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Host Cell Tropism and Adaptation of Blood-Stage Malaria Parasites: Challenges for Malaria Elimination

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Plasmodium falciparum and *Plasmodium vivax* account for most of the mortality and morbidity associated with malaria in humans. Research and control efforts have focused on infections caused by *P. falciparum* and *P. vivax*, but have neglected other malaria parasite species that infect humans. Additionally, many related malaria parasite species infect nonhuman primates (NHPs), and have the potential for transmission to humans. For malaria elimination, the varied and specific challenges of all of these *Plasmodium* species will need to be considered. Recent advances in molecular genetics and genomics have increased our knowledge of the prevalence and existing diversity of the human and NHP *Plasmodium* species. We are beginning to identify the extent of the reservoirs of each parasite species in humans and NHPs, revealing their origins as well as potential for adaptation in humans. Here, we focus on the red blood cell stage of human infection and the host cell tropism of each human *Plasmodium* species. Determinants of tropism are unique among malaria parasite species, presenting a complex challenge for malaria elimination.

More than 60% of known infectious organisms are zoonotic, and they account for 75% of emerging human diseases (Taylor et al. 2001; Jones et al. 2008). The predominant species of malaria parasites infecting humans, *Plasmodium falciparum* and *Plasmodium vivax*, are anthroponotic in human populations; however, these species also originated from a transmission event from African great apes to humans (Liu et al. 2010, 2014). In addition, four other malaria parasite species from the genus *Plasmodium* infect humans: *Plasmodium malariae*, *Plasmodium ovale curtisi*, *Plasmodium ovale wallikeri*, thought to be transmitted within humans, and *Plasmodium knowlesi*, a zoonosis of humans from macaque monkeys. All *Plasmodium* species are characterized by a complex life cycle with several stages of differentiation through its anopheline mosquito vector and the vertebrate host. In humans, following a primary stage of infection and multiplication in the liver, parasites are released into the bloodstream. The clinical symptoms of malaria are associated with the blood stage, when parasites proliferate asexually by invasion of red blood cells (RBCs), replication, egress from the infected cell, and reinvasion of an uninfected cell in a cyclical fashion. A subset of parasites can leave this asexual cycle to develop into sexual forms known as gametocytes, which are taken up by mosquitoes,

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in which sexual recombination and development occur to form parasites that can be reintroduced into the host, completing the life cycle.

In contrast to *Plasmodium* species that infect lizards and birds, the host range for primate *Plasmodium* species was thought to be highly restricted with only rare instances of zoonotic transmission. Recently, however, this dogma of strict host tropism in nature has been challenged, particularly with the emergence of *P. knowlesi* in the human population. Restrictions to infection of the host can occur at many points throughout the life cycle of *Plasmodium* parasites. Because host and vector habitat and behavior are difficult to study in natural settings, host–cell tropism at the RBC invasion step has been the most studied at the molecular and cellular level. Furthermore, with advances in molecular genomic and genetic tools, we now have a greater understanding of parasite species diversity present also in animal host populations. This increased knowledge raises several questions and concerns as the research agenda shifts toward the eradication of malaria. How large and deep is the repertoire of existing *Plasmodium* species? Is the range of *Plasmodium* species to which humans are susceptible fully known? Is the zoonotic reservoir significant today as a means of transmission?

Here, we review the molecular determinants at the RBC invasion step that regulate host-cell tropism, discussing how these factors may influence the ability of *Plasmodium* parasites to breach species barriers and expand host range, impeding efforts to eliminate malaria.

MOLECULAR MEDIATORS OF RBC INVASION AND TROPISM

For successful RBC invasion, an extracellular *Plasmodium* parasite must initiate and execute a complex, well-ordered series of molecular interactions with the plasma membrane surface of the host cell. At each step of invasion, parasite proteins or invasion ligands bind to native receptors on the RBC surface or secreted parasite receptors (Cowman et al. 2012). The two superfamilies of *Plasmodium* invasion ligands that mediate the most specific interactions with receptors on the RBC surface, and therefore thought to be primary determinants of tropism, are the Duffy binding-protein ligand (DBL) family and the reticulocyte-binding protein homolog (RBL) family (Fig. 1) (Cowman and Crabb 2006; Tham et al. 2012; Wright and Rayner 2014; Paul et al. 2015).

The DBL invasion ligands all contain a well-characterized Duffy-binding-like receptor-binding domain, named for the founding members of this family—the *P. vivax* Duffy-binding protein (PvDBP) (Wertheimer and Barnwell 1989) and the orthologous *P. knowlesi* Duffy-binding protein α (PkDBPα) (Haynes et al. 1988). The interaction of PvDBP or PkDBPα with the RBC Duffy antigen receptor for chemokines (DARC) is the major determinant of human infection by these *Plasmodium* species (Miller et al. 1975; Singh et al. 2005). *P. falciparum* has an even larger repertoire of DBL invasion ligands, all of which use sialic acid–containing receptors on the human RBC surface to mediate invasion. At least one of these invasion ligands, PfEBA-175 has previously been implicated in host tropism (Martin et al. 2005).

The *P. vivax* proteins PvRBP1 and PvRBP2, for which the RBL family is named, are thought to restrict *P. vivax* to reticulocytes by virtue of their specific binding to reticulocytes (Galinski et al. 1992). Despite the kinship between the two species, the RBL ligands in *P. knowlesi*, PkNBPXa and PkNBPXb, are strongly divergent from their *P. vivax* counterparts (Mayer et al. 2009); their role in human infection is not known. In *P. falciparum,* PfRh proteins have been shown to underlie preferences for specific receptor repertoires (PfRh4; Stubbs et al. 2005; Gaur et al. 2006), as well as preference of RBCs from different host species (Rh5; Hayton et al. 2008, 2013).

A more detailed review of factors involved in invasion of host RBCs by the parasites can be found in Singh and Chitnis (2016).

Plasmodium knowlesi: AN ESTABLISHED ZOONOSIS

A Zoonosis Happening Today

P. knowlesi is currently the only clearly zoonotic malaria parasite. Although the first natural case

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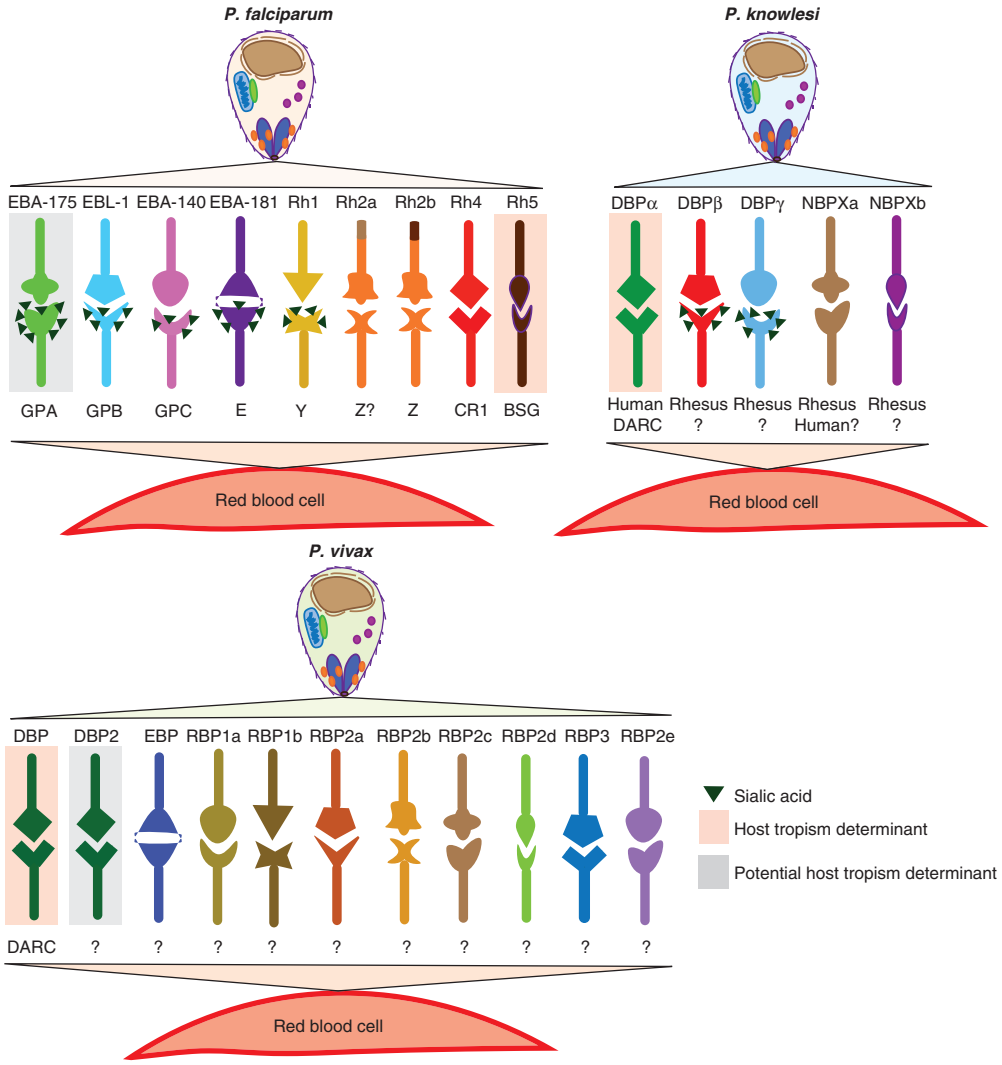

Figure 1. Specific ligand–receptor interactions mediate *Plasmodium* invasion of red blood cells (RBCs) that can determine host tropism. Parasite invasion ligands and their cognate RBC receptors are shown for *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium knowlesi*. A question mark or letter indicates receptors that are yet to be identified. Orange shading highlights known host tropism determinants, whereas grey shading indicates potential host tropism determinants. The triangles on some RBC receptors denote sialic acid residues. For *P. vivax*, DBP2 represents the duplicated DBP that has been implicated in *P. vivax* invasion of Duffy antigen receptor for chemokines (DARC)-negative individuals (Ménard et al. 2013). The expanded RBP family identified through whole-genome sequencing (WGS) of the *P. vivax* SalI reference strain (Carlton et al. 2008) comprises RBP1a, RBP1b, RBP2a, RBP2b, RBP2c, RBP2d, and RBP3, whereas EBP and RBP2e represent the predicted Duffy-binding protein ligand (DBL) and reticulocyte-binding protein homolog (RBL) orthologs identified through WGS of a *P. vivax* field isolate (Hester et al. 2013). *P. knowlesi* DBPα-DARC invasion pathway functions in invasion of macaque RBCs; however, it does not appear to be a tropism determinant for the macaque host population. NBPXa has been shown to bind human RBCs in vitro, but its role in invasion of human RBCs remains to be defined.

of *P. knowlesi* malaria was reported as early as 1965 (Chin et al. 1965), it was only in 2004 that Singh et al. conclusively showed that the majority, if not all, of cases diagnosed as *P. malariae* on the basis of morphology in the Kapit division of Malaysia from 2000 to 2002 were in fact *P. knowlesi* (Singh et al. 2004). A follow-up study examined archival blood films from 1996 and confirmed that misdiagnosis of *P. knowlesi* for *P. malariae* had been common earlier than realized. The investigators also speculated that an earlier survey conducted in 1952 may also have misidentified *P. knowlesi* cases as *P. malariae*, challenging the idea that it is a newly emerging zoonosis (Lee et al. 2009). Since then, cases have been reported in various regions of Southeast Asia and *P. knowlesi* is now the leading cause of malaria in some parts of Malaysia (Singh and Daneshvar 2013). Although there is significant evidence suggesting that most transmission is zoonotic (Singh et al. 2004; Daneshvar et al. 2009), a recent study reports cases, albeit limited, of infection within families, without notable interaction with potential zoonotic hosts (Barber et al. 2012). Intriguingly, another recent study suggests that the prevalence may be higher than previously thought, with a potentially large asymptomatic population harboring the parasite (Fornace et al. 2015). In any case, human encroachment on macaque habitats (Cox Singh and Culleton 2015) will only increase the opportunity for the parasite to evolve and adapt to humans. To date, there is little evidence of direct transmission among humans (Singh et al. 2004; Daneshvar et al. 2009).

P. knowlesi Diversity

Considerable diversity has been found within *P. knowlesi* strains isolated in human populations. Parasitemia and disease severity in human patients were found to be associated with a specific allele of the PkNBPXb invasion ligand (Ahmed et al. 2014). A subsequent whole-genome study found dimorphism in the natural *P. knowlesi* population (Pinheiro et al. 2015). A larger study soon supported the possibility of two distinct *P. knowlesi* populations in circulation (Assefa et al. 2015; Divis et al. 2015). These studies, comprising isolates from two natural macaque hosts (long-tailed and pig-tailed macaques), as well as humans, showed that there are two distinct sympatric clusters of *P. knowlesi* matching to the two natural macaque hosts. The genetic diversity between these clusters was great enough to suspect subspeciation. Strikingly, although interaction between the clusters is likely limited because of different geographical and ecological niches of the macaque hosts, both of the clusters are found in humans. Introgression between subspecies provides opportunities for a parasite to adapt to a new environment and potentially new host organisms. The possibility of hybridization in the human host was speculated, based on genetic mosaicism observed across the genome. Further, through genomic analyses of human isolates several genes were found to be under strong positive selection in the human population. These data indicate the potential for *P. knowlesi* to adapt and evolve within the human population.

Adaptation of *P. knowlesi* to Human RBCs

Consistent with *P. knowlesi* being a zoonosis of humans derived by transmission from macaque monkeys, in vitro culture of *P. knowlesi* has always been performed in macaque blood, because the low replication rate in human blood has impeded continuous propagation (Kocken et al. 2002). Interestingly, it was found that *P. knowlesi* parasites used in malaria therapy of neurosyphilis human patients resulted in high parasitemia infections and pathogenesis with increased passage through humans, suggesting an adaptation toward virulence (van Rooyen and Pile 1935; Ciuca et al. 1955). Recently, both our group and another group successfully obtained *P. knowlesi* lines adapted to grow efficiently in human RBCs (Lim et al. 2013; Moon et al. 2013). In both cases, the parental *P. knowlesi* H strain was in culture for an extended period of time in a mixture of human and macaque RBCs, until it was able to be maintained in purely human RBCs. The increased invasion efficiency observed in the human-adapted lines remains reliant on the PkDBPα–DARC interaction (Moon et al. 2013). Interest-

ingly, we found that although the parental strain showed a strong preference for the very young fraction of circulating human RBCs, the human-adapted line had circumvented this specific tropism, permitting invasion of an expanded pool of RBCs of varying age (Lim et al. 2013). Whether this mechanism mirrors the natural mode of adaptation in the field is yet to be determined. Increased numbers of human infections, particularly those of high density, may serve as a sentinel of increased adaptation of *P. knowlesi* to the human population.

P. vivax: STRENGTH IN DIVERSITY

Diversity and Origin of *P. vivax*

Despite being the most widely distributed of the human malaria parasites, *P. vivax* has long been considered benign and has not received as much attention as the demonstrably lethal *P. falciparum*. It is becoming apparent that *P. vivax* parasites are considerably more diverse than *P. falciparum* (Rosenberg et al. 1989; Qari et al. 1991, 1992; Cui et al. 2003; Neafsey et al. 2012; Carlton et al. 2013). In a recent study, next-generation sequencing of four geographically distinct *P. vivax* isolates (Neafsey et al. 2012) revealed a high rate of single-nucleotide polymorphisms (SNPs). *P. vivax* therefore has a larger effective population size compared with *P. falciparum* that has not gone through a recent bottleneck or drug-driven sweep in selection.

Analysis of a growing number of samples from different geographical locations has also led to the discovery of "*P. vivax*–like" parasites such as *Plasmodium simium* (Coatney et al. 1971; Costa et al. 2014). *P. simium*, found in New World monkeys, is considered to be morphologically and genetically indistinguishable from *P. vivax* (Collins et al. 1969; Coatney et al. 1971; Deane 1988). A recent study showed that wild monkeys infected with *P. simium* showed high levels of seropositivity against *P. vivax* antigens (Camargos Costa et al. 2015). Sequencing of the PsDBP gene revealed only four polymorphic sites compared with PvDBP, highlighting the remarkable similarity between *P. vivax* and *P. simium* and suggesting a potentially large sylvatic reservoir for *P. vivax* or *P. vivax*–like parasites.

Two other *Plasmodium* species closely related to *P. vivax* have also been identified—*Plasmodium cynomolgi* and *Plasmodium simiovale*. Antibodies against the circumsporozoite protein of *P. simiovale* were detected in human population studies (Qari et al. 1993; Udhayakumar et al. 1994; Marrelli et al. 1998); however, an independent study could not confirm the presence of *P. simiovale* in the human population (Gopinath et al. 1994) and experimental infection of humans with *P. simiovale* in an early study was not successful (Dissanaike 1965). A recent analysis of the sequence of merozoite surface protein 9 (MSP-9) from diverse *Plasmodium* species suggests that *P. simiovale* and the macaque parasite, *Plasmodium fieldi*, form a clade, whereas *P. vivax* and *P. cynomolgi* form another (Chenet et al. 2013), arguing for a more in-depth phylogenetic analysis of this species. Interestingly, a case of *P. cynomolgi* human infection has been reported recently in Malaysia, showing the biological ability of this parasite to naturally infect humans (Ta et al. 2014).

Where does *P. vivax* originally come from? The position of *P. vivax* within a clade of related parasites that includes *P. cynomolgi*, which infects Asian primates, led to the widely held view that *P. vivax* originated in Asia. This theory, however, was at odds with the near-fixation of the DARC-negative allele in sub-Saharan Africa, which largely confers protection against *P. vivax* (Young et al. 1955; Miller et al. 1975). Several recent studies have identified *P. vivax*–like parasites in African great apes (Kaiser et al. 2010; Krief et al. 2010; Prugnolle et al. 2010). A large-scale study by Liu et al. (2014) found a much greater diversity of *P. vivax* in African great apes than found within the human population. The extant African ape reservoir of *P. vivax* likely descended from an ancient parasite pool, which served as a source for a single zoonotic transfer that has given rise to modern human *P. vivax*. Interestingly, the ape *P. vivax*–like parasites can infect both gorillas and chimpanzees alike, suggesting frequent transmission between these species. The reduced diversity of extant human *P. vivax* likely results from a bottlenecked line-

age that spread from Africa, where, thereafter, it became severely restricted within Africa as DARC negativity spread among the human population.

The PvDBP–DARC Interaction: Indispensable for *P. vivax?*

Three invasion ligands had been identified for *P. vivax* (PvDBP, PvRBP1, and PvRBP2) (Wertheimer and Barnwell 1989; Galinski et al. 1992) before the assembly of the full genome, which thereafter revealed the presence of several more members of the RBL family (Fig. 1) (Carlton et al. 2008). The interaction of PvDBP with the DARC receptor, however, had been shown to be essential for human infection (Miller et al. 1975; Wertheimer and Barnwell 1989).

De novo assembly of additional *P. vivax* isolates revealed that the SalI reference strain, which had been extensively passaged in vivo in monkey models, was missing several large genomic regions including loci for putative invasion ligands of both the RBL and DBL family (Hester et al. 2013). In addition, a duplication of PvDBP (Ménard et al. 2013) was observed in several field isolates, an apparently recent event, based on the similarity of the two DBP loci. In any case, *P. vivax* possesses a large set of invasion ligands that could be used to confer phenotypic diversity.

A number of reports have recently documented *P. vivax* infection in several DARC-negative individuals (Ménard et al. 2010a,b; Woldearegai et al. 2013). Additionally, cases of high parasitemia and severe disease caused by *P. vivax* have been increasing over the past decade (White et al. 2014). These discoveries suggest a diversity in the ability of *P. vivax* isolates to infect human RBCs.

A Hidden Pool of *P. vivax* in Plain Sight?

P. vivax can consistently be detected in the few DARC-positive individuals that are surveyed in largely DARC-negative populations (Culleton and Carter 2012). DARC-negative individuals also show significant exposure in populations of almost exclusive DARC negativity. Strikingly, samples collected by passive case detection from 13% of individuals attending a clinic in the Republic of Congo had antibodies to the preerythrocytic stage of *P. vivax* (Culleton et al. 2009). These studies support the hypothesis that there is ongoing transmission of *P. vivax* in sub-Saharan Africa, although the extent of this "transmission" has not been fully assessed. It has been suggested that the small population of DARC-positive individuals (<5%) may be sufficient to sustain this highly transmissible species. Further, it is possible that there exists a large reservoir of *P. vivax* in humans with a very low circulating parasitemia, which, nevertheless, can be transmitted to humans. The growing number of *P. vivax* cases in DARC-negative individuals adds yet another challenge. The discovery of *P. vivax* in gorillas and chimpanzees now provides the additional complexity of an animal reservoir. In fact, an expanded NHP reservoir could exist in many geographical areas as *P. vivax* and related species have also been found in various South American monkeys, including howler monkeys (Costa et al. 2014). A human traveler from the Central African Republic infected with ape *P. vivax* (Prugnolle et al. 2013), the lone case of a modern zoonotic transfer of this parasite species, suggests that if interaction between the human and NHP populations increases, risk of animal-borne *P. vivax* may increase in the human population.

Challenges for Establishing a *P. vivax* Blood-Stage Vaccine

Efforts to develop preventative measures against malaria have been heavily skewed toward *P. falciparum* (discussed later in this review) (Reyes-Sandoval 2013). It is becoming apparent that this lag in research in *P. vivax* control and prevention will be a major and persistent challenge in our goal toward malaria elimination (WHO 2015). The search for an ideal blood-stage vaccine target has centered on PvDBP because of its necessity in invading human RBCs. Antibodies raised against the binding region of PvDBP, called region II (RII), have been shown to be efficient at inhibiting the binding of PvDBP to DARC (Chitnis and Sharma 2008).

PvRII has also shown promise in preclinical trials in animal models (Mueller et al. 2009). However, the high diversity among *P. vivax* strains and in the PvDBP alleles has led to conflicting reports on whether a PvRII-based vaccine would confer protection against all strains (de Sousa et al. 2014). Although there are a few more antigens under consideration as blood-stage vaccine targets (such as merozoite surface protein 1 [MSP-1] and apical membrane antigen 1 [AMA-1]), there is clearly a need to screen a larger set of antigens to find a more suitable one (Valencia et al. 2011). In addition, as the community decides how and where to concentrate vaccine development resources, several unique features of *P. vivax* should be considered, such as its higher transmissibility compared with *P. falciparum* (Brown et al. 2009) and its tendency to cause relapse in patients as a result of the elusive hypnozoite stage. Other life-cycle stages such as the preerythrocytic or transmission stages may thus be better to target in developing an efficient vaccine for *P. vivax*; indeed, efforts to interrupt these stages have been more successful (Reyes-Sandoval 2013).

P. falciparum: THE MALIGNANT MALARIA

Origin of *P. falciparum*

The origin of the deadly *P. falciparum* has been the subject of intense study. Until recently, the only identified parasite species closely related to *P. falciparum* in the *Laverania* sublineage was the chimpanzee parasite *Plasmodium reichenowi*. There were various hypotheses offered about the origin of *P. falciparum* with genetic evidence suggesting that it arose from *P. reichenowi*, likely by a single host transfer from chimpanzees to humans (Rich et al. 2009). Subsequent studies identified two other *P. falciparum*–related chimpanzee parasites *Plasmodium gaboni* (Ollomo et al. 2009) and *Plasmodium billcollinsi* (Krief et al. 2010). Nevertheless, seminal work by Liu et al. (2010), revealed that *P. falciparum* originated from a single host transfer from gorillas and not chimpanzees. *P. falciparum* was found to be most closely related to a *Plasmodium* species isolated from Western gorillas, later termed *Plasmodium praefalciparum* (Rayner et al. 2011). This study, which relied on amplification of mitochondrial sequences from single copies of *Plasmodium* genomes from ape fecal samples, also revealed a diversity of closely related *Laverania* species displaying specificity for either gorilla or chimpanzee hosts (Fig. 2).

The potential for closely related great ape parasites such as *P. praefalciparum* to infect humans does not presently appear to be significant (Sundararaman et al. 2013; Délicat-Loembet et al. 2015), with *P. falciparum* clearly emerging from a single ancient transmission event. Conversely, *P. falciparum* has been found to infect bonobos and gorillas as an anthroponosis, which raises concerns about potential animal reservoirs of *P. falciparum*. However, most of the *P. falciparum*–infected great apes were captive and living in close proximity to humans (Krief et al. 2010; Prugnolle et al. 2010), suggesting that such a threat is low and will not undermine elimination efforts, as long as humans continue to live apart from great apes or treat captive apes with antimalarials.

Diversity in Invasion Ligands

A comparison of the *P. reichenowi* and *P. falciparum* genome sequences implicates RBL and DBL invasion ligands as factors associated with adaptation to a host species. Although the two genomes are highly similar, the loci for several orthologous invasion ligands in the two species are extensively differentiated between species, as evidenced by pseudogenization, disruptions in gene synteny, and sequence divergence (Otto et al. 2014). Variant expression of invasion ligands in laboratory-adapted and field strains has shown the differential usage of ligand–receptor interactions between *P. falciparum* isolates for the invasion of RBCs (Reed et al. 2000; Duraisingh et al. 2003a,b; Nery et al. 2006; Bei et al. 2007; Jennings et al. 2007; Gomez-Escobar et al. 2010). The use of diverse ligand–receptor pairs is thought to be both a mechanism of immune evasion and a means to invade diverse RBC subtypes within an individ-

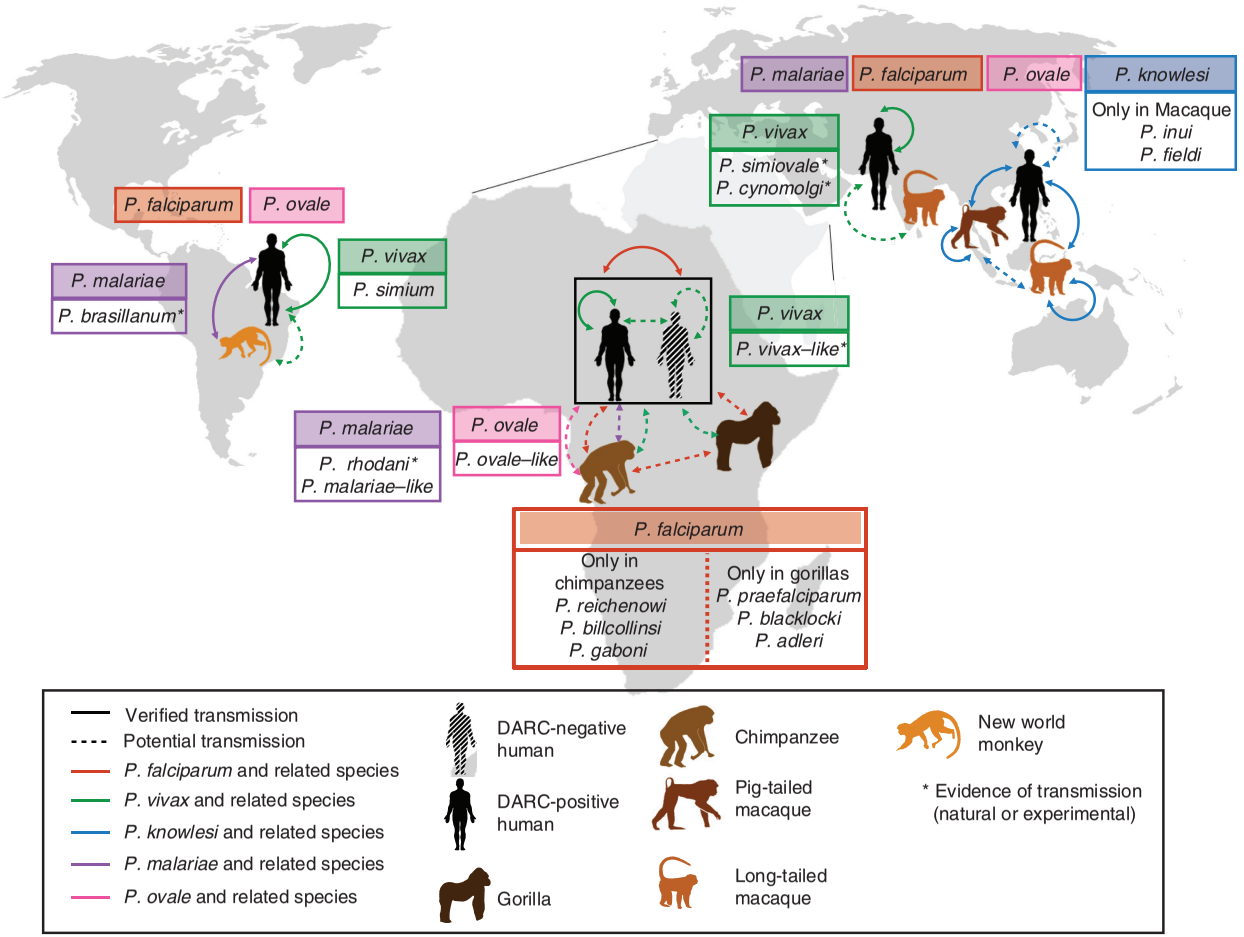

Figure 2. Global distribution and transmission of *Plasmodium* species in human and animal populations. *Plasmodium falciparum* (red), *Plasmodium vivax* (green), *Plasmodium malariae* (purple) and the two species of *Plasmodium ovale* (pink) can be found in all malaria-endemic areas to varying extents. Additionally, *Plasmodium knowlesi* (blue) is found in Southeast Asia. Arrows between the different hosts indicate established transmission of the different parasite species within the human population, and/or between or from nonhuman primates. The dotted arrows show potential transmission of parasites to look out for, based on population surveys. All arrows are similarly color-coded. *Plasmodium* species related to the main human species are in boxes in corresponding colors and species for which cases of human infection have been observed (experimentally or naturally) are marked with an asterisk. DARC, Duffy antigen receptor for chemokines.

ual, between individuals, and even potentially between host species.

Sialic Acid and Host Specificity

The sialic acid on RBCs appears to be key to the binding of many invasion ligands of *P. falciparum*, including the DBL invasion ligands, PfEBA-175, PfEBL-1, PfEBA-140, and PfEBA-181, and the RBL invasion ligand Rh1 (Orlandi et al. 1992; Gilberger 2003; Lobo 2003; Maier et al. 2003; Triglia et al. 2005; Mayer et al. 2009). Some strains are highly dependent on the presence of sialic acid for successful invasion, whereas other strains can invade in a sialic acid–independent fashion (Stubbs et al. 2005; Gaur et al. 2006). Humans express only the *N*-acetylneuraminic acid (Neu5Ac) form of sialic acid, because of an inactivating mutation in the enzyme cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (CMAH), which otherwise converts Neu5Ac to *N*-glycolylneuraminic acid (Neu5Gc) (Chou et al. 1998; Irie et al. 1998; Hayakawa et al. 2001). In contrast to humans, all great apes have an intact *CMAH* gene and express a high degree of Neu5Gc on their RBC surface (Muchmore et al. 1998).

It has been postulated that this chemical difference in sialic acid between humans and other great apes might influence the specificity of *Laverania* parasites for their hosts. Chimpanzees can be experimentally infected with *P. falciparum* (Blacklock and Adler 1922; Daubersies et al. 2000); however, natural infections of wild-living chimpanzees have not been observed (Liu et al. 2010). Evidence to date suggests that *P. reichenowi* does not infect humans (Blacklock and Adler 1922; Bruce-Chwatt et al. 1970). One study showed that PfEBA-175 and *P. reichenowi* EBA-175 preferentially bind to RBCs of their own host species and erythroid-like cells expressing the host-specific sialic acid (Martin et al. 2005). The investigators further showed that *Aotus* monkeys, which serve as model organisms for *P. falciparum* (Herrera et al. 2002) express Neu5Ac, potentially explaining their susceptibility to *P. falciparum* infection. However, it was subsequently observed that PfEBA-175 binds to both Neu5Ac and Neu5Gc (Wanaguru et al. 2013), contradicting previous findings (Martin et al. 2005), but potentially providing an explanation for the ability of Neu5Gc to potently inhibit binding of PfEBA-175 to human RBCs (Orlandi et al. 1992). Indeed, homologs of EBA-175 from *P. reichenowi* and *P. billcollinsi*, another chimpanzee parasite, bind human RBCs as well as human glycophorin A with similar affinities, suggesting that perhaps EBA-175 is not a major tropism determinant for these species (Wanaguru et al. 2013).

PfRh5 as a Host Restriction Factor

Of the RBL and DBL invasion ligands, PfRh5 is the only established invasion ligand that has been found to be essential for RBC invasion by all *P. falciparum* strains tested to date (Crosnier et al. 2011). Interestingly, polymorphisms in this molecule are associated with invasion into *Aotus* RBCs, through mapping using the progeny of a genetic cross (Hayton et al. 2008, 2013). Subsequently, it was found that PfRh5 binds chimpanzee and gorilla basigin (BSG) at much reduced levels compared with human BSG, suggesting that the molecule might be critical in defining the specificity of *P. falciparum* for human RBCs. Specific amino acid residues in BSG were identified that contribute to recognition of human BSG by PfRh5. Notably, two of these residues, F27 and K191, were identified as targets of positive selection in a study using population genetics and phylogenetics (Forni et al. 2015), providing further evidence that this key receptor is under selection pressure both within the human lineage and during NHP evolution.

Targeting the Tropism Ligands of *P. falciparum* for Vaccine Development

Sterile immunity to *P. falciparum* infection—the ultimate goal of a malaria vaccine—does not occur in naturally exposed human populations. Instead, individuals acquire partial immunity with age (Persson et al. 2008; Badiane et al. 2013), likely a result of continual exposure to *Plasmodium* infections and gradual acquisi-

tion of antibodies against parasite antigens, including many invasion ligands. The merozoite invasion ligands have been proposed as vaccine candidates. However, inclusion of multiple antigens in an invasion-blocking vaccine would be necessary to effectively counter the ability of *P. falciparum* to use different invasion pathways and overcome sequence polymorphism of invasion ligands (Nery et al. 2006; Bowyer et al. 2015; Mensah-Brown et al. 2015). Many studies have reported the presence of invasion-inhibitory antibodies acquired toward *P. falciparum* DBL ligands, (PfEBA-175, PfEBA-140, PfEBA-181) and RBL ligands (PfRh2 and PfRh4) (Ford et al. 2007; Persson et al. 2008; Reiling et al. 2010; Reiling et al. 2012; Badiane et al. 2013). Recent studies have shown that simultaneous blockade of multiple invasion ligand–receptor interactions can synergistically inhibit invasion (Lopaticki et al. 2011; Williams et al. 2012; Pandey et al. 2013), showing the potential of such a vaccine strategy. Recently, several in vitro–based culture studies have shown the strong potential of the essential invasion ligand PfRh5 as an antigenic target for inhibition (Douglas et al. 2011, 2014; Patel et al. 2013; Reddy et al. 2014). Additionally, administration of a PfRh5-based experimental vaccine blocks *P. falciparum* infection in *Aotus* monkeys following parasite inoculation (Douglas et al. 2015). It is possible that a major challenge to elimination of *P. falciparum* by vaccine strategies targeting invasion ligands is the polymorphism and redundancy that might allow the parasites to persist in reservoirs such as young RBCs that can be invaded using hitherto unidentified ligand–receptor interactions.

THE OTHER *PLASMODIA*: AN UNEXPECTED DIVERSITY

Although *P. ovale* and *P. malariae* are understudied relative to other human malaria parasites, they contribute significantly to the global malaria burden. The distribution of *P. ovale* is thought to be limited to some tropical areas in Africa, New Guinea, and parts of the Philippines and Indonesia (Mueller et al. 2007). Its global burden may, however, be an underestimation, as *P. ovale* presents with low parasitemia and is easy to miss or misdiagnose as the morphologically similar and more prevalent *P. vivax*. Diagnosis became more sensitive and accurate with the development of a species-specific polymerase chain reaction (PCR) method (Snounou et al. 1993). However, some cases identified as *P. ovale* by light microscopy could not be detected by this method because of strong genetic variation (Tachibana et al. 2002; Win et al. 2004; Calderaro et al. 2007) among analyzed samples. It took several years after these observations for Sutherland et al. (2010) to show that the "classic" and "variant" types of *P. ovale* are in fact two distinct subspecies that are nonrecombining but sympatric in endemic regions.

Similarly, variant forms of *P. malariae,* not detectable through the standard species-specific PCR, have been observed in distinct endemic regions, such as China and Southeast Asia (Kawamoto et al. 1999). The same variant sequence was found in distinct geographical regions, indicating the presence of a stable and common form of *P. malariae*. Further investigation will determine whether this is another existing or ongoing speciation event.

Both *P. ovale*–like and *P. malariae*–like species have been detected in African great apes, albeit at a much lower frequency than the *Laverania* clade, in the study conducted by Liu and colleagues (2010). This discovery warrants further investigation to determine whether these neglected species are also more widely prevalent than assumed. In addition, historical observations suggest that *P. malariae* may be able to infect a larger range of host species; *P. brasilianum,* a parasite in South American monkeys and *P. rhodaini*, found in African chimpanzees are morphologically similar to *P. malariae* and show similar disease progression. They can both be transmitted to humans experimentally, and it has been suggested that this species might indeed be *P. malariae* (Coatney 1968; Rayner 2015). Genetic analysis now suggests that *P. malariae* can infect Old and New World monkeys as well as humans, presenting a formidable zoonotic reservoir for *P. malariae* (Lalremruata et al. 2015).

PERSPECTIVES: WHAT WILL WE NEED TO REACH ELIMINATION?

Defining the Extent of Zoonotic Reservoirs: Continuous Surveying and Sampling

Emergence of new zoonoses would rely on a number of criteria being favorable, including the probability of contact between human and animal host, shared mosquito vectors, and RBC-stage infectivity. This is well-discussed in a review, in which J.K. Baird, writing before the discovery of multiple *Laverania* species, assesses the risk of 18 non-*Laverania* NHP species and one *Laverania* species found in different geographic regions to cause human infection (Baird 2009). He concludes that only three species have great potential to be zoonotic—*P. knowlesi*, which is already well established as a human parasite, *P. cynomolgi*, for which there has been one report of a natural human infection (Ta et al. 2014), and *Plasmodium inui*, which is often found in the same natural hosts and vectors as *P. knowlesi*.

Today, our knowledge of the extent to which we are exposed to *Plasmodium* species has come primarily from direct surveys of individuals, animal hosts, and mosquito vectors in malaria-endemic regions. Modern-day efforts to determine the bounds of the *P. knowlesi* zoonotic reservoir have relied on sampling of wild macaques (Lee et al. 2011; Moyes et al. 2014), which helps prioritize areas of high-transmission risk, but as the investigators note, the animal reservoir may extend beyond the known natural hosts. It is also important to obtain whole-genome sequences of *P. knowlesi* from macaque reservoirs to fully detect evidence of selection and potential host switching. Although *Laverania* parasites, appear to have limited potential to cause zoonotic infections in the populations studied (Sundararaman et al. 2013; Délicat-Loembet et al. 2015), longitudinal surveys covering wider regions may provide more definitive evidence. Further sensitive and specific detection methods will be crucial in defining the extent of the reservoir.

Although not discussed in this review, vector surveys and studies will also be required to provide evidence for transmission to humans under favorable conditions (Vythilingam 2010; Paupy et al. 2013; Maeno et al. 2015).

Critical Review of Hospital Records and Case Studies for Early Detection of Unusual Cases

Although host and vector sampling are very useful in assessing the risk of zoonotic infections, they require extensive resources and can be impractical. Hospital records have proven invaluable in documenting cases of interest. Indeed, case reports and hospital records have led to many of the important reevaluations of dogma discussed in this review, including cases of *P. vivax* in DARC-negative individuals (Rubio et al. 1999), possible zoonotic *P. vivax* (Prugnolle et al. 2013), and the first case of *P. cynomolgi* human infection (Ta et al. 2014). Many of these findings, however, rely on correct diagnosis on site. Diagnosis based on morphology is prone to mistakes (*P. knowlesi* misdiagnosed as *P. malariae,* or *P. ovale* and possibly *P. cynomolgi* as *P. vivax*) and it is critical that molecular tools be used to definitively identify *Plasmodium* species. Increased awareness of the presence of these parasites in endemic regions will be important in early detection of unusual cases. This should also be accompanied with development of new rapid diagnostic tests that can detect and discriminate a larger range of species, as well as training of local public health staff.

The Promise of In Vitro Experimental Advances

The robust in vitro culture system accounts for our disproportionately greater knowledge of *P. falciparum* above other human *Plasmodium* species. Although advances in genomic tools are lending greater insight into the more neglected species, in vitro experiments will remain the gold standard to understand the mechanisms of invasion and host tropism. There have been substantial advances in studying *P. vivax* ex vivo (Russell et al. 2012) and even potential for genetic manipulation in vivo (Moraes Barros et al. 2014). There are reports of short-term culture of *P. vivax* in vitro (Golenda et al. 1997) and of *P. malariae* (Lingnau et al. 1994), but efforts to

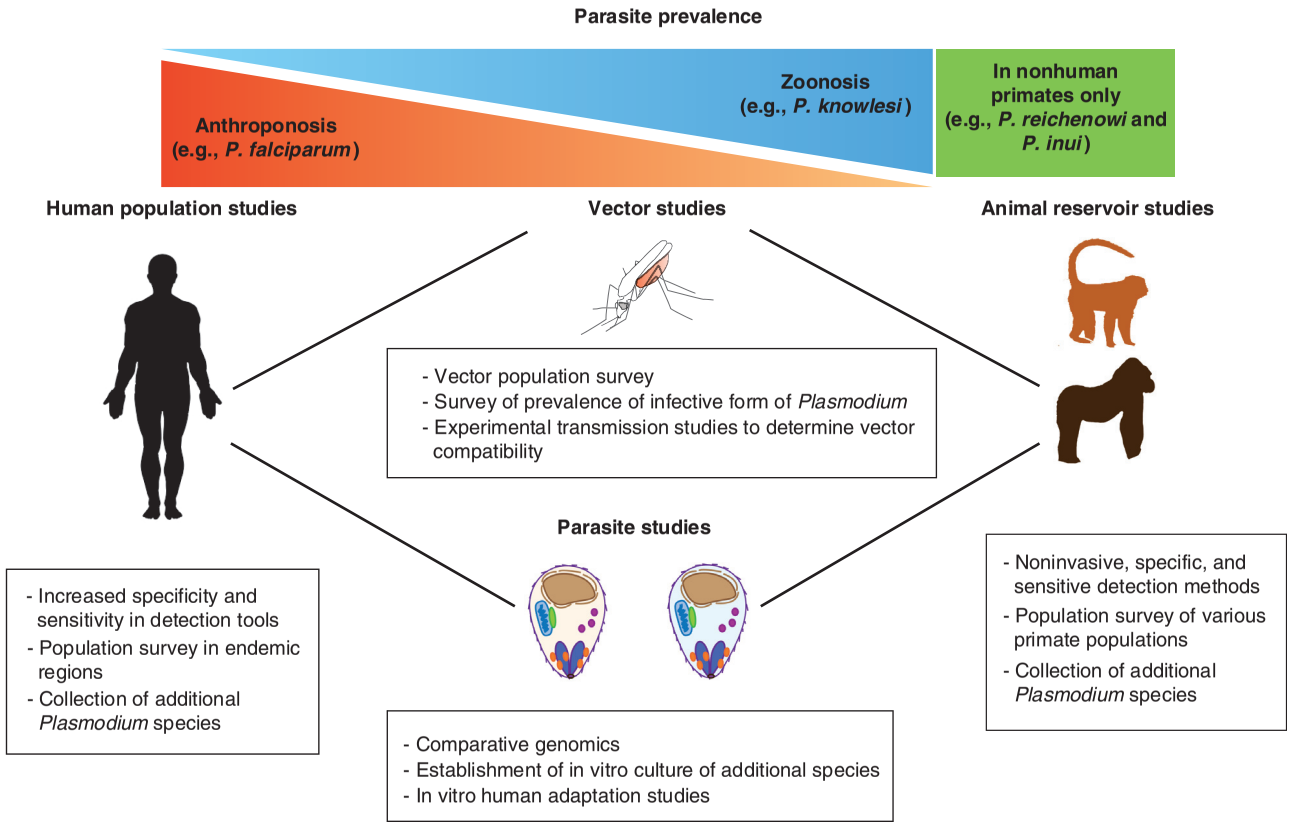

Figure 3. Toward reaching the goal of malaria elimination. Studies addressing several aspects of the ecology and biology of *Plasmodium* parasites can shed light on their evolving tropism and host adaptation. Vector, human host, and animal host population studies can help determine the diversity of *Plasmodium* species to which humans are exposed. Experimental studies can determine compatibility between parasites and hosts and elucidate the molecular mechanisms behind differences in cell tropism.

establish a reliable in vitro culture system should be renewed for these species, as well as *P. ovale*. In vitro culture will also facilitate initial screening of blood-stage vaccine targets for *P. vivax* and help identify promising candidates to pursue further. The success of adapting *P. knowlesi* to human blood has opened new doors in investigating mechanisms of host switch and adaptation relevant in the field, and has also provided the community with a more accessible tool for genetic manipulation because it obviates the need for macaque blood (Lim et al. 2013; Moon et al. 2013). Isolation of the newly identified parasites will be a daunting task but will provide a much-needed resource as we hopefully approach control if not eradication of the human *Plasmodium* species. Figure 3 summarizes studies that in conjunction will provide the necessary information for more efficient and relevant eradication strategies.

CONCLUDING REMARKS

Control of malaria has been a goal for the scientific community for several decades, while there is now a renewed emphasis on elimination. Most effort to date has focused mainly on *P. falciparum* and *P. vivax*, leaving the burden and prevalence of the lesser-studied species unclear. Having been comparatively understudied, the true extent of these parasites in humans and potential zoonotic reservoirs is not known. Expansion of current surveillance efforts to include all potential reservoirs might be needed. Methodologies with increased sensitivity will be essential for the detection of low parasitemia infections that are associated with the less-studied *Plasmodium* species. Continuous and more sensitive sampling and sequencing of human and animal *Plasmodium* species will keep us informed on the existing diversity and influence the elimination strategies to implement. *Plasmodium* parasites are continuously evolving, and molecular determinants leading to changes and expansion in host tropism will be key factors to investigate, which in some cases might identify critical molecules for development as vaccine candidates. Finally, it is also important to consider that human development is dramatically changing the ecology of infection, as human encroachment may create new and greater opportunities for potential animal reservoirs to transmit *Plasmodium* parasites.

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