microbiol.* **26**,
      32–40 https://doi.org/10.1016/j.mib.2015.04.007

104   Campos-Salinas, J., León-Guerrero, D., González-Rey, E., Delgado, M., Castanys, S. et al. (2013) LABCG2, a new ABC transporter implicated in
      phosphatidylserine exposure, is involved in the infectivity and pathogenicity of *Leishmania. PLoS Negl. Trop. Dis.* **7**, 101371 https://doi.org/10.1371/
      journal.pntd.0002179

105   Weingärtner, A., Drobot, B., Herrmann, A., Sanchez-Canete, M.P., Gamarro, F., Castanys, S. et al. (2010) Disruption of the lipid-transporting
      LdMT-LdRos3 complex in *Leishmania donovani* affects membrane lipid asymmetry but not host cell ínvasion. *PLoS One* **5**, e12443 https://doi.org/10.
      1371/journal.pone.0012443

106   Weingärtner, A., Kemmer, G., Müller, F.D., Zampieri, R.A., dos Santos, M.G., Schiller, J. et al. (2012) *Leishmania* promastigotes lack phosphatidylserine
      but bind annexin V upon permeabilization or miltefosine treatment. *PLoS One* **7**, e42070 https://doi.org/10.1371/journal.pone.0042070

107   Vermes, I., Hahnen, C., Steffens-Nakken, H. and Reutellingsperger, C. (1995) A novel assay for apoptosis: flow cytometric detection of
      phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J. Immunol. Methods* **184**, 39–51 https://doi.org/10.1016/
      0022-1759(95)00072-I

108   Weingärtner, A., dos Santos, M.G., Drobot, B. and Pomorski, T.G. (2011) Ca^{2+}-activated transbilayer movement of plasma membrane phospholipids in
      Leishmania donovani during ionomycin or thapsigargin stimulation. *Mol. Biochem. Parasitol.* **179**, 59–68 https://doi.org/10.1016/j.molbiopara.2011.05.
      006

109   dos Santos, M.G., Muxel, S.M., Zampieri, R.A., Pomorski, T.G. and Floeter-Winter, L.M. (2013) Transbilayer dynamics of phospholipids in the plasma
      membrane of the *Leishmania* genus. *PLoS One* **8**, e55604 https://doi.org/10.1371/journal.pone.0055604

110   Aslett, M., Aurrecoechea, C., Berriman, M., Brestelli, J., Brunk, B.P., Carrington, M. et al. (2010) TriTrypDB: a functional genomic resource for the
      Trypanosomatidae. *Nucleic Acids Res.* **38**, D457–D462 https://doi.org/10.1093/nar/gkp851

111   Seabra, S.H., de Souza, W. and Damatta, R.A. (2004) *Toxoplasma gondii* exposes phosphatidylserine inducing a TGF-β1 autocrine effect orchestrating
      macrophage evasion. *Biochem. Biophys. Res. Commun.* **324**, 744–752 https://doi.org/10.1016/j.bbrc.2004.09.114

112   DaMatta, R.A., Seabra, S.H., Deolindo, P., Arnholdt, A.C.V., Manhães, L., Goldenberg, S. et al. (2007) *Trypanosoma cruzi* exposes phosphatidylserine as
      an evasion mechanism. *FEMS Microbiol. Lett.* **266**, 29–33 https://doi.org/10.1111/j.1574-6968.2006.00495.x

113   Lourido, S. (2019) *Toxoplasma gondii*. *Trends Parasitol.* **35**, 944–945 https://doi.org/10.1016/j.pt.2019.07.001

114   Chen, K., Günay-Esiyok, Ö., Klingeberg, M., Marquardt, S., Pomorski, T.G. and Gupta, N. (2021) Aminoglycerophospholipid flipping and P4-ATPases in
      Toxoplasma gondii. J. Biol. Chem. **296**, 100315 https://doi.org/10.1016/j.jbc.2021.100315

115   Bisio, H., Krishnan, A., Marq, J.B. and Soldati-Favre, D. (2022) *Toxoplasma gondii* phosphatidylserine flippase complex ATP2B-CDC50.4 critically
      participates in microneme exocytosis. *PLoS Pathog.* **18**, e1010438 https://doi.org/10.1371/journal.ppat.1010438

116   Gajria, B., Bahl, A., Brestelli, J., Dommer, J., Fischer, S., Gao, X. et al. (2008) ToxoDB: an integrated *Toxoplasma gondii* database resource. *Nucleic
      Acids Res.* **36**, D553–D556 https://doi.org/10.1093/nar/gkm981