August, 2014

The Plasmodium Bottleneck: Malaria Parasite Losses in the Mosquito Vector

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Nearly one million people are killed every year by the malaria parasite Plasmodium. *Although the disease-causing forms of the parasite exist only in the human blood, mosquitoes of the genus* Anopheles *are the obligate vector for transmission. Here, we review the parasite life cycle in the vector and highlight the human and mosquito contributions that limit malaria parasite development in the mosquito host. We address parasite killing in its mosquito host and bottlenecks in parasite numbers that might guide intervention strategies to prevent transmission.*

Key words: malaria - mosquito - *Plasmodium* - innate immunity

Malaria continues to be one of the world's most devastating diseases and is caused by parasites of the genus *Plasmodium*. In 2010, there were an estimated 219 million cases of malaria resulting in around one million fatalities, mostly in children under five years old (Murray et al. 2012, WHO 2012). Increased awareness of the devastating impacts of malaria has led to a significant reduction of malaria cases and fatalities in the recent past. However, these achievements are threatened by a reduction in the distribution of insecticide treated nets, the resistance of mosquitoes to insecticides, parasite resistance to antimalarial drugs (including the 1st-line drug artemisinin) together with the levelling of funding for malaria control efforts (WHO 2012). For these reasons, there is an urgent need to improve current malaria control efforts and, importantly, to develop new strategies to eliminate and eventually eradicate the disease. Different from the other two major infectious disease killers, human immunodeficiency virus and tuberculosis, malaria is unique because it requires a mosquito vector for transmission to occur. Thus, the mosquito stages of the malaria parasite development have the potential to provide important targets for the control of transmission.

Parasite development in its vector starts when a mosquito ingests an infected blood meal containing *Plasmodium* sexual forms, known as gametocytes (Fig. 1). Within ~15 min, gametocytes round-up (in case of *Plasmodium falciparum*), egress from the red blood cell (RBC) and differentiate into gametes. Male gametocytes undergo a drastic transformation known as exflagella-

tion by which the DNA replicates to 8N followed by the formation of eight haploid microgametes. Microgametes detach from the exflagellation centre and actively search for female gametes to fertilise. Fertilisation gives rise to a diploid zygote that subsequently undergoes one round of DNA replication to become tetraploid. Zygotes differentiate into motile ookinetes that migrate in the blood bolus to invade and traverse the mosquito midgut epithelium. The ookinete may traverse multiple epithelial cells before emerging from the basal side facing the haemocoel, where it lodges beneath the basal lamina and differentiates into a round oocyst. Within the next 10-14 days each oocyst grows in size and undergoes sporogony to produce thousands of sporozoites. Upon oocyst maturation, sporozoites are released into the haemolymph where they circulate with the haemolymph and specifically invade the salivary glands. Following invasion, sporozoites lodge in the lumen of the salivary gland. When an infected mosquito feeds on a human host, sporozoites are released with the saliva and deposited in the skin, thus closing the transmission cycle.

Malaria parasites undergo dramatic losses during their development in the mosquito vector (Fig. 1). Population reduction occurs at each developmental step, from the formation of gametocytes in the human host to oocyst formation, resulting in very low parasite numbers. In fact, even in high transmission areas, the majority of the mosquitoes are not infected by the parasite (Chege & Beier 1990, Mbogo et al. 1993, Mendis et al. 2000, Gouagna et al. 2010). This reduction in numbers in the mosquito midgut is mediated in part by the transition of the parasite from an intracellular (RBC) to extracellular forms, thus exposing the parasites to both human and mosquito components that are deleterious to the parasite (Figs 1-3). It has been estimated that out of the thousands of gametocytes that a female *Anopheles* mosquito typically ingests in a blood meal, only 50-100 develop into ookinetes and only around five survive to form oocysts (Gouagna et al. 1998, Sinden & Billingsley 2001, Whitten et al. 2006). A study performed in *Anopheles gambiae* mosquitoes with blood from *P. falciparum*-infected patients showed that on average, from 433.5

gametocytes detected in patient blood, only 12.6 round forms, 5.5 ookinetes, 1.8 early oocysts and two mid-size oocyst were detected in the mosquito (Gouagna et al. 1998) (Fig. 1). More importantly, this study also shows that prevalence (the proportion of mosquitoes that carry at least 1 parasite) was only 38%, meaning that 62% of the mosquitoes that fed on the infected blood never got infected (Gouagna et al. 1998). In the entire *Plasmodium* life cycle (in both human and mosquito hosts), parasite numbers are lowest during the oocyst stage and then quickly expand when each oocyst releases thousands of sporozoites. For this reason, the midgut stages of parasite development constitute prime targets for strategies aiming to block malaria transmission.

Here we discuss the multiple mechanisms that limit parasite survival in the mosquito, starting with events occurring in the human host, and then in the mosquito midgut lumen (Fig. 2), and finally during invasion of the mosquito midgut epithelium (Fig. 3). In addition, we describe the mechanisms that the parasite has evolved to evade some of these antiplasmodial responses. Finally, we address strategies that are under consideration to target *Plasmodium* development in the mosquito and discuss future challenges that need to be overcome in order to succeed in any malaria transmission-blocking (TB) strategy.

Human factors - Cytokines and reactive nitrogen species (RNS) - Evidence suggests that cytokines produced during malaria infections can mediate the killing of
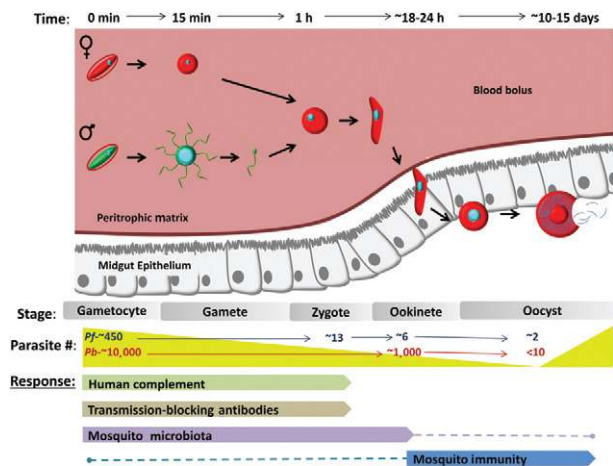

Fig. 1: *Plasmodium* bottlenecks in the mosquito host. Shortly after ingesting an infectious blood meal, *Plasmodium* gametocytes are activated within the *Anopheles* midgut resulting in the emergence of male and female gametes. Following fertilisation, the resulting zygote differentiates into a motile ookinete. The ookinete must then penetrate the peritrophic matrix that completely surrounds the blood meal and invade the midgut epithelium. After traversal, ookinetes attach to the basal surface of the epithelium and differentiate into sessile oocysts that grow and produce thousands of sporozoites over an approximate two-week period. Upon maturation, sporozoites are released into the haemolymph from where they invade the salivary glands. The cycle is completed when the mosquito feeds on a new host and delivers sporozoites with the saliva. The illustration indicates development time, approximate parasite numbers during each stage of development (yellow bar) and the timing of anti-*Plasmodium* responses (bottom).

gametocytes circulating in the blood stream of the host (Naotunne et al. 1991, Karunaweera et al. 1992) (Fig. 2). Infections of *Plasmodium cynomolgi* in toque monkeys or *Plasmodium vivax* in humans cause a period known as "crisis" or clinical paroxysms that are characterised by acute fever preceded by chills and rigor that coincide with the release of asexual parasites from RBCs (Naotunne et al. 1991, Karunaweera et al. 2003). During this period, *Plasmodium* gametocytes lose infectivity to mosquitoes due to an increase of the pro-inflammatory cytokines tumour necrosis factor (TNF)-α and interferon (IFN)-γ produced by the host immune system (Naotunne et al. 1991, Karunaweera et al. 1992). Although not completely understood, two proposed mechanisms by which TNF-α and IFN-γ could affect *Plasmodium* gametocytes in the human host are through the induction of phagocytosis or by increased nitric oxide (NO) production by leukocytes (Naotunne et al. 1993, Muniz-Junqueira et al. 2001).

Similar to the effects of pro-inflammatory cytokines, the levels of RNS have also been shown to increase during the paroxysm periods of *P. vivax* infections (Naotunne et al. 1993, Cao et al. 1998). Induced by unknown parasite factors, the activation of leukocytes results in an up-regulation of NO synthase (NOS) expression and increased NO production (Naotunne et al. 1993). NO is a highly reactive molecule and its reaction products (RNS) induce damage to DNA, proteins and lipids that ultimately result in cell death. Supporting the role of NO, Naotunne et al. (1993) demonstrate that TNF-α-mediated gametocyte inactivation is dependent on leukocyte activation and the production of NO. The inhibition of NO synthesis restored the infectivity of *P. falciparum* and *P. vivax* gametocytes to mosquitoes (Motard et al. 1993, Naotunne et al. 1993). Similarly, in mouse infections with *Plasmodium yoelii*, gametocyte infectivity to mosquitoes is highly impaired when mice experience crisis (4-5 days after infection) (Cao et al. 1998). During this time, treatment of the mice with the NOS inhibitor L-NMMA partially restored gametocyte infectivity suggesting that RNS are involved in this inhibitory effect.

One important aspect of these studies is that serum from semi-immune patients, which present mild clinical symptoms, did not affect *P. vivax* gametocyte infectivity (Karunaweera et al. 1992). This would suggest that clinical immunity is an adaptation in which parasite-killing factors (e.g., TNF-α) are reduced in order to diminish disease pathology (Karunaweera et al. 1992), where lower levels of circulating TNF-α would have little effect on circulating gametocytes. This is in contrast to the high TNF-α levels and increased NO production by a non-immune malaria-infected host (e.g., a child under 5 years old), which reduces gametocyte infectivity of the mosquito (Mshana et al. 1991, Othoro et al. 1999, Lyke et al. 2004).

In summary, these data suggest that human factors that limit gametocyte infectivity are probably the first contributors to the reduction of malaria parasite numbers in the mosquito.

Mosquito midgut lumen - Cytokines - In addition to their effects in the human host, antimalarial blood stream components can also target the parasite in the

mosquito midgut lumen (Fig. 2). Blood components, including white blood cells (WBCs), cytokines, complement proteins, RNS and other factors, remain active for several hours in the mosquito midgut after blood ingestion (Lensen et al. 1997, Margos et al. 2001, Simon et al. 2013). TNF-α was shown to reduce the formation of *Plasmodium berghei* ookinetes through the RNS-mediated reduction of exflagellating males and subsequent ookinete formation (Ramiro et al. 2011). Although the mechanism by which TNF-α enhances RNS inhibition of male gamete exflagellation is unknown, it has been proposed that the reduction in ookinete numbers could be the result of TNF-α-induction of leukocyte phagocytosis of sexual stage parasites (Muniz-Junqueira et al. 2001). In support of this theory, phagocytosis of *P. falciparum* and *P. berghei* gametocytes/gametes by lymphocytes was shown to occur in vitro and in vivo in the midgut of *An. gambiae* mosquitoes after ingestion of an infected blood meal (Sinden & Smalley 1976, Lensen et al. 1997).

RNS - RNS have been shown to affect the development of the malaria parasite inside the mosquito midgut lumen (Fig. 2). Pre-incubation of *P. yoelii* gametocytes with NOC5, a NO donor, inhibits gametogenesis and zygote formation (Cao et al. 1998). In addition, it has been reported that up to 50% of *P. berghei* ookinetes

developing inside the midgut of *Anopheles stephensi* mosquitoes show markers of apoptosis. It was hypothesised that these killing effects could be achieved in newly formed ookinetes when exposed to RNS or reactive oxygen species (ROS) donors (Ali et al. 2010). Supporting this theory, the removal of WBCs or treatment with the NOS inhibitor L-NAME significantly reduced the number of apoptotic ookinetes (Ali et al. 2010). In addition, Ramiro et al. (2011) showed that RNS can affect *P. berghei* male gamete exflagellation, fertilisation, as well as ookinete development.

Activated WBCs are believed to be one of the primary sources of NO that affect the sexual stages of the parasite in the human host and in the mosquito midgut. However, additional sources of NO and RNS come into play after a mosquito takes a blood meal.

Upon ingestion of a malaria infected blood meal, mosquito midgut levels of NO and nitrates significantly increase (Luckhart et al. 2003, Peterson et al. 2007). Digestion of RBCs by mosquito proteases release haemoglobin which comprises ~90% of the total ingested blood mass (Briegel & Rezzonico 1985). Oxyhaemoglobin and haeme which persist throughout blood digestion in the mosquito midgut can react with NO and ROS to produce toxic NO metabolites which in turn can affect malaria parasite development (Peterson et al. 2007).
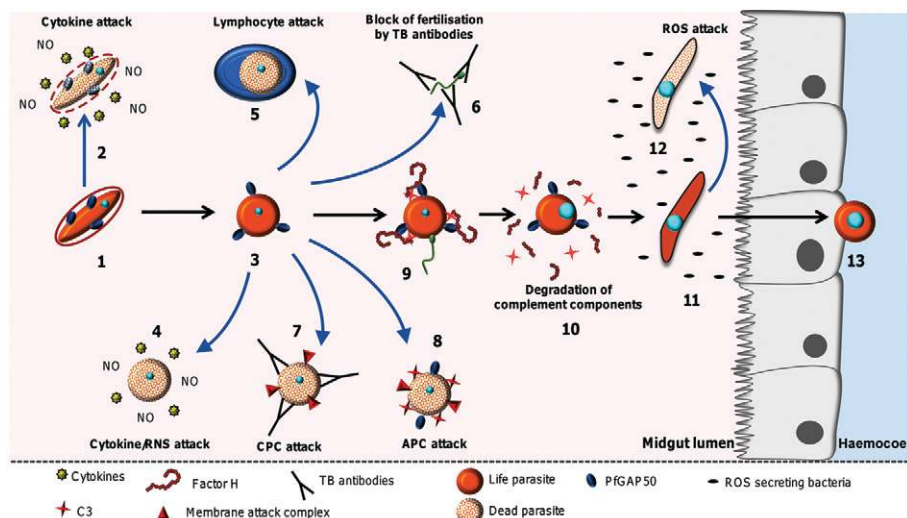

Fig. 2: factors that influence malaria parasite development in the mosquito midgut lumen. When a female *Anopheles* mosquito feeds on a malaria-infected person, it ingests sexual forms of the parasite: male and female gametocytes (1). Some of these gametocytes may be dead or non-infectious to the mosquito due to exposure to cytokines or nitric oxide (NO) produced in the infected human host (2). After the gametes egress from the red blood cell (RBC) (3) they become exposed to factors from the human blood that may negatively affect parasite development. These include damage caused by serum cytokines and NO (4), phagocytosis by lymphocytes (5), inhibition of fertilisation by transmission-blocking (TB) antibodies (6) and the attack of the vertebrate complement system (7, 8). The attack by the complement system can be initiated by two mechanisms: activation of the classical pathway (CPC) by opsonising antibodies (against Pfs230) that bind to gamete surface proteins (7) or activation of the alternative pathway (APC) by binding of C3 to the surface of the gamete (8). In both cases, lysis occurs after the formation of a membrane-attack complex on the parasite membrane. To evade the activation of the alternative complement pathway, the parasite uses the surface protein PfGAP50 to recruit factor H from the blood serum, thus inhibiting further activation of the system (9). Proteins from the complement system are degraded approximately 6 h after blood-feeding (10). Parasites that escape further develop into ookinetes which shares its niche with the midgut bacteria that multiplied exponentially after the ingestion of the blood (11). These bacteria may secrete antimalarial compounds, including reactive oxygen species (ROS), which impact ookinete viability (12). The ookinetes that survive invade and traverse the midgut epithelium after which they form oocysts on the basal side of the midgut epithelium (13). RNS: reactive nitrogen species.

In addition, the mosquito midgut epithelium can also be a source of NO. The up-regulation of NOS has been observed in midguts of *An. gambiae* and *An. stephensi* mosquitoes upon ingestion of a malaria infected blood meal (Dimopoulos et al. 1998, Luckhart et al. 1998). Blood components have also been shown to increase mosquito midgut NOS expression. The cytokine transforming growth factor-β1 (TGF-β1), a component of the human serum, at low concentrations induces *An. stephensi* NOS and reduces the mosquito parasite burden (Luckhart et al. 2003). In addition, *P. falciparum* glycosylphosphatidylinositol and parasite-derived haemozoin pigments also appear to induce *An. stephensi* NOS expression in the mosquito midgut epithelium (Lim et al. 2005, Akman-Anderson et al. 2007). However, the impact that each of these NO sources (especially midgut-derived NO) have on each parasite stage in the midgut lumen remains to be determined.

The two main antioxidant systems of the malaria parasite, the thioredoxin and the glutathione (GSH) redox systems, play a protective role during the parasite development in the mosquito. Interruption of the GSH redox pathway by disruption of the gamma-glutamyl-cysteine synthetase gene (the rate limiting enzyme in GSH biosynthesis) or alternatively, the GSH reductase gene, results in the formation of fewer ookinetes and stunted oocysts that fail to fully develop in *An. stephensi* mosquitoes (Vega-Rodríguez et al. 2009, Pastrana-Mena et al. 2011). Likewise, *P. berghei* oocysts lacking thioredoxin-dependent 2-Cys peroxidase 1 expression, which is part of the thioredoxin redox system, produce fewer sporozoites (Yano et al. 2008). Moreover, *P. berghei* ookinetes increase the expression of antioxidant peroxiredoxins after exposure to increased levels of oxidative stress (Turturice et al. 2013). It is conceivable that the malaria parasite regulates its antioxidant defense systems to overcome the damage cause by RNS in the mosquito midgut lumen. The mosquito peritrophic matrix (PM), a cellular layer that completely surrounds the blood meal and is secreted by the midgut epithelium in response to feeding, may constitute another protective mechanism from RNS damage. Proteins from the PM have been shown to bind and consequently remove haeme produced by haemoglobin digestion, thus reducing its capacity to form additional RNS (Pascoa et al. 2002, Devenport et al. 2006). The protective properties of the PM have yet to be determined, but presumably this structure confers some level of protection for parasites located at the periphery of the blood bolus where active digestion is taking place.

Host complement system - The complement system is part of the innate immune defense in vertebrates and is a first-line defense against pathogens including bacteria, fungi and protozoans. After a foreign organism is detected by the vertebrate host, the complement system is activated within seconds through either the classical, lectin or alternative pathways [reviewed in Walport (2001)]. This complex system is made up of around 30 abundant proteins in the blood plasma. Once complement has been activated, ultimate destruction of the foreign organism can be achieved by several mechanisms including ingestion by phagocytes or by the assembly of a membrane attack complex on the pathogen surface that ultimately results in its killing.

Infection of the human host by malaria parasites activates both the classical and alternative complement pathways (ACP) (Adam et al. 1981, Wenisch et al. 1997, Goka et al. 2001, Roestenberg et al. 2007). By comparing the infectivity of *Plasmodium* parasites to mosquitoes by feeding gametocytes in either native or in heat-inactivated serum (heat destroys complement activity), parasites in inactivated serum produced significantly more oocysts than those in native serum (Grotendorst et al. 1986, Tsuboi et al. 1995, Margos et al. 2001, Simon et al. 2013). Furthermore, Grotendorst et al. (1986) demonstrated that the ACP is responsible for this reduction in parasite numbers. When factor B (a specific component of the alternative pathway) is removed from serum, parasite viability is increased. Similar experiments, in which components specific to the classical pathway were also removed from serum, did not alter parasite viability. Interestingly, the early mosquito stages of the parasite (gametocytes, gametes and early zygotes) are more protected from complement attack than later stages (late zygotes and ookinetes) (Grotendorst et al. 1986, Tsuboi et al. 1995, Margos et al. 2001, Simon et al. 2013), suggesting that malaria parasites may have evolved a mechanism of protection during their most vulnerable stages.

Immediately after the female mosquito feeds on blood, the midgut starts secreting digestive enzymes that degrade proteins from the serum and the RBCs. Host complement proteins are also exposed to mosquito proteases which raise the question of how long does the complement system from the human serum remain active after the mosquito has taken a blood meal? Two different studies addressed this question by measuring the products of C3 activation, which results in the formation of C3a (Simon et al. 2013) and C3b (Margos et al. 2001). Both reports show that the peak of complement activity in the midgut is during the first hour post-blood-feeding and remains active up to 6 h after feeding. This loss of complement activity in the mosquito blood bolus also coincides with the transition of the parasite from complement-resistant stages (gametocytes, gametes and early zygotes) to those that are complement-sensitive (late zygotes and ookinetes) (Fig. 2) (Grotendorst et al. 1986, Tsuboi et al. 1995, Margos et al. 2001, Simon et al. 2013). However, digestion of the blood bolus is not uniform and occurs from the outside in. Therefore, it can be predicted that the complement molecules closer to the periphery of the blood bolus will be inactivated faster and consequently that parasites located in these areas will be protected from complement attack much earlier.

Evasion of the complement system - To survive, pathogenic microorganisms have evolved different mechanisms to evade the attack from the host complement system. To date, three evasion mechanisms have been described: inactivation of complement by pathogen-derived proteases, inactivation by complement-binding (neutralising) proteins and the acquisition of complement regulatory

proteins. There is evidence to suggest that *Plasmodium* has evolved to acquire a complement regulatory protein needed to evade complement attack during its development in the mosquito blood bolus (Fig. 2).

Parasites became sensitive to complement attack following treatment of *Plasmodium gallinaceum* gametes with trypsin, implying that a parasite surface protein is responsible for the protection from complement (Grotendorst et al. 1986). This was further examined in a recent report showing that *Plasmodium* gametes and zygotes bind on their surface factor H (FH) from the host serum. FH regulates ACP activation by binding to C3b thus preventing the formation of the C3 convertase and accelerating its decay [reviewed in de Córdoba et al. (2004)]. When mosquitoes were fed with *P. falciparum* gametocytes in native human serum together with anti-FH antibodies, mosquitoes were rendered resistant as parasite numbers were effectively reduced to zero (Simon et al. 2013). Co-immunoprecipitation assays with anti-FH antibodies on protein extracts from activated gametes in native human serum identified PfGAP50 as a parasite receptor for FH (Simon et al. 2013). However, the authors suggest that there are additional unknown FH receptors on the surface of the gametes as anti-PfGAP50 antibodies only reduced the infectivity to mosquitoes by 38-60%. This hypothesis is plausible as other pathogens including *Streptococcus pyogenes* and *Borrelia burgdorferi* use more than one surface protein to capture FH from the host and evade the ACP response [reviewed in Zipfel et al. (2007)].

TB antibodies - Naturally acquired immunity against malaria asexual stages can confer partial protection against the disease [reviewed in Doolan et al. (2009)]. In addition to the asexual stages, gametocytes also circulate in the blood. If a gametocyte is not ingested by a mosquito during blood-feeding, it decays and evidence suggests that it is eventually removed from circulation by the host immune system resulting in the production of gametocyte-specific antibodies (Baird et al. 1991, Taylor & Read 1997, Saeed et al. 2008). Such antibodies would also be ingested by the mosquito and could interfere with progression of the developmental cycle in the midgut lumen (Fig. 2).

Immune sera of individuals living in malaria endemic regions have TB activity against the mosquito midgut stages of both *P. falciparum* and *P. vivax* (Baird et al. 1991, Mulder et al. 1994, Lensen et al. 1998, Arévalo-Herrera et al. 2005, Bousema et al. 2011). Specifically, antibodies against the gametocyte/gamete proteins Pfs48/45 and Pfs230 show a negative correlation between antibody activity and parasite development in the mosquito (Healer et al. 1999, Drakeley et al. 2004, van der Kolk et al. 2006, Bousema et al. 2007, 2010). Both Pfs48/45 and Pfs230 participate in gamete fertilisation and are members of a 6-cysteine protein family containing adhesive domains (van Dijk et al. 2001, 2010, Eksi et al. 2006). These proteins are stored in the parasitophorous vacuole membrane of the gametocyte and relocate to the surface of the gamete during activation in the mosquito midgut (Williamson et al. 1996). Gene disruption of P48/45 and P230 in *P. berghei* and Pfs230 in *P. falciparum* drastically reduces infectivity to mosquitoes by interfering with fertilisation (van Dijk et al. 2001, 2010, Eksi et al. 2006).

It appears that anti-Pfs230 antibodies hinder fertilisation by a mechanism different from interference with gamete adhesion. Early TB studies reported that antibodies against Pfs48/45, but not Pfs230 inhibited *P. falciparum* development in the mosquito, a finding that discouraged the use of Pfs230 as a potential TB vaccine antigen (Vermeulen et al. 1985). Later studies showed that anti-Pfs230 antibodies inhibit *P. falciparum* development in the mosquito in the presence of active serum complement (Quakyi et al. 1987, Read et al. 1994, Healer et al. 1997). These antibodies activate the classical complement pathway resulting in the formation of the membrane-attack complex causing lysis of the parasite (Fig. 2).

In summary, these studies highlight the possible applications of an antibody-based TB vaccine to reduce parasite development in its mosquito host and could have a profound influence on the transmission of malaria in endemic countries.

Midgut microbiota - Mosquitoes, as all higher organisms including humans, carry an intestinal microbiota that is mostly composed of bacteria and yeast [reviewed in Dillon and Dillon (2004)]. There is evidence to suggest that the symbiotic relationship between bacteria and the mosquito confers to the latter some protection against invading pathogens like malaria. Several studies using laboratory-reared and field-captured *Anopheles* mosquitoes have shown that midgut bacteria (primarily Gram-negative bacteria) have a negative effect on *P. falciparum* and *P. vivax* development in the mosquito (Seitz et al. 1987, Pumpuni et al. 1993, 1996, Straif et al. 1998, Gonzalez-Ceron et al. 2003, Dong et al. 2009, Cirimotich et al. 2011). For instance, infections after removal of endogenous microbiota by antibiotic treatment lead to higher parasite numbers when compared with infections of untreated mosquitoes (Beier et al. 1994, Dong et al. 2009, Meister et al. 2009). In addition, when antisera raised against *An. gambiae* midgut lysates were fed to *An. gambiae* mosquitoes together with *P. falciparum* cultured gametocytes, there was an increase in the number of parasites that developed into oocysts relative to controls and this effect was attributed to antibacteria antibodies in the serum (Noden et al. 2011). These results are in contrast to previous reports where similar experiments resulted into a reduced parasite load due to antibodies targeting mosquito proteins required for parasite development (Srikrishnaraj et al. 1995, Lal et al. 2001, Suneja et al. 2003, Chugh et al. 2011). The reasons for these discrepancies are unclear and require further investigation.

Recent studies have shown that certain variants of *Serratia marcescens*, a bacterium commonly found in the midgut of laboratory and field mosquitoes, are able to inhibit *Plasmodium* in the mosquito (Bando et al. 2013). Increased *Plasmodium* inhibition was correlated to increased flagella length and abundance and with reduced ability of the ookinete to invade the midgut (Bando et

al. 2013). The authors hypothesise that a physical barrier imposed by the flagella may contribute to the decreased success of ookinetes to invade the midgut epithelium.

Recent evidence has also emerged that bacteria may influence *Plasmodium* development directly through the production of antimalarial compounds (Fig. 2). Cirimotich et al. (2011) isolated an *Enterobacter* bacterium (*Esp_Z*) from Zambian populations of *Anopheles arabiensis* mosquitoes. Co-feeding of *Esp_Z* and *P. falciparum*-infected blood to mosquitoes reduced the parasite burden in a dose-dependent manner. The antiparasitic effect of this bacterium, which is observed at the zygote to ookinete transition, can be rescued by addition of the antioxidant vitamin C to the infectious blood meal. This result suggests that ROS secreted by *Esp_Z* bacteria is responsible for the reduction in parasite numbers (Cirimotich et al. 2011). These findings have important implications as a naturally occurring bacterium like *Esp_Z* could be exploited to develop new malaria TB alternatives. However, as mentioned above, the parasite's antioxidant systems are highly active during development in the mosquito and can be regulated according to the redox state of the environment were the parasite is developing (Yano et al. 2008, Vega-Rodríguez et al. 2009, Pastrana-Mena et al. 2011).

Additional evidence suggests that the midgut microbiota play a direct role in the activation of the mosquito immune response. Shortly after blood ingestion, the resident microbiota undergo dramatic proliferation (about 2 orders of magnitude) peaking at about 24 h (Pumpuni et al. 1996). This strong bacterial proliferation is likely to result in an immune response independent of parasite presence. Dong et al. (2009) reported that the mosquito midgut bacteria induce a basal level of immunity that enhances the expression of antimicrobial immune genes that also have antiplasmodial activity. This is true for the induction of SRPN6 in response to *Enterobacter* infection, which in turn contributes to the anti-*Plasmodium* response of the mosquito (Eappen et al. 2013). Moreover, Rodrigues et al. (2010) reported that bacteria are necessary for immune priming.

A delicate balance exists between the commensal gut microbiota and its mosquito host to limit bacterial overproliferation and the subsequent immune response that may have negative effects towards mosquito fitness. To reduce hyperactivation of the mosquito immune system by the midgut bacteria, the mosquito is thought to form a dityrosine network that restricts contact of bacteria with the midgut epithelium, thus reducing the antimicrobial response (Kumar et al. 2010). As a result, proliferation of the normal gut flora is not impaired and malaria parasite development is unhindered (Kumar et al. 2010). In addition, dual oxidase expression by the mosquito midgut epithelium likely results in ROS production, playing a similar role to that in *Drosophila* of limiting bacterial numbers (Ha et al. 2009). Moreover, it was proposed that when *Plasmodium* ookinetes breach the PM and the protective dityrosine network lining the luminal surface of the midgut epithelium, bacteria "leak" through the open spaces thus activating an antibacterial immune response that is also harmful to invading ookinetes (Kumar et al. 2010, Rodrigues et al. 2010).

Physical barriers to Plasmodium development - The PM - The distension of the mosquito midgut by a blood meal induces midgut epithelial cells to secrete components of an extracellular layer, known as the PM, which completely surrounds the ingested blood. The PM is composed of proteins, glycoproteins and proteoglycans that are structurally linked by chitin (Shao et al. 2001, Dinglasan et al. 2009). The PM constitutes a physical barrier that prevents direct contact of commensal bacteria and of components of the blood meal with the midgut epithelium. Initially soft and fragile, the PM polymerises and gradually thickens reaching maximal rigidity at about 24 h after the blood meal (Shao et al. 2001). As the PM completely surrounds the blood meal, it constitutes the first significant physical barrier to *Plasmodium* development in the mosquito. As ookinetes mature (approximately 16-20 h after blood ingestion) they migrate to the periphery of the blood bolus, presumably guided by environmental or sensory cues. Upon contact with the PM, the ookinete secretes a chitinase (and possibly other proteases) from its micronemes to locally disrupt and penetrate the chitinous PM. Inactivation of the chitinase genes dramatically reduces the ability of the ookinete to traverse the PM (Dessens et al. 2001, Tsai et al. 2001). In some parasite species, ookinetes secrete a chitinase precursor (or zymogen) that is activated by mosquito midgut proteases (Shahabuddin et al. 1993, Shahabuddin & Kaslow 1994), indicating that the parasite has adapted to the protease-rich environment of the mosquito midgut to facilitate its own development.

The midgut epithelium - After traversal of the PM it is believed that ookinetes display extensive gliding motility along the lumenal surface of the midgut epithelium. This movement may be important to initiate midgut invasion (Zieler & Dvorak 2000), possibly through the interaction with one or more of the numerous glycoproteins that comprise the glycocalyx of the midgut epithelium (Shen et al. 1999, Wilkins & Billingsley 2001, Dinglasan et al. 2007a). Carbohydrate moieties on the midgut epithelium seem to play an important role in ookinete binding to the midgut (Zieler et al. 1999, Zieler & Dvorak 2000). Moreover, it appears that the interaction between mosquito sugars and parasite lectins are required to establish invasion (Zieler & Dvorak 2000, Dinglasan et al. 2007a, b). However, very little is known regarding the specific protein-protein interactions that mediate this process since these carbohydrate moieties are post-translational modifications of yet unknown proteins. Given the implications as possible TB vaccine targets, identifying these specific interactions is a high priority area of research.

Using a phage display library, a peptide termed SM1 was identified that interacts with the lumenal surface of the mosquito midgut epithelium (Ghosh et al. 2001) and has greatly increased our understanding of the process of midgut invasion. The SM1 dodecapeptide strongly inhibits *P. berghei* ookinete invasion via competitive binding to a putative mosquito midgut receptor (Ghosh et al. 2001). Transgenic mosquitoes engineered to express SM1 in the midgut following blood-feeding are impaired in the transmission of the malaria parasite (Ito et al. 2002). Further studies have identified that SM1 is a mimotope

(its conformation resembles) of *Plasmodium* enolase, a protein secreted onto the surface of the ookinete where it acts as an invasion ligand (Ghosh et al. 2011). Ookinete surface enolase also interacts with plasminogen from the blood serum to locally promote its conversion into the proteolytically active plasmin that in turn, is required for midgut invasion (Ghosh et al. 2011). Recently, the mosquito midgut receptor for SM1 and ookinete surface enolase was identified as enolase binding protein (EBP) (Vega-Rodríguez et al. 2014). EBP is expressed on the lumenal surface of the mosquito midgut and is required for *P. berghei* ookinete midgut invasion through its interaction with surface enolase. This interaction can be disrupted through competition with excess SM1, thus inhibiting ookinete midgut invasion (Vega-Rodríguez et al. 2014). However, some ookinetes still invade the mosquito midgut in the presence of excess SM1 and this served as the basis for the selection of SM1-resistant parasites that do not require EBP for midgut invasion. This is the first evidence demonstrating that mosquito midgut invasion by *Plasmodium* ookinetes, similar to merozoite invasion of the RBC, can occur through multiple invasion pathways (Vega-Rodríguez et al. 2014). While further work is needed to characterise additional midgut receptor(s), these findings suggest that the process of ookinete midgut invasion is complex and involves multiple parasite-mosquito interactions. Characterisation of these interactions remains a major goal for future research and has the potential to lead to novel intervention strategies.

The process of ookinete invasion is thought to produce severe damage to the midgut as the ookinete traverses multiple epithelial cells during its journey to the basal lamina. Invaded cells undergo dramatic cytoskeletal changes and increase the production of reactive oxygen and nitrogen species that trigger apoptosis and cell death (Han et al. 2000). During this time ookinetes are likely exposed to a highly toxic cellular environment. To ensure its survival, the ookinete must rapidly escape before it is damaged or before the damaged cell is extruded from the midgut epithelium. This evasion process occurs by invasion of neighbouring naïve cells or by exiting the epithelium to rest at its final extracellular destination between the basal side of the epithelium and the basal lamina. While it remains unclear how ookinete invasion triggers programmed cell death, this is likely a general response to remove damaged epithelial cells and may not be specific to parasite invasion (Baton & Ranford-Cartwright 2005, Okuda et al. 2007).

Recent evidence further supports the concept that midgut traversal is a critical step in the mosquito anti-*Plasmodium* response. Invaded cells produce high levels of NOS and peroxidases that in turn increase the levels of midgut nitration (Kumar et al. 2004). Further nitration is increased by the activation of the haeme peroxidase 2 (HPX2) and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 5 (NOX5), processes that are regulated by the c-Jun N-terminal kinase (JNK) pathway (Oliveira et al. 2012, Garver et al. 2013). This epithelial nitration response is believed to modify ookinetes, thus "marking" them for immune recognition by the mosquito complement system (Oliveira et al. 2012, Garver et al. 2013). Thus, midgut nitration appears to be a major determinant of *Plasmodium* survival, yet many questions remain regarding how nitration mediates immune recognition. What proteins on the surface of the ookinete undergo modifications as a result of increased nitration? How are these modifications subsequently recognised by the mosquito complement system?

Components of the anti-Plasmodium immune response - A great deal of effort has been invested in the identification of components that contribute to parasite developmental success in the mosquito in the hope that this knowledge can be translated to the development of alternative strategies for parasite killing. Through the use of dsRNA-mediated gene-silencing, several genes have been described that positively or negatively influence parasite development in the mosquito (Blandin et al. 2008). While more research is required to fully understand the contributions of individual mosquito genes to anti-*Plasmodium* immunity, evidence suggests that parasite killing after traversal of the midgut epithelium occurs primarily during two separate stages or phases of parasite development (Fig. 3). The first phase, or "early-phase", occurs during the ookinete to oocyst transition immediately after exiting the midgut epithelium. A second, or "late-phase", is thought to act on developing oocysts.

The "early-phase" immune response - Approximately 18-24 h after blood-feeding, *Plasmodium* ookinetes invade the lumenal side of the midgut epithelium and within minutes reach the basal surface that is believed to be the major site of ookinete killing (Shiao et al. 2006). At this point, ookinetes are exposed to the haemocoel and to the complement-like soluble immune proteins that circulate in the haemolymph (Blandin et al. 2004, Shiao et al. 2006).

The thioester protein 1 (TEP1) was initially identified in view of its high similarity to the vertebrate complement factor C3 and to the related family of alpha-2-macroglobulins (Levashina et al. 2001). Similar to the role of C3 in the complement pathway of mammals, TEP1 behaves as an opsonin promoting the phagocytosis of bacteria (Levashina et al. 2001) and binds to the ookinete surface to mediate parasite lysis or melanisation (Blandin et al. 2004). In the haemolymph, TEP1 is expressed constitutively as full-length (inactive) and processed (active) forms that require the scaffolding proteins leucine-rich immune molecule 1 (LRIM1) and *Anopheles Plasmodium*-responsive leucine-rich repeat protein 1 (APL1) to direct TEP1 to pathogen surfaces and prevent self-recognition (Fraiture et al. 2009, Povelones et al. 2009). Loss of TEP1, LRIM1, or APL1 prevents ookinete recognition and dramatically increases parasite numbers (Fraiture et al. 2009, Povelones et al. 2009). Interestingly, members of the APL1 family (APL1A, APL1B, APL1C) appear to confer pathogen recognition specificity to the TEP1/LRIM1/APL1 complex. APL1A expression is required for protection against *P. falciparum* parasites, while APL1C is needed for protection against the rodent malaria parasites *P. berghei* and *P. yoelli* (Mitri et al. 2009). Although it is unclear how the mosquito immune

system is able to distinguish their targets, it is reasonable to assume that members of the APL1 gene family have important functions in parasite recognition as part of the mosquito complement-like pathway.

Upon binding to the ookinete, TEP1 is thought to promote parasite killing through lysis or melanisation (Blandin et al. 2004). Recent evidence has identified that a non-catalytic protease, SPCLIP1, may function to amplify complement activation by binding to TEP1 on the pathogen surface and promote the recruitment of additional TEP1 (Povelones et al. 2013). A similar amplification of the complement response is also seen in the vertebrate complement system where C3b, the cleavage product of C3 (equivalent to TEP1), binds factor B on the surface of the pathogen. This complex is then activated by factor D into the C3 convertase that in turn mediates the recruitment of additional C3b molecules to the pathogen surface [reviewed in Walport (2001)]. However, our knowledge of this process in the mosquito is incomplete and additional proteins that promote parasite killing may be recruited to the ookinete surface, as is the case for the vertebrate complement system. Multiple proteins and co-factors are known to assemble onto pathogen surfaces as part of the mammalian complement pathways and

Fig. 3: mechanisms of "early-phase" and "late-phase" immunity. Parasite development after traversal of the midgut epithelium is subjected to two "phases" of the mosquito innate immune response. An "early-phase" limits the ookinete survival before or at the transition to oocyst differentiation. As ookinetes traverse the midgut epithelium they undergo nitration (red dots) and in this way are "marked" for immune recognition by complement-like proteins circulating in the mosquito haemolymph [including thioester protein 1 (TEP1)]. Following recognition, TEP1 binds to the ookinete surface to initiate lysis or melanisation that result in parasite killing. A second, "late-phase" immune response limits oocyst survival and involves the production of nitric oxide (NO) by the signal transducer and activator of transcription (STAT) pathway leading to parasite killing. While increased levels of NO have been implicated in this process, it is unclear to what extent the midgut, fat body and possibly haemocytes may contribute to the "late-phase" response. This figure was adapted from Gupta et al. (2009) and Fraiture et al. (2009). APL1: *Anopheles Plasmodium*-responsive leucine-rich repeat protein 1; BL: basal lamina; LRIM1: leucine-rich immune molecule 1; ME: midgut epithelium; NOS: NO synthase; PM: peritrophic matrix.

further investigation of the components of the mosquito complement-like pathway remains an important goal for future investigation.

Furthermore, many questions remain regarding the mechanisms involved in directing TEP1 binding to the ookinete surface. As mentioned previously, midgut nitration appears to be a critical determinant of parasite recognition by the mosquito complement-like pathway (Oliveira et al. 2012). However, it is unclear if TEP1 directly recognises these protein modifications on the parasite surface or if this is mediated by other supporting molecules.

"Late-phase" immunity - Ookinetes that survive the process of midgut invasion and the early immune responses are subjected to a second or "late-phase" immune response that further limits oocyst numbers. First proposed by Gupta et al. (2009), the signal transducer and activator of transcription (STAT)-A or STAT-B significantly increases parasite survival without altering the number of early oocysts. Further experiments determined that STAT-A or STAT-B silencing increased oocyst survival through decreased production of NOS (Gupta et al. 2009).

In *An. gambiae*, STAT-B regulates *stat-A* mRNA expression and STAT-A mediates the transcriptional activation of *nos* in response to infection (Gupta et al. 2009). This transcriptional cascade can be manipulated through suppressor of cytokine signalling-3 (SOCS-3)-silencing (an inhibitor of STAT-A) resulting in the constitutive activation of STAT-A signalling and increased NOS expression (Gupta et al. 2009). This results in a near refractory phenotype that elevates NOS expression in the mosquito midgut and carcass (Gupta et al. 2009).

In addition to this study, there is a great deal of evidence suggesting that NOS and subsequent NO production are important determinants of oocyst development (Luckhart et al. 1998, Bahia et al. 2011, Vijay et al. 2011). NOS expression appears to be induced throughout the entire mosquito in response to *Plasmodium* infection (Luckhart et al. 1998, Gupta et al. 2009) suggesting that NOS activation is a generalised, rather than local, epithelial response to infection (Gupta et al. 2009). Much work remains to be done to better define the mechanisms of late-phase immune response and how it interferes with oocyst development.

Immune pathways that limit parasite development - Much of our knowledge of the mosquito innate immune response stems from *Drosophila* research and from other insect systems. Orthologous immune signalling pathways have been described in mosquitoes that respond to a variety of pathogens and regulate anti-*Plasmodium* immunity. However, compared to *Drosophila*, there have been rapid expansions of mosquito immune gene families, suggesting a functional broadening of the mosquito defense systems (Waterhouse et al. 2007). As a result, the mosquito represents a unique model to study host-parasite interactions and the innate immune response.

In *Drosophila*, the Toll and immunodeficiency (IMD) pathways have traditionally been associated with the production of antimicrobial peptides (such as cecropin
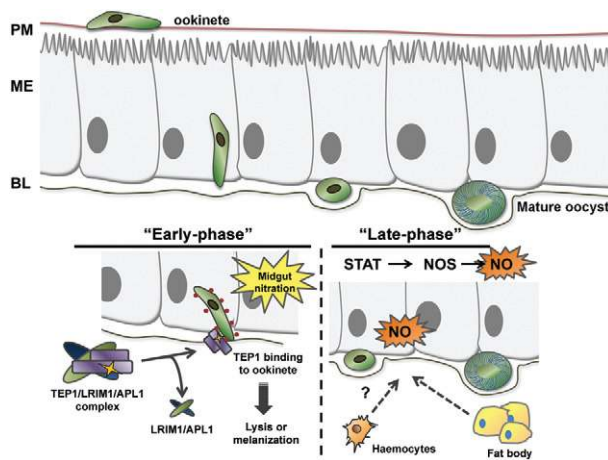
and defensin) in response to bacterial infection, while the JAK-STAT pathway has been implicated in antiviral immunity (Lemaitre & Hoffmann 2007). While these pathways certainly exist in mosquitoes [reviewed by Cirimotich et al. (2010)], it appears that these immune pathways also mediate anti-*Plasmodium* defenses. However, given current technology that relies primarily on systemic gene-silencing, the contributions of individual mosquito tissues (midgut, haemocytes, fat body) to the immune defenses has been difficult to ascertain. Three major insect immune pathways have been described: Toll, IMD and JAK-STAT.

The Toll pathway - In *Drosophila*, the involvement of the Toll pathway has been well described in the host response to Gram-positive bacteria, fungi and viruses (Lemaitre & Hoffmann 2007). Similar experiments in *Anopheles* suggest that this pathway is evolutionarily conserved in mosquitoes (Frolet et al. 2006, Garver et al. 2009).

REL1, originally described as Gambif1, is an anopheline nuclear factor kappa B-like transcription factor orthologous to *Drosophila* Dorsal (Barillas-Mury et al. 1996). Upon immune activation, Toll signalling results in the directed degradation of Cactus, a negative regulator of REL1, thus allowing translocation of REL1 to the nucleus and the expression of its downstream effector genes (Frolet et al. 2006). Silencing *cactus* expression by dsRNA injection results in the constitutive activation of the Toll pathway, even without immune challenge (Frolet et al. 2006). Frolet et al. (2006) demonstrate that Toll activation significantly impairs *P. berghei* development, although the exact mechanism is not well understood. Recent evidence implicates mosquito haemocytes as critical mediators of this anti-*Plasmodium* response (Ramirez et al. 2014).

Further studies suggest that Toll activation may be more efficient at limiting *P. berghei* (a rodent parasite) development than *P. falciparum* (the human malaria parasite) in multiple mosquito vectors (Garver et al. 2009). This would imply that immune recognition of rodent and human malaria parasites may occur through different mechanisms (Dong et al. 2006, Garver et al. 2009). How the mosquito distinguishes these two parasites is a very interesting question that remains to be answered.

The IMD pathway - The IMD pathway of mosquitoes is analogous to the TNF signalling pathway in mammals. Pathogen recognition is mediated by peptidoglycan recognition protein LC and the adaptor protein IMD, triggering the cleavage of the transcription factor REL2 (*Drosophila* Relish), which results in its nuclear translocation (Meister et al. 2005, 2009, Luna et al. 2006). This cascade of events is commonly referred to as the canonical IMD pathway, yet additional signalling events are activated through other IMD pathway components that include TGF-ß-activated protein kinase 1 (TAK1), which mediates the JNK signalling pathway in *Drosophila* (Silverman et al. 2003, Delaney et al. 2006). Currently, it is unclear what role the IMD pathway plays in TAK1 signalling and mitogen-activated protein kinase (MAPK) activation in mosquitoes, however recent evidence implies that JNK activation plays a key role in

the mosquito immune response to *Plasmodium* (Garver et al. 2013). As a result, the *Anopheles* IMD pathway appears to be highly complex and further research is required to fully understand its intricacies.

Based on *Drosophila* research, the *Anopheles* IMD pathway is likely regulated at several different steps [reviewed by Cirimotich et al. (2010)]. In mosquitoes, it has been suggested that immune regulation of one IMD pathway component occurs through the differential splicing of the transcription factor REL2 (Meister et al. 2005, Luna et al. 2006). A short form (REL2-S) lacking the inhibitory ankyrin domain and a full-length form (REL2-F) are constitutively expressed throughout development (Meister et al. 2005). Whereas the short form is constitutively active and thought to be responsible for basal immune function, REL2-F is localised in the cytoplasm and thus transcriptionally inactive (Meister et al. 2005, Luna et al. 2006). Upon immune activation, IMD signalling stimulates DREDD-dependent cleavage of REL2-F exposing its nuclear translocation signal, resulting in nuclear translocation and transcriptional activation of REL2-dependent genes (Kim et al. 2006). Due to the inability to distinguish the REL2 short and full-length forms by RNAi, their effect on parasite infection have been difficult to elucidate (Meister et al. 2005, Luna et al. 2006). Nevertheless, the anti-*Plasmodium* effects are likely due to REL2-F processing (Meister et al. 2005).

The most striking evidence that IMD signalling is involved in directing anti-*Plasmodium* immunity was obtained by silencing *caspar*, an inhibitor of IMD signalling through DREDD-dependent cleavage of REL2-F (Kim et al. 2006). Caspar-silencing renders mosquitoes refractory to malaria parasites and it appears that activation of the IMD pathway is more efficient in limiting *P. falciparum* infection than that of the murine malaria parasite, *P. berghei* (Garver et al. 2009). However, additional work is needed to understand the precise mechanisms of REL2 activation and the contributions made by the various mosquito immune tissues to this process.

The JAK-STAT pathway - The JAK-STAT (or STAT) pathway has been the least investigated of the three major signalling pathways in mosquitoes and as such, much of our knowledge is based on JAK-STAT signalling from vertebrate or *Drosophila* model systems. In *Drosophila*, the STAT pathway regulates several aspects of development, epithelial renewal, the immune response to bacterial and viral infections and haemocyte differentiation/proliferation (Arbouzova & Zeidler 2006, Buchon et al. 2009). Similar to *Drosophila*, the mosquito JAK-STAT pathway has also been implicated in the immune response to bacteria (Barillas-Mury et al. 1999, Gupta et al. 2009), viruses (Souza-Neto et al. 2009) and *Plasmodium* parasites (Gupta et al. 2009, Bahia et al. 2011).

In *An. gambiae*, two STAT transcription factors (STAT-A and STAT-B) have been identified (Barillas-Mury et al. 1999, Gupta et al. 2009), where STAT-B is thought to regulate the transcription of the *stat-A* gene upon activation (Gupta et al. 2009). This is in contrast to *Drosophila* and other mosquito species that contain only a single STAT transcription factor (Gupta et al. 2009,

Souza-Neto et al. 2009, Bahia et al. 2011). Following immune activation, STAT is phosphorylated leading to its translocation to the nucleus and activation of downstream effector genes. Silencing of STAT leads to increased *P. berghei* and *P. falciparum* survival in *An. gambiae* (Gupta et al. 2009), as well as *P. vivax* in the Brazilian vector *Anopheles aquasalis* (Bahia et al. 2011).

STAT signalling is tightly regulated by the inhibitors suppressors of cytokine signalling (SOCS) and protein inhibitors of activated STAT, which respectively prevent STAT phosphorylation or promote degradation. Expression of SOCS is mediated by STAT activation and thus serves to shut off STAT signalling through a negative feedback loop (Gupta et al. 2009). Gupta et al. (2009) demonstrated that SOCS-silencing dramatically reduces parasite numbers and that this response is mediated by increased levels of NOS as a result of constitutive STAT activation.

Other pathways - Although the Toll, IMD and JAK-STAT pathways have been the most investigated in mosquitoes, other less characterised pathways may also contribute to the mosquito immune response to *Plasmodium*.

Recently, a lipopolysaccharide-induced TNF-α factor-like transcription factor (LL3) was described in *An. gambiae* that mediates a potent anti-*Plasmodium* immune response against both *P. berghei* and *P. falciparum* parasites (Smith et al. 2012). LL3 expression is strongly up-regulated in response to ookinete invasion of the midgut and directly influences the expression of SRPN6 (Smith et al. 2012), a serine protease inhibitor implicated in the anti-*Plasmodium* immune response (Abraham et al. 2005, Pinto et al. 2008, Eappen et al. 2013). However, because SRPN6-silencing in susceptible lines of *An. gambiae* does not impact infection intensity (Abraham et al. 2005), the large increase in oocyst numbers following LL3 knockdown likely extends beyond the regulation of SRPN6 in the mosquito anti-*Plasmodium* response. Clarification of the mechanisms of LL3 activation and the role of LL3 in the overall context of mosquito immunity are the subjects of further study.

Emerging evidence suggests that components of the ingested blood meal also affect mosquito immune function (Pakpour et al. 2013). TGF-ß1 and insulin in the ingested blood are believed to activate insulin/insulin growth factor 1 (IGF1) signalling (IIS) and MAPK signalling cascades in the mosquito thus increasing mosquito susceptibility to parasite infection (Surachetpong et al. 2009, 2011, Pakpour et al. 2012). In contrast, another blood component, human IGF1, reduces malaria parasite infection (Drexler et al. 2013). It appears that a number of factors contribute to IIS and MAPK signalling and that this delicate balance determines *Plasmodium* developmental success in the mosquito host. However, there is much more to learn on how the mosquito IIS and MAPK pathways contribute to mosquito immunity.

Recent evidence suggests that components of the IIS pathway can be manipulated in transgenic *An. stephensi* to reduce parasite infection and decrease mosquito lifespan through the over-expression of an activated Akt molecule in the mosquito midgut (Corby-Harris et al.

2010). However, these effects appear to be mediated by a disruption of mitochondrial dynamics that perturb midgut homoeostasis and are independent of IIS signalling (Luckhart et al. 2013).

Contributions of mosquito tissues to the anti-Plasmodium immune response - The immune responses that limit *Plasmodium* development are multi-faceted, involving multiple mosquito tissues that exert both individual and concerted responses over several days as the parasite journeys through the mosquito. While the midgut serves as the initial barrier and recognition site for invading ookinetes, immune responses in the fat body at the time of ookinete invasion suggest that pathogen recognition triggers a systemic humoral response (Dimopoulos et al. 1997). While cellular and humoral mosquito immunity effectively limit parasite development in the mosquito, the role of each immune tissue have been difficult to ascertain due to limitations in mosquito genetics. Thus, the contribution of each mosquito immune tissue to the overall anti-*Plasmodium* defense is an important question that has yet to be fully addressed. Once identified, it may be possible to harness the most effective responses to limit *Plasmodium* development via genetic engineering or chemical inhibitors.

Although incomplete, our current understanding of the role of each respective tissue (midgut, haemocytes, fat body) is summarised below.

Midgut - The mosquito midgut is an important component of the mosquito immune response, serving as a physical barrier to parasite development and the initial site of pathogen recognition. There is evidence that the mosquito can sense the presence of malaria parasites shortly after the ingestion of the blood meal (Vlachou et al. 2005, Dong et al. 2006), but it appears that the primary midgut immune response is triggered in response to ookinete invasion (Vlachou et al. 2005, Dong et al. 2006, Smith et al. 2012).

Several groups have identified immune components that are up-regulated in response to *Plasmodium* ookinete invasion (Vlachou et al. 2005, Dong et al. 2006, Mendes et al. 2011). However, it is unclear to what extent these immune effectors are produced in the midgut itself. It has been suggested that the increased TEP1 transcript expression in the midgut following ookinete invasion may actually be the result of increased haemocyte attachment to the midgut epithelium (haemocytes strongly express TEP1) (Blandin et al. 2004, Vlachou et al. 2005). Further experiments are needed to clarify if the increased expression of other immune effectors and antimicrobial peptides are also the result of haemocyte attachment or *de novo* synthesis in the midgut epithelium.

Parasite invasion also induces dramatic physiological changes in cells of the midgut epithelium (Han et al. 2000). NOS is up-regulated, which in turn leads to the formation of nitrites and peroxides that promote apoptosis (Han et al. 2000, Kumar et al. 2004, Kumar & Barillas-Mury 2005, Herrera-Ortiz et al. 2011). This likely produces a hostile environment for the invading ookinete that it must escape before damage to itself and before the damaged cell is extruded from the midgut

epithelium, to ensure its own survival (Han et al. 2000). While it remains unclear to what extent the "time bomb" model limits parasite development, some have suggested that this is likely a general response to remove damaged epithelial cells and is not specific to parasite invasion (Baton & Ranford-Cartwright 2005).

New evidence suggests that the process of ookinete invasion does not directly limit parasite numbers, but rather "marks" ookinetes for later destruction by components of the haemolymph (Oliveira et al. 2012). Invading ookinetes are thought to become labelled by epithelial nitration mediated by HPX2 and NADPH NOX5, thus marking the ookinete for recognition by TEP1 and ultimate lysis or melanisation (Oliveira et al. 2012). Recent work implicates the JNK pathway in the induction of HPX2 and NOX5 (Garver et al. 2013), thus modulating the levels of epithelial nitration in response to midgut invasion that is required for mosquito complement recognition and subsequent parasite destruction (Oliveira et al. 2012, Garver et al. 2013).

Importantly, ROS balance in different tissues of the mosquito, including the midgut, haemolymph and fat body, is an important determinant of parasite survival. For example, the refractory mosquito L35 strain is in a chronic state of oxidative stress when compared to the susceptible S or G3 strains, resulting in a deleterious environment that promotes parasite killing (Kumar et al. 2003). Reducing oxidative stress in refractory mosquitoes by dietary supplementation with antioxidants abrogates the ROS-mediated killing effect (Kumar et al. 2003). Silencing of the ROS detoxification enzymes, catalase or oxidation resistance 1, increase oxidative stress and greatly reduce parasite survival (Molina-Cruz et al. 2008, Jaramillo-Gutierrez et al. 2010). Taken together, evidence would suggest that the levels of ROS are tightly controlled by the mosquito to enhance parasite killing during ookinete traversal of the midgut (Kumar et al. 2003, Molina-Cruz et al. 2008).

Haemocytes - The role of haemocytes, or circulating blood cells, has been largely unexplored in the context of their contributions to mosquito innate immunity. Much like their *Drosophila* counterparts, mosquito haemocytes are believed to be the primary phagocytic cells that directly eliminate bacterial pathogens in the haemocoel (Lavine & Strand 2002). In addition, haemocytes are thought to produce several immune components found in the mosquito haemolymph, such as TEP1 (Blandin et al. 2004, Frolet et al. 2006), which mediate the immune response and pathogen clearance. However, very little is known regarding their role in anti-*Plasmodium* immunity.

Transcriptional profiling of *An. gambiae* haemocytes reveal specific responses to *Plasmodium* parasites (Baton et al. 2009, Pinto et al. 2009) and bacterial pathogens (Baton et al. 2009), suggesting that haemocytes may have an integral role in regulating mosquito innate immune responses. However, these studies did not distinguish among the three haemocyte sub-types (prohaemocytes, oenocytoids and granulocytes) thus far characterised in *Anopheles* (Castillo et al. 2006). These haemocyte populations are dynamic with changes in overall numbers and

proportions in response to age (Hillyer et al. 2005, King & Hillyer 2013), feeding status (Castillo et al. 2011) and bacterial (King & Hillyer 2013) or *Plasmodium* infection (Rodrigues et al. 2010, Ramirez et al. 2014).

Rodrigues et al. (2010) have shown that the proportion of circulating granulocytes increases in response to ookinete invasion rendering the mosquito more resistant to *Plasmodium* infection upon further challenge. Interestingly, the haemolymph of *Plasmodium*-infected mosquitoes (in the presence of midgut microbiota) contains a soluble factor that promotes haemocyte differentiation and is able to confer *Plasmodium* resistance when transferred to naïve mosquitoes (Rodrigues et al. 2010, Ramirez et al. 2014). Although the identity of this haemocyte differentiation factor is at present unknown, its production is independent of the major mosquito immune pathways (Toll, IMD, JAK-STAT) (Ramirez et al. 2014).

Several questions regarding mosquito haemocyte biology and function have yet to be addressed and is further confounded by the lack of agreement on the methodology for haemocyte isolation and analysis. However, these challenges precipitate the need for reliable cell-type specific haemocyte markers to better understand their function and identify the contributions of each cell type to anti-*Plasmodium* immunity.

Fat body - Based on *Drosophila* research, the mosquito fat body is thought to play a central role in the regulation of humoral immunity through the production of antimicrobial peptides (Lemaitre & Hoffmann 2007). However, given the systemic nature of dsRNA mediated silencing commonly used in mosquito research, the specific contributions of the fat body to immune signalling require further examination.

Transcriptional analysis of the mosquito carcass (that includes the fat body) reveals significant changes in response to a blood meal, as well as specific transcriptional profiles characteristic of rodent and human malaria parasites (Dong et al. 2006).

Additional evidence also suggests that two major nutrient transporters, lipophorin and vitellogenin, produced by the fat body during the process of vitellogenesis (or egg production), also influence parasite survival (Rono et al. 2010). Silencing of lipophorin reduces *Plasmodium* oocyst size and impairs development, while loss of vitellogenin also results in reduced parasite numbers. These results suggest that developing parasites may be able to capture nutrients circulating in the mosquito haemolymph to facilitate its own development. Moreover, it has been suggested that these proteins may protect *Plasmodium* against non-self recognition by the mosquito complement-like cascade (Rono et al. 2010).

Mechanisms of immune evasion - There has been significant evolutionary pressure on malaria parasites to adapt to their mosquito host to ensure their survival and subsequent transmission to a new host. As a result, malaria parasites have evolved mechanisms to evade the mosquito immune response in their natural mosquito vectors, a process that may be specific to different geographical regions. Collins et al. (1986) found that African *P. falciparum* parasites strains were significantly

more proficient in evading the melanisation response of refractory *An. gambiae* than those of New World or Asian origin.

More recently, light has been cast on the mechanism for this unique phenotype by determining that African strains of *P. falciparum* can evade TEP1-mediated lysis by the mosquito complement-like system of refractory *An. gambiae* mosquitoes, while Brazilian 7G8 *P. falciparum* isolates are efficiently targeted and melanised by this system (Molina-Cruz et al. 2012). Through quantitative trait *locus* analysis of genetic crosses between the African and Brazilian parasite strains, Molina-Cruz et al. (2013) identified *Pfs47* as the candidate gene mediating this process. Present on the ookinete surface, *Pfs47* is thought to prevent the induction of the mosquito midgut nitration responses that mark parasites for TEP1-mediated lysis by a yet unknown mechanism. The highly polymorphic nature of *Pfs47* and its geographic distribution suggest that *P. falciparum* parasites have adapted to different *Anopheles* mosquitoes to ensure their survival (Molina-Cruz et al. 2013).

While details of host-parasite co-evolution are only beginning to emerge, these studies highlight the ability of the parasite to adapt to its vector host to ensure its survival. Mosquito factors have also been described that may protect parasites from immune recognition (Osta et al. 2004) or have been co-opted by parasites to facilitate invasion (Rodrigues et al. 2012). However, the precise role of these components, and possibly others, requires future investigation.

Intervention strategies - The bottleneck in malaria parasite numbers within its mosquito host (Fig. 1) argues that the midgut is an optimal target for intervention strategies to prevent malaria transmission.

For many years, a large effort has been invested into the identification of TB vaccine antigens that target the sexual stages of the parasite in the mosquito midgut, including Pfs230, Pbs48/45, HAP2, Pfs25 and PfCHT1 [reviewed by Pradel (2007) and Blagborough and Sinden (2009)]. In addition, it has also been proposed that antibodies targeting mosquito components may also serve as promising TB targets (Dinglasan & Jacobs-Lorena 2008). One major advantage of this strategy is that using a conserved mosquito TB vaccine target could be applied for all anopheline mosquitoes, thus obviating the need to develop specific targets for each parasite-host combination. Proof-of-principle experiments targeting mosquito midgut ligands involved in ookinete invasion (Dinglasan et al. 2007a) or that regulate mosquito immunity (Williams et al. 2013) have been explored.

Transgenic technology has also been proposed to genetically modify mosquito vectors to render them incapable of malaria parasite transmission. Through the production of synthetic peptides, effector proteins or methods to increase the mosquito immune response, several approaches have been used to confer anti-*Plasmodium* resistance in laboratory and natural host-pathogen combinations [reviewed by Wang and Jacobs-Lorena (2013)]. Although these experiments are promising, significant hurdles remain regarding release strategies and required drive-mechanisms to ensure that these resistant mosquitoes can spread into wild mosquito populations.

Wolbachia is an endosymbiont commonly found in arthropods and can spread into populations by transmission through the germ line (Werren et al. 2008). A surprising and important discovery was that when *Aedes aegypti* harbour *Wolbachia* it becomes resistant to viral and *Plasmodium* infections (Moreira et al. 2009). In *An. gambiae*, somatic, non-heritable *Wolbachia* infections were shown to also confer similar anti-*Plasmodium* properties (Kambris et al. 2010, Hughes et al. 2011). Recently, stable maternally-inherited *Wolbachia* was introduced into *An. stephensi* and was found to limit *P. falciparum* development, as well as confers cytoplasmic incompatibility (Bian et al. 2013). However, *Wolbachia* imposed a significant fitness load to these mosquitoes. While promising, further challenges remain regarding the mechanism by which *Wolbachia* confers anti-*Plasmodium* immunity, as well as its introduction into other anopheline vectors.

An alternative approach has recently been described involving the engineering of the mosquito microbiota to secrete antimalarial effector genes, an approach known as paratransgenesis (Wang et al. 2012). Genetically engineered *Pantoea agglomerans*, a common resident of the mosquito midgut, were able to confer resistance to *P. falciparum* and *P. berghei* and dramatically reduce infection prevalence independent of the anopheline vector (Wang et al. 2012). Future research must address issues of how to introduce the modified bacteria into field populations and, importantly, resolve issues relating to the release of genetically modified organisms in nature.

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