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Deletion of the Complement Phagocytic Receptors CR3 and CR4 Does Not Alter Susceptibility to Experimental Cerebral Malaria

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Summary

Complement receptors for C3-derived fragments (CR1-4) play critical roles in innate and adaptive immune responses. Of these receptors, CR3 and CR4 are important in binding and phagocytosis of complement-opsonized pathogens including parasites. The role of CR3 and CR4 in malaria or in cerebral malaria has received little attention and remains poorly understood in both human disease and rodent models of malaria. CR3 and CR4 are members of the β_2-integrin family of adhesion molecules and are expressed on all leukocytes that participate in the development of cerebral malaria (CM), most importantly as it relates to parasite phagocytosis (monocytes/macrophages) and antigen processing and presentation (dendritic cells). Thus it is possible that these receptors might play an important role in disease development. To address this question, we examined the role of $CR3^{-/-}$ and $CR4^{-/-}$ mice in experimental cerebral malaria (ECM). We found that both $CR3^{-/-}$ and $CR4^{-/-}$ mice were fully susceptible to ECM and developed disease comparable to wild type mice. Our results indicate that CR3 and CR4 are not critical to the pathogenesis of ECM despite their role in elimination of complement-opsonized pathogens. These findings support recent studies indicating the importance of the terminal complement pathway and the membrane attack complex in ECM pathogenesis.

Keywords

cerebral malaria; complement phagocytic receptors; β_2-integrins

Of the complement C3 receptors, only the complement receptor 1 (CR1, CD35) has an established role in the pathophysiology of malaria. CR1 serves as a host erythrocyte receptor for *Plasmodium falciparum* through its binding to PfRh4 (1-3) and polymorphic variants of CR1 associate with susceptibility to, and/or resistance to, severe malaria and cerebral malaria (CM) (reviewed in (4)). By contrast, the remaining complement C3 receptors, CR2, CR3 and CR4, have poorly defined roles in the development and progression of malaria infection and CM. Based on *in vitro* studies, C3dg, the ligand for CR2, is generated in large amounts and deposited on red blood cells in an alternative pathway-specific mechanism in murine malaria infections (5). The relevance of this observation to human cerebral malaria

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remains unclear, especially in light of studies demonstrating that coupling of C3d to malaria antigens in murine vaccine studies does not provide enhanced immunogenicity (6-8). The remaining two receptors, CR3 and CR4, are well known for their role in the phagocytosis of iC3b-opsonized pathogens (reviewed in (9-11)). However, the contribution of CR3 and CR4 to parasite killing and/or clearance via phagocytosis in both human and murine uncomplicated malaria and in CM is not known.

Complement receptor 3 (a.k.a., $\alpha M\beta_2$, CD11b/CD18) and CR4 (a.k.a., $\alpha X\beta_2$, CD11c/CD18) are members of the β_2-integrin family of adhesion molecules that play important roles in tissue-specific homing of leukocytes during inflammation, leukocyte activation in the immune response and phagocytosis (12-14). Both receptors bind multiple ligands and are widely expressed on all leukocytes (15) including neutrophils and macrophages that aid in clearance of malaria parasites and, dendritic cells which process antigen after ingesting parasite-infected red blood cells. The extent to which CR3 and CR4 contribute to these essential immune functions during malaria has received little attention. Instead CR3 and CR4 are primarily used as cell surface markers to distinguish between myeloid subsets or followed for changes in expression during the course of malaria infection (16-20). Treatment with anti-CR3 antibody reportedly had no effect on the course of experimental cerebral malaria (ECM) (21, 22). However, technical limitations of blocking antibody experiments require cautious interpretation as many variables affect experimental outcome (e.g., differing antibody affinities and avidities, and variability with respect to dosing, timing, and antibody half-life). To avoid these technical limitations and directly determine if CR3 and or CR4 are critical for the development and progression of ECM, we used mice deficient in these receptors.

We compared susceptibility and clinical severity of $CR3^{-/-}$(23), $CR4^{-/-}$(24) and wild type mice in *P. berghei* ANKA-induced ECM as previously described (25). All mice used in this study were on the C57Bl/6 background. For these studies, *P. berghei* ANKA was maintained by passage in BALB/c mice (26). ECM was induced by injecting mice i.p. with 5×10^5 PbA-infected RBCs. Peripheral parasitemia was monitored on day 6 post-infection by Giemsa-stained, thin-blood smears. Mice were monitored twice daily for clinical signs of neurologic disease, using the following scoring scale: 0, asymptomatic; 1, symptomatic (ruffled fur); 2, mild disease (slow righting); 3, moderate disease (difficulty righting); 4, severe disease (ataxia, seizures, coma); 5, dead. Mice observed having seizures were given a score of 4 regardless of other clinical signs of disease. Moribund animals were scored 4.5 and humanely sacrificed. Mice were classified as having ECM if they displayed these symptoms between days 6-9 post-infection, had positive thin-blood smears, and had a corresponding drop in external body temperature or succumbed to infection.

We found that $CR3^{-/-}$ and $CR4^{-/-}$mice did not survive significantly longer than wild type mice ($p>0.05$, Log rank test; Figure 1a and d) and that all three groups of mice succumbed to infection at the same rate. Disease severity in $CR3^{-/-}$ and $CR4^{-/-}$mice was identical compared to wild type mice and corresponded well to survival (Figure 1b and e). Interestingly, peripheral parasitemia was significantly elevated in $CR3^{-/-}$ ($p=0.0028$, unpaired Student's t-test), but not in $CR4^{-/-}$ mice compared to wild type mice (Figure 1c and f). The latter results suggest a minor role for CR3 in parasite clearance, but not in survival or disease severity.

The absence of an altered disease phenotype in $CR3^{-/-}$ and $CR4^{-/-}$ mice raised questions regarding the role of other β_2-integrin adhesion molecules in ECM. Previous studies have reported minimal differences in the course of ECM through day 10 in $CD11d^{-/-}$ ($\alpha D\beta_2$) mice (27) not unlike what we report here for CR3 and CR4. In contrast, LFA-1 (CD11a, ($\alpha L\beta_2$), also a member of the β_2-integrin family, is thought to play a key role in the development of ECM based on studies demonstrating significant protection from the development of ECM on treatment with anti-LFA-1 antibodies (21, 22, 28). To our knowledge no one has directly assessed the role of LFA-1 in ECM using $LFA\text{-}1^{-/-}$ mice to verify these reports. Therefore, we performed ECM using $LFA\text{-}1^{-/-}$ mice (29). We found that $LFA\text{-}1^{-/-}$ mice were significantly protected from ECM (p=0.0001, Log rank test) developing only mild clinical disease (p<0.05, days 6-10, Wilcoxon rank-sum test) and that peripheral parasite levels were similar to wild type mice (p>0.05, Student's t-test) (Figure 2a-c).

Our original hypothesis was that deletion of either CR3 or CR4 would potentiate disease development by virtue of impaired parasite clearance thus leading to a more severe course of ECM compared to wild type mice. To our surprise, there was no difference in survival or clinical disease between the complement receptor mutants and wild type mice. An alternative outcome may have been reduced disease severity due to altered leukocyte trafficking in the absence of either receptor, mostly due to loss of interaction with ICAM-1 (30-32), which is expressed at high levels on endothelial surfaces in the CNS during CM and ECM (22, 33). Thus loss of CR3 and CR4 expression on T cells and macrophages could reasonably be expected to reduce adherence and subsequent vascular occlusion, both characteristic features of CM. We cannot rule out the possibility of compensatory changes in receptor expression during ECM in either receptor-deficient mouse, however we have not observed such changes in other CNS inflammatory disease models using these mice (D.C. Bullard and S.R. Barnum, unpublished observations). The finding that $LFA\text{-}1^{-/-}$ mice are significantly resistant to the development of ECM, while $CR3^{-/-}$ and $CR4^{-/-}$ mice are not, indicates that, of the β_2-integrin family members, LFA-1 plays the most critical role in ECM.

Regardless of the potential roles for CR3 and CR4 in ECM pathophysiology, the data we present here supports a developing story indicating that, of the complement pathways and components, the complement terminal pathway and the membrane attack complex (MAC) are most important in ECM development. Previous studies have shown that deletion of C5 results in marked increase in resistance to ECM and that inhibition of C9 (and therefore the MAC) is protective in ECM (25, 34). More recently we have shown that inhibition of the classical or alternative complement pathways does not alter the course of ECM. Furthermore, deletion of C3 does not prevent C5 cleavage indicating that the canonical C5 convertases are not wholly responsible for C5 cleavage during ECM (T.N. Ramos et al., In press). The data we present here indicate that the opsonophagocytic functions of the complement system at the level of C3-derived fragments is also not critical for the development and progression of ECM. Thus in the murine cerebral malaria model system, biological functions of the complement system derived from components and activation pathways prior to C5 cleavage play a minor role in ECM pathophysiology. Taken together,

these data indicate that targeting C5 or components of the MAC may offer a new therapeutic avenue for cerebral malaria.

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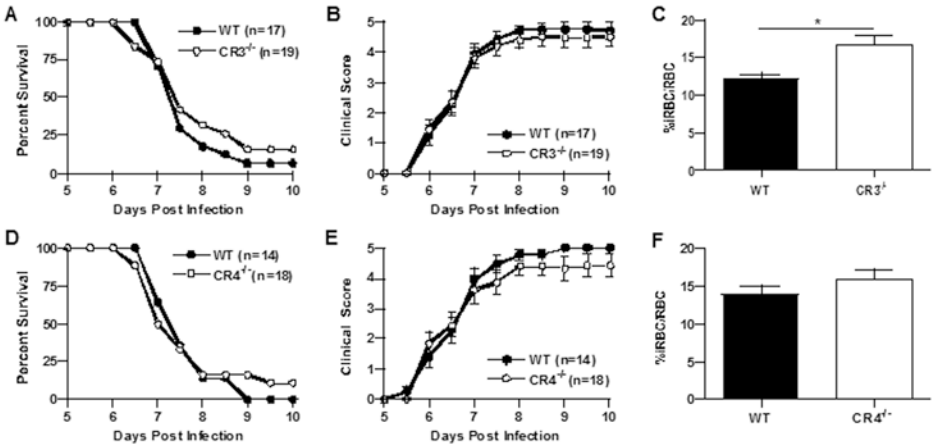

Figure 1.

Cerebral malaria in CR3$^{-/-}$ and CR4$^{-/-}$ mice is comparable to that of wild type mice. Wild type and complement receptor-deficient mice were injected i.p. with 5×10^5 PbA-iRBC and clinical scores and survival were monitored twice daily for ten days as previously described. CR3$^{-/-}$ mice (n=19) were fully susceptible to disease-induced mortality (p>0.50, Log rank test) compared to wild type mice (n=17) (a) and had similar disease severity from day 6 through 10 (b). CR4$^{-/-}$ mice (n=18) were as susceptible to disease-induced mortality (p>0.50, Log rank test) as wild type mice (n=14) (d) and had similar disease severity from day 6 through 10 (e). Peripheral parasitemia, assessed at day 6 after infection, was significantly elevated in CR3$^{-/-}$ mice compared to wild type mice (12.1 vs. 16.7 %iRBCs/total RBC, wild type vs. CR3$^{/-}$ mice respectively, p=0.0028, Student's t-test) (c), but not for CR4$^{-/-}$ mice (13.9 vs. 16 %iRBCs/total RBC, wild type vs. CR4$^{-/-}$ mice respectively, p=0.23) (f). The data shown for all panels are the mean +/- SE pooled from four independent experiments.

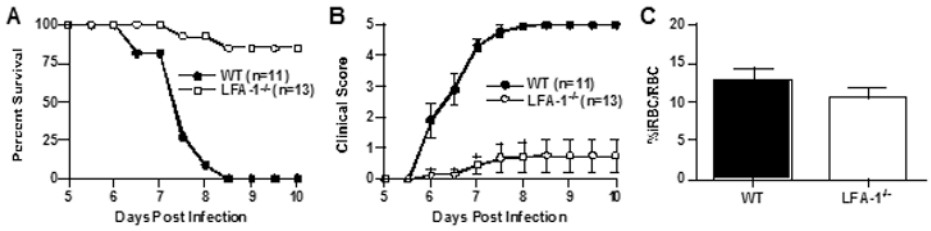

Figure 2.

LFA-1$^{-/-}$ mice are highly resistant to cerebral malaria. LFA-1$^{-/-}$ mice (n=13) were significantly resistant to disease-induced mortality (p=0.0001, Log rank test) compared to wild type mice (n=11) (a) and had reduced disease severity clinical on days 6 through 10 (p<0.05, Wilcoxon rank-sum test) (b). There was no significant difference in peripheral parasitemia between LFA-1$^{-/-}$ and wild type mice (p>0.05, Student's t test) (c). The data shown for all panels are the mean +/- SE pooled from three independent experiments.