Commentary

Understanding *Anopheles* and *Plasmodium* interactions: lessons from the real world

Malaria remains one of the major infectious diseases in the world, causing between 1 and 2 million deaths worldwide (WHO 2002). The disease prevails in most of the African continent and is caused by the bite of a female *Anopheles gambiae* mosquito infected with the parasite *Plasmodium falciparum*. Transmission of malaria depends on the completion of the *Plasmodium* life cycle in the mosquito. Biological strategies to control malaria include drugs against the vector and parasite, protection against the parasite (vaccine), and blocking transmission (of the parasite). Previously, molecular mechanisms which controlled the parasite load in mosquitoes and factors involved in innate immunity were intractable. However, in the light of several recent breakthroughs such as transformation of the *Anopheline* vectors, RNAi mediated gene silencing, DNA microarray data and completion of whole genome sequences of *P. falciparum* and *A. gambiae*, it is now possible to study the interactions between parasite and mosquito at a molecular level and find targets for transmission blocking. A comprehensive review by Michel and Kafatos (2005) summarizes recent advances in this field. We describe here studies dealing with some of the components of mosquito innate immunity and their impact on *Plasmodium* development. We also show that the laboratory data generated using model organisms may have limitations in interpretation, since the natural host–parasite relationship is a product of an evolutionary association and is optimised for delicate survival balance.

After the female mosquito ingests infected blood, the parasite forms an invasive stage, the ookinete, which crosses the mid-gut epithelium and upon reaching the basal lamina develops into oocytes. *Plasmodium* parasites need to overcome the immune system of the mosquito before invading the mid-gut tissue. Evading this mid-gut barrier is one of the big hurdles for the parasite and is a potential transmission blocking site in the mosquito. In order to understand the immune response of the mosquito to parasite infection (after mid-gut invasion), functional screens using double stranded RNA (dsRNA) have been carried out (Blandin *et al* 2002, Osta *et al* 2004). Of the several genes studied in such screens, a small set of genes having a drastic effect on the survival of *Plasmodium* were chosen for further analysis: two agonists belonging to the C-type lectins (*CTL4* and *CTLMA2*) and the antagonist *LRIM1* (leucine-rich repeat immune protein 1) which is a pattern recognition receptor (Osta *et al* 2004). dsRNA of these genes was injected in the body cavity of newly emerged females of *Anopheles gambiae* G3 strain which are susceptible to the rodent parasite *Plasmodium berghei.* The knockdown efficiency of the genes was found to be 96.8% (*CTLMA2*), 86.1% (*LRIM1*) and 70% (*CTL4*), as measured 4 days later by RT-PCR. These mosquitoes were fed with blood containing transgenic parasites in the ookinete and oocyst stages. The parasites expressed GFP (green florescent protein) so that they could be scored by phenotype (fluorescent or melanized non-fluorescent oocysts) 7 days later. Confirming their role as agonists, knockdown of genes *CTL4* and *CTLMA2* had pronounced effects on melanization of the ookinetes (97% and 53%, respectively). In the *CTL4/CTLMA2* double knockdown, a much higher melanization (of the oocytes) would be expected. However, the knockdown resulted in a melanization phenotype similar to that with *CTL4* (88%). The *LRIM1* knockdown had a positive effect on oocyst numbers, showing an average 3.6-fold increase in the number of ookinetes as compared with the control. Interestingly, lack of *LRIM1* in the *CTL4/CTLMA2* knockdowns resulted in negligible melanization and the number of oocysts were similar to those obtained with the *LRIM1* knockdown alone, indicating an epistatic action of the gene. In contrast, activity of *LRIM1* is needed to bring about melanization in the *CTL* knockouts. In order to understand the dynamics of gene expression in the mid-gut and carcass 24 h after infection (stage when the ookinetes invade the mid-gut epithelium), RT-PCR was carried out. Both *LRIM1*and *CTLs* were up-regulated in comparison with the uninfected controls and in both cases more

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expression was observed in the carcass than the mid-gut. An interesting outcome of this study concerns the role of *CTL*s in protection of the parasite. In previous reports, secreted CTLs were implicated in the elimination of pathogens in both vertebrates (Holmskov *et al* 1994) and invertebrates (Yu and Kanost 2003). During the course of its long association with the host, the parasite seems to have subverted host proteins for its own ends. A similar phenomenon is also observed in vertebrates (Hemingway and Craig 2004). This data was generated by using the rodent parasite *P. berghei* which, because of its higher rate of infection (resulting in more number of oocysts), is more amenable to experimentation than infection by *P. falciparum,* which results in a lower number of oocysts. However, *A. gambiae* is not the natural host of *P. berghei* and hence there might not be any association between these two under natural conditions and no co-evolution (Boëte 2005). It was also argued whether the immune responses in *P. falciparum*, which needs a higher temperature (~27–30ºC) for infection, would be similar to those obtained with *P. berghei* which needs a lower temperature for infection (~21ºC). A report by Dong *et al* (2006) corroborates this view. In a laboratory study, it was shown that immune responses to *P. falciparum* and *P. berghei* infection in *A. gambiae* are diverse. Thus, in order to confirm whether these 3 genes are indeed the targets for parasite blocking, experiments carried out under natural conditions to study the interactions between *A. gambiae and P. falciparum* were essential.

Cohuet *et al* (2006) studied the role of genes *CTL4, CTLMA2* and *LRIM1* in the development of *P. falciparum* using a previously described gene-silencing method in field samples of *A. gambiae*. The experiments were carried using the Yaoundé strain (derived from field specimens near Yaoundé), which is a representative of the local vector population in the Cameroon. The mosquitoes were fed with *P. falciparum*-infected blood samples collected from volunteers carrying asymptomatic gametocytes. Gene silencing was carried out in the females of the Yaoundé strain as described previously with either the gene (experimental) or GFP (control). The knockdown values averaged 81.5% (*CTL4*), 96.9% (*CTLMA2*) and 96.8% (*LRIM1*) and were similar to those obtained in the experiments of Osta *et al* (2004). Control and experimental mosquitoes were membrane fed with human blood carrying a varying number of gametocytes per microlitre. In order to minimize the impact of human factors that might block immunity, serum from donor blood was replaced with an equal volume of 'uninfected' serum. The mosquitoes were dissected and stained mid-guts were scored for oocysts 7 days later. Contrary to the results obtained earlier, there was no statistical difference in oocyst numbers between the *CTL4*, *CTLMA2* and *LRIM1* knockdowns. In order to rule out the effect of different host strains, similar experiments were performed using *P. berghei*. The results confirmed a pronounced effect of gene silencing similar to those obtained when the G3 strain was used. Thus, the effect was independent of the strain used in the study and seemed to be due to the parasite species. It should be noted here that the average number of oocysts obtained in the control groups was ~5, in contrast to ~300 oocysts obtained in the experimental group infected with *P. berghei.* This corroborates the view that there might be inherent differences in the way the two parasite species infect the host, resulting in different host–parasite interactions. Although there was no pronounced effect of gene silencing, all three genes were up-regulated 24 h after blood feeding as revealed by RT-PCR. Similar results were obtained in previous infection with *P. berghei.* Thus, the transcriptional up-regulation of the genes did not correlate with the effects observed upon gene silencing. It can be noted here that the genes did not show any significant up-regulation in the mid-gut tissue following infection, contrary to the previous findings in *P. berghei* where the genes showed up-regulation in the mid-gut tissue, though not as much as that in the carcass. Further studies are warranted to understand the significance of these observations.

In conclusion, there are significant differences in response to gene silencing of immune genes in different species of the *Plasmodium* parasite. Genes which had a dramatic effect on the development of the rodent parasite *P. berghei* upon silencing failed to show any significant effect on the development of *P. falciparum.* As described previously, these effects may be due to the inherent differences in the parasite and/or host–parasite interactions. Similar results were reported by Ito *et al* (2002) wherein the authors showed that transgenic *A. stephensi* expressing the dodecapeptide SM1 impaired the transmission of *P. berghei* but not the relevant human parasite. Although the authors argue that the differences in the effects of gene silencing may be due to the lower infection levels of the parasite in *A. gambiae*, a point which needs further investigation by modulating the infectivity of both the parasites, they favour the hypothesis that the observed differences might be due to co-evolution of the human parasite and its natural vector (Cohuet *et al* 2006). Reduced infection (a low parasitic load) might in itself be a co-adaptation mechanism to achieve a delicate equilibrium between the mosquito and the parasite, as a high immune response might

be detrimental to the host (Schmid-Hempel 2003). One way to prove this is by eliciting a more specific immune response. The studies by Dong *et al* (2006) support this assumption. Therefore, although gene silencing approaches open up a gateway for finding new targets to block parasites in the mosquito, the results should be accepted only after thorough analysis. Not only are the results dependent on biological variations, small and often 'unimportant' procedures such as the gene delivery route might induce some genetic changes causing still unknown responses (Hemingway and Craig 2004). One way to effeciently introduce genes into the vector without altering the immune response would be by using a transposon-mediated approach. This way the dsRNA could then be expressed under regulation of a tissue-specific promoter and thus the exact time and developmental stage of expression would make it possible to achieve the desired effect. Another possibility which would have to be considered is the effect of the parasite genotype on vector competence as well as the role of human genotypes in malaria prevalence (Lambrechts *et al* 2005; Fowkes *et al* 2006). Recently, genomes of a number of laboratory and field samples of *P. falciparum* have been described in the context of generating a malaria haploid map (Carlton 2007). These data would lead to better understanding of the genes involved in drug resistance and infectivity of the parasite and might also highlight the adaptation of the parasite with respect to its host. Thus, in spite of these apparent complexities, recent advances in functional tools in all the three species provide new approaches to our fight against malaria.

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