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Cytoadherence, pathogenesis and the infected red cell surface in *Plasmodium falciparum*

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Abstract

The particular virulence of *Plasmodium falciparum* compared with the other malaria species which naturally infect humans is thought to be due to the way in which the parasite modifies the surface of the infected red cell. Approximately 16 hours into the asexual cycle, parasite encoded proteins appear on the red cell surface which mediate adherence to a variety of host tissues. Binding of infected red cells to vascular endothelium, a process which occurs in all infections, is thought to be an important factor in the pathogenesis of severe disease where concentration of organisms in particular organs such as the brain occurs. Binding to uninfected red cells to form erythrocyte rosettes, a property of some isolates, is linked to disease severity. Here we summarise the data on the molecular basis of these interactions on both the host and parasite surfaces and review the evidence for the involvement of particular receptors in specific disease syndromes. Finally we discuss the relevance of these data to the development of new treatments for malaria.

1. Introduction

Plasmodium falciparum is by far the most virulent of the four malaria species that have humans as their primary vertebrate host and this has usually been explained in terms of the adherent properties of the infected r.b.c. Approximately 16 h after invasion of the r.b.c., changes occur in the surface of parasitised r.b.c. which render them adherent to endothelial cells [1] resulting in the sequestration of parasites in the deep vasculature or to uninfected red cells leading to the formation of rosettes. Sequestration of parasites in the brain is thought to be the precipitating event in the development of cerebral malaria [2] and the rosetting phenotype has been clearly associated with severe disease in several studies [3, 4]. The pathophysiological mechanisms which underlie conditions such as cerebral malaria are still the subject of some debate. Various hypotheses have been put forward centering either round reduced tissue perfusion caused by the pre-

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sence of sequestered parasites, or on the local release of mediators such as Tumour Necrosis Factor or Nitric Oxide [5–7]. While the truth is probably a combination of these explanations, there is general agreement that tissue specific sequestration is an early and probably necessary event. Unravelling the molecular mechanisms responsible for these adherence properties may therefore be an important step in the development of new strategies for the prevention and treatment of severe disease. Firstly, we will discuss current knowledge of the host receptors involved and their potential role in the development of severe disease, and then go on to address the parasite encoded proteins which mediate these interactions. Finally, we will discuss ways in which this information may be used to explore new avenues for the control of the disease rather than the parasite.

2. Endothelial receptors and severe malaria–host molecules

Over the past 10 years a number of molecules expressed on the surface of endothelial cells have been shown to bind r.b.c. infected with *P. falciparum*. In chronological order these are Thrombospondin (TSP), CD36, ICAM-1, VCAM, E-selectin, chondroitin sulphate A (CSA), CD31 and P-selectin [8–14]. Indirect evidence also suggests a role for the $\alpha_v\beta_3$ integrin [15]. Some of these receptors (CD36 and TSP) appear to bind most if not all parasites from infected patients [16] whereas the others bind only a subset of isolates. Chondroitin sulphate A appears to be a special case in that recent data suggest that this molecule is responsible for the concentration of parasites in the placenta [17], a particular problem in endemic areas for the foetus in primagravidae. These data are consistent with the possibility that binding to CSA is a rare property of infected cells which is magnified by selection in the placenta in primagravidae. Having experienced these organisms, it is possible that the woman mounts an effective antibody response which prevents multiplication of these phenotypes in subsequent pregnancies.

With the identification of so many other receptors and such a wide spectrum of clinical outcomes, a number of investigators have, over the years, attempted to correlate the ability of parasites to bind to a given receptor to the disease syndrome in the patient. These studies have involved relatively small numbers of isolates and a subset of potential receptors. They have shown first that there exists a wide range of binding avidities in different parasites and second that there was either no correlation of receptor-binding with disease, or an association of high levels of binding to CD36 with severe, non-cerebral malaria (see, for example, [18, 19]). In view of these findings we carried out a much larger study in which we examined quantitatively the ability of 200 isolates to adhere to CD36, ICAM-1, E-selectin, VCAM and CD31, the latter being a pan-endothelial marker and included at the time as a negative control. We used material derived from a large case-control study in Kilifi, Kenya and examined parasites from 50 patients in each of four clinical groups; community controls, severe malarial anaemia, non-severe malaria and cerebral malaria. The results demonstrated the following:

1. that binding to CD36 was identical in cerebral malaria patients and community controls and slightly but significantly higher in non-severe disease;
2. that CD36 was quantitatively the most important receptor;
3. that 80% of isolates had significant adherence to ICAM-1; that binding to ICAM-1 was significantly elevated in non-anaemic disease and was highest in cerebral malaria patients;
4. that binding to the other receptors tested with the possible exception of VCAM was too low to be of general significance suggesting that they may not be relevant in the field.

As an alternative means of investigating the same phenomenon, we carried out an extensive immuno-histopathological study of post-mortem material from individuals who had died of cerebral malaria together with controls [20].

These experiments revealed widespread endothelial activation in all organs in the malaria patients and in addition showed:

1. that there was very little CD36 expression in brain endothelium;
2. that when scored quantitatively for co-localisation of parasitised r.b.c. and individual receptors in cerebral vessels, the presence of ICAM-1 in a vessel was by far the best predictor of the presence of parasites in the same vessel.

Taken together these two sets of data are not consistent with a role for enhanced binding to CD36 in the development of cerebral malaria and are consistent with, but do not prove, a role for enhanced binding to ICAM-1.

It is doubtful whether further experiments along these lines will be any more conclusive since there are a number of problems with these types of investigations which are difficult to overcome. From a purely practical point of view, as more receptors are identified it will be necessary to repeat such large field studies in order both to assess the relevance of the receptors to adherence in wild isolates and also to assess their role in particular disease states. These studies are expensive, extremely time consuming and require access to good quality field material. Quantitative binding studies suffer from the problems of case definition and parasite sampling. It is never possible to ascertain whether or not a patient presenting early with non-severe disease may have gone on to develop severe complications and so labelling an individual isolate as non-severe will be subject to a significant error rate. Also one is forced always to sample parasites from the peripheral blood which are not those currently sequestered in the target organ. Immuno-histochemical studies are hampered by the problems in obtaining post-mortem material sufficiently rapidly to be representative of the situation at time of death and by the fact that patients can die many days after drug treatment has commenced so that parasites are no longer present. An alternative explanation for the results in this latter study could be that the presence of parasites in a particular site by themselves causes endothelial activation and that this explains the co-localisation of ICAM-1 and parasitised r.b.c. Finally, these studies were carried out using single receptors and static adhesion assays, a situation far removed from that in the small vasculature. We and others [21, 14] showed that under flow, parasitised r.b.c. roll on ICAM-1, yet are immobilised from flow on CD36. This could lead to situations where the two molecules could act synergistically in tissue beds such as the brain where CD36 expression is very low. Evidence for such synergy has been obtained in both static assays [22] and under flow conditions [14]. The adhesion parameters to a variety of other receptors are known to differ significantly under flow [14, 23, 24] and it is also possible that additional synergistic-binding occurs in other combinations of receptors.

Because of these considerations, which would be difficult to eliminate in future studies of sufficient size and power, we explored additional avenues to investigate the relationship between receptor-binding and disease. If any one particular receptor is implicated in the development of severe disease then the high selection pressure from malaria should select for protective polymorphisms in that receptor providing that they do not compromise survival more than infection with *P. falciparum*. Since we and others had mapped the parasitised r.b.c.-binding site on ICAM-1 to an area not involved in other receptor functions of the molecule [25, 26], such mutations could conceivably occur without compromising normal cellular interactions. Sequencing of domain one of African ICAM-1 revealed a high frequency mutation (K29/M, termed $ICAM^{Kilifi}$) present in East and West Africa but absent from Europeans [27]. When the effect of this mutation on susceptibility to severe malaria was examined by genotyping samples from a large case-control study carried out in Kenya, homozygotes for $ICAM^{Kilifi}$ were seen to be at a two-fold higher risk of cerebral malaria compared with controls. This result is apparently at odds with the original hypothesis but is still consistent with a role for binding to ICAM-1 in cerebral malaria. To determine the functional

consequences of this polymorphism, the mutant and wild-type sequences were expressed either as soluble proteins or on the surface of COS cells and the ability to bind infected r.b.c.s tested. Using a high avidity ICAM-1-binding clone (ITG-ICAM), no difference between the two proteins was evident. However, with a clone of lesser avidity, significantly higher levels of binding occurred with the wild-type than to the Kilifi variant (A Craig and C Newbold, unpublished). At this time, therefore, several explanations for the current gene frequency of $ICAM^{Kilifi}$ exist:

a) that this is actually a neutral mutation at current frequencies due to genetic drift. This implies that the susceptibility to severe malaria was a chance result;
b) selection for the mutation has occurred by other pathogen or disease syndrome with a mortality comparable or greater to that from *P. falciparum* and that the current frequency reflects a balanced overall risk;
c) that the mutation was originally selected because of resistance to severe malaria but has now resulted in the in-vivo selection of higher affinity ICAM-1-binding organisms and is therefore a susceptibility allele in populations with a long history of intense malaria exposure.

In view of the binding results, (c) is clearly a possibility and would predict that in other areas of Africa the allele could be protective or neutral with regard to severe malaria. The only other likely disease syndrome with a comparable mortality to malaria in Africa is Acute Respiratory Infection (ARI). Since ICAM-1 is a receptor for the major human serogroup of rhinovirus [28] and since the viral-binding site is in domain one in an area that overlaps the K29 residue [29], protection from rhinovirus is a possibility. Clearly rhinovirus infection per se is not life threatening, but the resulting predisposition to secondary bacterial infections may be sufficient to select the Kilifi allele to its current frequency. We are currently investigating the binding of rhinovirus to the African allele to address this point.

In summary, current data are still consistent with a role for ICAM-1 in the development of cerebral malaria but the results are not conclusive. Searching for polymorphisms in putative receptors is an alternative but still indirect method for determining their relevance in severe disease. Indeed, the hypothesis that tissue specific expression of combinations of cytoadherence receptors is the primary determinant of conditions such as cerebral malaria still relies primarily on indirect evidence. The identification of the parasite ligands involved (see later) opens up the possibility of carrying out in situ RT-PCR of parasite RNA isolated from different organs to establish this point.

3. Rosetting and severe malaria–host molecules

Rosetting, the ability of infected r.b.c.s to selectively bind uninfected r.b.c.s, is a phenotypically variable property and thus is also highly divergent between different genotypes or isolates. Most (e.g. [3, 4]), but not all [30] studies which have examined the relationship between rosetting and disease suggest that parasites of the rosetting phenotype are more likely to occur in patients with severe disease. There is disagreement as to whether the increased frequency of rosetting parasites is confined to cerebral malaria patients or all cases of severe disease. These differences may reflect methodological variation, genuine differences attributable to geography or something more fundamental concerning the relationship of rosetting and disease. It has also been reported in some [3] but not all [31] studies that antibodies, which disrupt rosettes, are less common in patients with disease. Further support for the association between rosetting and severe disease can be found in the reports that suggest that rosette size and frequency is diminished when parasites are grown in cells exhibiting haemoglobinopathies known to be protective against severe malaria [32]. Some relationship between the rosetting phenotype and disease severity, however, clearly exists but at present it is impossible to determine whether this is causal (e.g. through further impairment of blood flow) or reflects an

additional endothelial-binding phenotype which itself is associated with a poor outcome. It does not appear that rosetting has been selected in-vivo to increase parasite re-invasion rates [33]. In terms of the molecules on uninfected cells which act as receptors/ligands, current data is inconclusive. Rosettes are generally smaller in cells of the O blood group compared with A or B and tend to be the largest in group A. Immunoglobulin is necessary for rosette formation in some parasite lines and is also a component of the fibrillar strands seen between parasitised r.b.c. and host cells [34]. Rosettes are disrupted by sulphated glycoconjugates [35, 36]. Recent data suggests a role for CR1 as a host receptor for infected r.b.c. in rosettes [37]. It is likely therefore that rosetting is a heterogeneous phenomenon with different receptors being more or less important in different parasite isolates.

4. Molecules on the infected red cell surface mediating adherence

What about the molecules on the parasitised r.b.c. surface that mediate adherence? A number of candidates have been identified including *P. falciparum* r.b.c. membrane protein 1 (PfEMP-1) [38, 39], sequestrin [40], modified r.b.c. band 3 [41], rosettins [42] and Pf332 [43]. Here we will concentrate on the role of PfEMP-1 which is the only parasite-derived protein for which full sequence information is available and is the only molecule which has been shown by several laboratories to be parasite encoded and on the parasitised r.b.c. surface. Direct [44] or indirect [45, 46] experiments by a number of investigators also show PfEMP1 to be involved in binding to diverse receptors.

5. Role of *P. falciparum* red blood cells membrane protein 1 in adherence

5.1. Endothelial-binding

P. falciparum r.b.c. membrane protein 1 was originally described as a strain specific, high mol. wt., highly polymorphic protein present on the surface of *P. falciparum*-infected r.b.c. [47]. Its biochemical characteristics of insolubility in non-ionic detergents and extreme sensitivity to proteases, together with its location and degree of antigenic diversity in field isolates suggested that it was the *P. falciparum* equivalent of the phenotypically variable antigen (SICA antigen, [48]) described in *Plasmodium knowlesi*. Work in monkeys [49] reinforced this view. Longitudinal studies in African children naturally exposed to *P. falciparum* showed that the antibody response to the infected r.b.c. surface (presumably directed against PfEMP1) correlated with protection from clinical malaria [50]. In the early nineties, in-vitro data from two laboratories showed that PfEMP1 did undergo antigenic variation in cloned isolates and that changes in antigenic type were accompanied by changes in the receptor-binding specificity [51, 52]. More recently, more longitudinal data have been amassed which suggest that the presence of antibodies to specific PfEMP-1 molecules protects against subsequent clinical infection with isolates expressing that variant [53].

Together, this both provided circumstantial evidence that PfEMP1 was the parasite ligand for endothelial-binding and suggested that the same molecule was implicated in immune evasion and the maintenance of chronic infection through antigenic variation. Further progress was hampered until 1995 when the gene(s) encoding PfEMP1 were identified and sequenced and shown to be expressed in a fashion consistent with each gene coding for a particular variant antigen type [54–56]. The genes were shown to be present at about 50 copies/haploid genome, to be highly polymorphic both within and between isolates but to have a similar basic structure. Each gene has two exons; a well-conserved 3′ exon which is rich in acidic aa and probably represents the intracellular portion of the protein, possibly interacting with basic submembranous proteins such as Histidine Rich Protein 1 (HRP1) and a highly diverse 5′ exon containing two to five copies of a motif denoted the Duffy Binding Ligand (DBL), (see Fig. 1). The DBL-like sequence had been identified previously in other malarial proteins such as the Duffy binding pro-

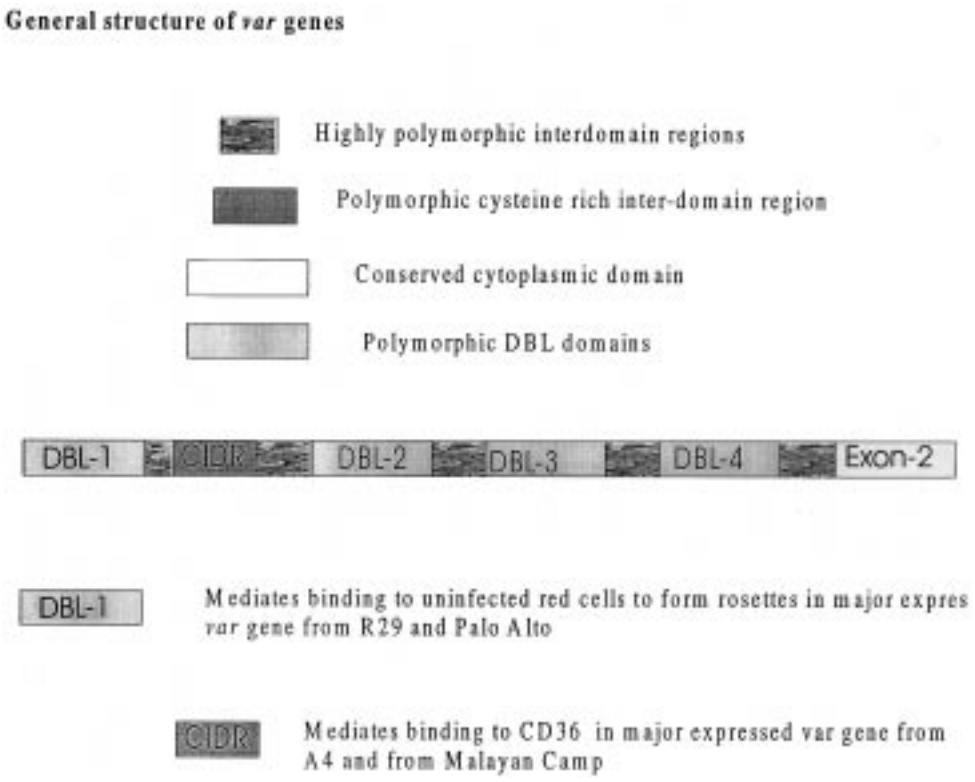

Fig. 1.

tein of *P. vivax* and the r.b.c.-binding antigen (EBA175) of *P. falciparum* [57, 58]. Heterologous expression studies had shown that the DBL domains bound the Duffy blood group determinant and glycophorin A, respectively, in these two proteins. The DBL domains are themselves highly diverse in sequence and identified only by the relative position of a number of key residues, particularly cysteines. In addition to the DBL domains, all *var* genes sequenced to date have an additional semi-conserved area immediately 3′ to the N-terminal or first DBL domain. This region is also characterised by the position of a number of cysteine residues and has therefore been termed the Cysteine Rich Inter Domain region (CIDR). Thus, PfEMP1 was shown to be a large and diverse multi-gene family, consistent with antigenic variation and yet to contain domains already proven to be capable of binding to a variety of cell surface structures. The clear conclusion from the sequence data therefore was that the DBL domains were likely to represent the endothelial-binding regions of the molecule. Studies to date have shown that this is not entirely true. Using an expressed *var* gene from the Malayan Camp (MC) parasite [56] the CIDR has been expressed as a glutathione-*S*-transferase fusion protein and shown to mediate binding to CD36.

In order to look more globally at the regions of *var* genes coding for receptor-binding, we sequenced the A4 *var* gene. A4 parasites bind to both CD36 and ICAM-1 and available data suggested that both functions were mediated by PfEMP-1. In common with other ICAM-1-binding PfEMP-1 molecules [45], this gene encoded a larger than average molecule containing five DBL domains (S Kyes and J Smith, unpublished). Constructs covering the entire extracellular domain were made and expressed in a variety

of systems. Only the CIDR bound to CD36. It is thus likely that this region is the one that is responsible for CD36-binding in all PfEMP-1 molecules, but further expression constructs from different *var* genes will have to be made to establish this point. The same set of constructs were used to try and identify the ICAM-1-binding region. Despite the use of several different constructs, multiple expression systems and a variety of different assays, it was not possible to achieve ICAM-1-binding with any of the expressed fragments (J Smith, A Craig, S Kyes, D Baruch, unpublished). We also generated antisera against each of the expressed domains. Despite the fact that 80% of the antisera reacted with the surface of unfixed infected cells of only the homologous variant type, suggesting that at least exposed linear epitopes and perhaps conformational epitopes were represented, none of the antisera reproducibly blocked binding to ICAM-1 (A Craig, T Fagan and C Newbold, unpublished). This suggests three possibilities. Firstly, the correct domain or combination of domains has not yet been expressed in an appropriate system to produce a functional fragment. This is particularly pertinent in the case of ICAM-1 as the interaction is known to produce rolling on cell surfaces and is therefore of low affinity. Secondly, it is possible that an additional or accessory molecule is required for adhesion to ICAM-1. Thirdly, ICAM-1-binding is mediated by a different molecule altogether. We believe the latter to be unlikely since previous data (see above) had strongly implicated the *var* gene products in ICAM-1-binding and in our own more recent studies, selection of parasites on ICAM-1 specifically selects for the expression of the A4 mRNA (S Kyes, unpublished). However, the possibility of accessory molecules needs to be considered as data on binding to other host receptors suggests that PfEMP-1 is not the only ligand. The case of thrombospondin is controversial. Baruch and colleagues [44] have used immobilised CD36, ICAM-1 and TSP to precipitate parasitised r.b.c. surface labelled molecules and tryptic fragments from them and conclude that PfEMP-1 is the ligand for all three of these receptors. We have exploited the set of cloned antigenic variants that we derived some years ago [51] to examine the protease sensitivity of ligand binding. We have shown that for ICAM-1 and CD36-binding, the sensitivity to sequence specific proteases is a variant specific phenomenon and that selection for a protease resistant binding phenotype also selects for the expression of a particular *var* gene. Binding to both of these receptors was reversed by immune serum. In addition, the specific binding activity as well as the expression of variant specific determinants at the cell surface appear simultaneously in the cell cycle. However, binding to TSP showed no variant specific characteristics, appeared 2 h earlier in the cell cycle and was not sensitive to immune serum. We suggested that these data were best interpreted by PfEMP1-mediating adherence to ICAM-1 and CD36 and another molecule (possibly modified band 3) being responsible for TSP binding [1]. Some support for these conclusions comes from data with parasites that have naturally lost HRP1 (and therefore knobs) or have had the gene disrupted by homologous recombination. In both cases, PfEMP1 appears to be expressed on the cell surface and binding to CD36 is normal in static adhesion assays. However, parasites lacking knobs fail to bind to TSP [59, 23]. Since the knob structure is the site of interaction of the infected red cell with endothelial receptors, these data are consistent with a role for band 3 in adherence to TSP.

In the case of CSA, parasites selected on this molecule appear to express a single dominant *var* gene, but this has not, to date, been shown to lead to the expression of PfEMP-1 on the cell surface, nor have direct binding experiments with expressed fragments been carried out. Moreover, parasites selected on CSA in vitro or parasites isolated from placenta seem not to bind to CD36 [17, 60]. Since binding to CD36 is a very common, if not universal, property of PfEMP-1, either a unique subset of abnormal *var* genes is involved, or other molecules on the infected r.b.c. surface may be important. As far as other receptors are concerned there is currently too little data for VCAM, E-selectin, P-selectin and CD31.

Clearly, these studies are still at an early stage. Defining the binding site for ICAM-1 or any of

the other receptors that may attach through PfEMP-1 is a major priority. It may then be possible to identify crucial residues that would serve as signatures for a particular ligand. Under these circumstances the role of individual receptors in tissue specific adhesion could be addressed directly by RT-PCR of organisms obtained at post-mortem.

5.2. Rosetting

Recent data [37] indicate that for the R29 rosetting clone from the IT lineage, rosetting is mediated by PfEMP-1. By deriving rosetting and non-rosetting lines from the clone, it has been shown that a single dominant *var* gene is expressed in rosetting but not non-rosetting organisms. This gene has been sequenced and fragments covering the whole ORF are expressed on the surface of COS cells. Uninfected r.b.c. bound to the first DBL domain with appropriate specificity but not to any other of the DBL constructs. Similar results were also obtained by another group using a different parasite genotype expressing a distinct rosetting-associated *var* gene [61]. Thus it seems likely that the N-terminal or first DBL domain of the expressed *var* gene product mediates rosetting in other parasite lines. However, because rosetting is such a heterogeneous phenomenon, it is dangerous to extrapolate this result to all rosetting lines of *P. falciparum*.

Wahlgren et al. [42] have assembled compelling evidence that low mol. wt. rosettins are expressed in clonally derived cultures in a manner consistent with their role in rosette formation. The identity and sequence of these molecules is awaited with interest.

6. Implications for novel interventions

The most promising line of research for the development of interventions in the short term is the role of CSA-binding in the placenta of primagravidae. If it can be shown that this phenotype is indeed mediated by an antigenically restricted subset of parasites (whether or not this is mediated by PfEMP-1) to which women learn to make a response after their first pregnancy, then it becomes feasible to consider immunising women of child bearing age against these organisms. Further research is needed: (a) to determine whether or not this binding is mediated by *var* genes and if so, how diverse they are antigenically; (b) to determine if women do develop a specific antibody response to a wide range of CSA-binding parasites after their first pregnancy; and (c) if *var* gene products are involved, to determine the primary sequence of a number of CSA-binding PfEMP-1 molecules.

With regard to organ specific endothelial adhesion, more information is required to establish which, if any, host receptors are critical in the development of syndromes such as cerebral malaria. This will be facilitated by human genetic studies, by RT-PCR from post mortem studies and by determining the regions and sequences of *var* genes, which are responsible for binding to each host receptor. At our current state of knowledge, strategies aimed at interfering with adhesion to CD36 must be viewed with caution. As it seems that higher avidity binding to this receptor is associated with non-severe disease, abolishing CD36 dependent adhesion could lead to the selection in-vivo of parasites with an affinity for a receptor which may lead to more virulent infection.

Current data would favour strategies directed at interfering with ICAM-1-binding. However, while early results suggested that this may be a possibility, our own studies suggest that it is extremely difficult to block binding to ICAM-1 coated surfaces, even with high concentrations of the soluble molecule [62]. This is not unexpected and may reflect the high on/off rates associated with the kinetics of a rolling interaction. For example, other approaches using mimotopes may be more successful but have yet to be tested.

Further work is also needed on the molecular basis of rosette formation. Whether the formation of rosettes themselves in vivo is a cause of severe disease, or whether rosette formation is a surrogate marker for a particular novel endothelial-binding phenotype, the fact that the correlation with disease severity is clear in most

studies suggests that a deeper understanding of the mechanisms involved may lead to new therapeutic avenues.

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