Experimental Cerebral Malaria Develops Independently of Endothelial Expression of Intercellular Adhesion Molecule-1 (ICAM-1)*

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Background: Endothelial-expressed intercellular adhesion molecule-1 (ICAM-1) is considered critical for the development of cerebral malaria (CM).

Results: ICAM-1 expression on leukocytes alone was sufficient for the development of experimental CM.

Conclusions: Endothelial expression of ICAM-1 is not required for development of CM.

Significance: Vascular occlusion in CM requires ICAM-1 expression on leukocytes but not endothelial cells.

SUMMARY

Cerebral malaria (CM) is a severe clinical complication of Plasmodium falciparum malaria infection and is characterized by a high fatality rate and neurological damage. Sequestration of parasite-infected red blood cells (iRBCs) in brain microvasculature utilizes host- and parasite-derived adhesion molecules and is an important factor in the development of CM. ICAM-1, an alternatively-spliced adhesion molecule, is believed to be critical on endothelial cells for iRBC sequestration in CM. Using ICAM-1 mutant mice, we found that the full-length ICAM-1 isoform is not required for development of murine experimental CM (ECM) and that ECM phenotype varies with the combination of ICAM-1 isoforms expressed. Furthermore, we observed development of ECM in transgenic mice expressing ICAM-1 only on leukocytes, indicating that endothelial cell expression of this adhesion molecule is not required for disease pathogenesis. We propose that ICAM-1-dependent cellular aggregation, independent of ICAM-1 expression on the cerebral microvasculature, contributes to ECM.

Cerebral malaria (CM)2 is thought to arise from a confluence of inflammatory events in which infected RBCs (iRBC), activated leukocytes, and platelets are sequestered on inflamed endothelium due to increased expression of adhesion molecules (1). Among the large number of adhesion molecules implicated in CM development, ICAM-1 has long been known to bind and retain iRBCs in the central nervous system (CNS) microvasculature (2-4). ICAM-1 binds to Plasmodium falciparum erythrocyte membrane protein 1 (PFEMP1) through its amino-terminal Ig domain (5-7) and to several members of the β2-integrin family of adhesion molecules including LFA-1, Mac-1, and p150,95 (8). ICAM-1 is expressed on essentially all cell types contributing to CM development including: lymphocytes, myeloid cells, platelets and endothelial cells (9) (8, 10, 11). The increased expression of ICAM-1 and other adhesion molecule receptor/ligand pairs within the microvasculature sets the stage for vessel occlusion under inflammatory conditions.
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The importance of ICAM-1 in CM is based on studies demonstrating the relationship between increased endothelial ICAM-1 expression and iRBC sequestration in human and murine CM (1, 14). In experimental cerebral malaria (ECM), the animal model of CM, treatment with anti-ICAM-1 antibodies markedly inhibited rolling and iRBC sequestration in the central nervous system (3, 4, 15). Anti-ICAM-1 antibodies also inhibited adherence and rolling of iRBCs on LPS-primed brain sections or ICAM-1 transfected cells (3). Furthermore, there are reports that ICAM-1 polymorphisms are associated with severe forms of malaria, particularly CM, although this remains controversial (16-18). Although these data indicate a critical role for ICAM-1 in iRBC binding to endothelium, no study has directly addressed the requirement of ICAM-1 in the development of ECM.

Multiple isoforms of ICAM-1, arising from alternative splicing, have been described in humans and mice (19-24). However their function, changes in expression, and relative expression on various cell types involved in the development of ECM remain unknown. We show here that ICAM-1 mutant mice deficient in all ICAM-1 isoforms (Icam1null) are highly resistant to the development of ECM. In contrast, mice expressing different combinations of three of the six known isoforms, but not the full-length molecule (Icam-1tm1Jcgr and Icam-1tm1Bay mice), are more susceptible to ECM. These data demonstrate that although the full-length ICAM-1 protein is not required for disease initiation, ECM is attenuated in its absence. We also report the unexpected finding that ICAM-1 expression on CNS microvasculature is not required for ECM development. For these studies, Icam1null mice were bred with newly developed transgenic mice that express the full length ICAM-1 isoform under the control of the leukocyte-specific promoter CD2. We observed that these transgenic mice, unlike Icam1null mice, were highly susceptible to ECM. These observations suggest that ICAM-1-mediated aggregation of leukocytes, platelets, and iRBC within the vascular space, independent of expression on the microvasculature, is critical for promoting vessel occlusion during the development of ECM.

EXPERIMENTAL PROCEDURES

Mice and treatment procedures. Icam-1null mutant mice (Icam1tm14hi) were backcrossed at least 12 generations onto C57BL/6 (25). Icam-1tm1Jcgr and Icam-1tm1Bay C57BL/6J (N10) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and have been previously described (26, 27). CD2-Icam1B/Icam1null mice were generated by inserting the full-length ICAM-1 cDNA into a CD2 minigene cassette vector (28, 29). Male and female mice between the ages of eight to twelve weeks were used for all experiments. Plasmodium berghei ANKA (PbA) was maintained by passage in BALB/c mice as previously described (30). ECM was induced by injecting mice i.p. with 5 x 10^5 PbA-infected RBCs and parasitemia and disease progression was monitored twice daily as previously described (31).

Flow cytometry. CD2-restricted expression of ICAM-1 on leukocytes was determined by flow cytometry as previously described (32). Inbred C57BL/6 mice were used as controls for all experiments. T cell infiltration into brains at day 6 post-infection was assessed by flow cytometry as previously described (33).

Statistical Analysis. Statistical significance of ECM survival was calculated using the log rank test using Prism 5 (GraphPad Software, Inc.). The Mann Whitney test was used to determine significant differences in ECM clinical scores. Data are shown as mean ± SEM. A value of p<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Icam1null are highly resistant to ECM, while Icam1tm1Jcgr and Icam1tm1Bay are partially resistant

Although changes in ICAM-1 expression are well documented in both severe and cerebral malaria (1, 14), no study has directly assessed ECM severity and development using mice deficient in all ICAM-1 isoforms. For these studies we used three different lines of ICAM-1 mutant mice, including Icam1null mice, which lack all known ICAM-1 isoforms (Figure 1A). We observed that Icam1null mice were highly resistant...
ICAM-1 expression on leukocytes is sufficient for ECM development

It has been assumed that ICAM-1 contributes to CM predominately, if not exclusively, via expression on endothelial cells based on its function in the classic rolling/firm adhesion/transmigration paradigm used by leukocytes for trafficking to sites of inflammation or into lymphoid tissues (35-38). To directly determine if ICAM-1 expression on the microvasculature is required for ECM development, we generated transgenic mice with an ICAM-1-deficient background that expressed only the full-length ICAM-1 isoform on leukocytes (CD2-Icam1fl/Icam1null). Expression of the full-length isoform on CD4+ and CD8+ T cells, CD19+ B cells and NK cells from CD2-Icam1fl/Icam1null mice was comparable to that seen on wild type mice (Figure 2A-D). We then performed ECM using wild type and CD2-Icam1fl/Icam1null mice. We observed that CD2-Icam1fl/Icam1null mice were fully susceptible to ECM, however they progressed to fatal ECM significantly slower than wild type mice (p<0.0001, Log rank test, ~2 day delay) with a corresponding decline in clinical scores (Figure 2E and F, Table 1). These results demonstrate that expression of a single ICAM-1 isoform, in this case the full length ICAM-1 isoform, on leukocytes alone is sufficient to drive development of ECM, independent of expression on endothelium.

Our results indicate that ICAM-1 is critical to the development of ECM, since deletion of all isoforms essentially prevents disease development. However, the disease phenotypes of the Icam1tm1Jcgr and Icam1tm1Bay mice raise two important points: 1) absence of the full length ICAM-1 isoform does not prevent the development of ECM and, 2) variable combinations of ICAM-1 isoforms in the absence of the full length isoform result in distinct ECM phenotypes, indicating that multiple isoforms can contribute to disease outcome. This is remarkable since Icam1tm1Jcgr and Icam1tm1Bay mice share two of the three expressed isoforms (Figure 1D and G). It is worth noting that in experimental autoimmune encephalomyelitis (EAE), the disease phenotype of Icam1tm1Jcgr and Icam1tm1Bay mice is reversed compared to what we report here for ECM (39). Thus Icam1tm1Bay mice developed severe EAE, while Icam1tm1Jcgr mice developed mild EAE. This contrast in disease phenotypes suggests differential utilization and/or signaling mechanisms based on the combination of ICAM-1 isoforms expressed on leukocytes and endothelium.

Perhaps the most interesting finding in our study was the observation that leukocyte expression of a single ICAM-1 isoform, in an otherwise Icam1null background, was sufficient for ECM development. These data, combined with the results of the Icam1tm1Jcgr and Icam1tm1Bay studies (Figure 1) demonstrate that expression of different ICAM-1 isoforms results in dramatically different ECM phenotypes, suggesting distinct effector functions for a given isoform or
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combination of isoforms. These effector functions likely result from the integration of isoform-specific signaling pathways initiated by binding to single or multiple receptors. In the setting of ECM, we hypothesize that leukocyte/platelet/iRBC aggregates form in an ICAM-1-dependent fashion through use of β2-integrins (LFA-1 and Mac-1), which are then further stabilized by fibrinogen receptor binding (gpIIa/IIIb; CD41/CD61) on platelets. Such aggregates could form under the inflammatory conditions characteristic of malaria and CM and potentially occlude microvessels irrespective of the adhesive state of the endothelium. The powerfully protective effect of anti-LFA-1 treatment in ECM suggests that LFA-1 is an important ICAM-1 counter receptor in aggregate formation (40-42). In addition, recent studies have suggested that blood brain barrier opening due to vascular leakage contributes significantly to the development of ECM (42). The data we report here suggests that both mechanisms may be simultaneously at work, with brain edema initiated and/or exacerbated by sporadic to widespread vessel occlusion. Taken together, our results indicate a more central role for ICAM-1 in CM than previously appreciated and suggest that ICAM-1-based therapeutics may be an effective treatment strategy in CM.
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2Abbreviations used: CM, cerebral malaria; CNS, central nervous system; ECM, experimental cerebral malaria; ICAM-1, intercellular adhesion molecule-1; iRBCs, infected RBCs PbA, Plasmodium berghei ANKA; PfEMP1, Plasmodium falciparum erythrocyte membrane protein 1

FIGURE LEGENDS
Figure 1. ICAM-1 mutant mice are resistant to the development of ECM. Wild type, Icam-1<sup>tm1Jcgr</sup> and Icam-1<sup>tm1Bay</sup> mice were injected i.p. with 5 x 10<sup>5</sup> PbA-iRBC and clinical scores and survival were monitored twice daily for ten days as described in (37). A, Schematic of ICAM-1 isoforms missing in Icam1<sup>null</sup> mice shown in light gray. B, Icam1<sup>null</sup> mice (n=15) were significantly resistant to disease-induced mortality (p=0.0001, Log rank test; 93% survival past day 10) compared to wild type mice (n=12). C, Icam1<sup>null</sup> mice had significantly reduced clinical signs of disease (p<0.0001 from day 6 onward, Mann Whitney test) compared to wild type mice. D, Schematic of ICAM-1 isoforms expressed by Icam1<sup>tm1Bay</sup> mice shown in black outline. E, Icam1<sup>tm1Bay</sup> mice (n=15) were significantly resistant to disease-induced mortality (p=0.0001, Log rank test; 60% survival past day 10) compared to wild type mice (n=16). F, Icam1<sup>tm1Bay</sup> mice had significantly reduced clinical signs of disease (p<0.0001 from day 6 onward, Mann Whitney test) compared to wild type mice. G, Schematic of ICAM-1 isoforms expressed by Icam1<sup>tm1Jcgr</sup> mice shown in black outline. H, Icam1<sup>tm1Jcgr</sup> mice (n=17) were significantly resistant to disease-induced mortality (p=0.0001, Log rank test; 41% survival past day 10) compared to wild type mice (n=17). I, Icam1<sup>tm1Jcgr</sup> mice had significantly reduced clinical signs of disease (p<0.0001 from day 6 onward, Mann Whitney test) compared to wild type mice. Shown is the mean ± SEM of three to four independent experiments for all groups of mice.

Figure 2. Leukocyte-specific expression of the full length ICAM-1 isoform in the Icam1<sup>null</sup> background leads to ECM development. A-D, Expression of the full-length ICAM-1 isoform on CD4<sup>+</sup> and CD8<sup>+</sup> T cells, CD19<sup>+</sup> B cells and NK cells from CD2-Icam1<sup>fl/Icam1null</sup> mice is comparable to expression on leukocytes isolated from wild type mice, as determined by flow cytometry (n=3 for each group). E, CD2-Icam1<sup>fl/Icam1null</sup> mice (n=18) were significantly resistant to disease-induced mortality (p<0.0001, Log rank test; 6% survival past day 10) compared to wild type mice (n=17). F, CD2-Icam1<sup>fl/Icam1null</sup> mice had significantly reduced clinical signs of disease (p<0.0001 from days 6-8.5, Mann Whitney test) compared to wild type mice. Shown is the mean ± SEM of three to four independent experiments for all groups of mice.
Table 1. Survival and clinical scores for wild type and ICAM-1 mutant mice with ECM.

| ICAM-1 Genotype          | % Survival A | Clinical Disease (WT vs. mutant) B |
|--------------------------|--------------|------------------------------------|
| Wild Type (n=12-18)      | 0            | -                                  |
| Icam1null (n=15)         | 93           | 18.3 vs. 1.4 C                     |
| Icam1tm1Bay (n=15)       | 60           | 19.4 vs. 5.5 C                     |
| Icam1tm1Jcgr (n=17)      | 41           | 19 vs. 9.4 C                       |
| CD2-Icam1fl/Icam1null (n=18) | 6           | 18.5 vs. 9.9 D                     |

A percentage of mice that survive to day 10 post-infection with PbA

B the area under the curve for the clinical scores from day 1 through 10

C $p<0.05$ days 6 through 10

D $p<0.05$ days 6 through 8.5
Figure 1

(A) Schematic representation of infection stages.

(B) Graph showing % survival over days post-infection for WT (n=12) and Icam1null (n=15).

(C) Graph showing clinical score over days post-infection for WT (n=16) and Icam1tm1Bay (n=15).

(D) Schematic representation of infection stages.

(E) Graph showing % survival over days post-infection for WT (n=16) and Icam1tm1Jcgr (n=17).

(F) Graph showing clinical score over days post-infection for WT (n=18) and Icam1tm1Jcgr (n=17).
Figure 2

A

% CD8+ T cells

0 25 50 75 100

WT

CD2-icam1fl/icam1null

B

% CD4+ T cells

0 25 50 75 100

WT

CD2-icam1fl/icam1null

C

% NK1.1+ T cells

0 25 50 75 100

WT

CD2-icam1fl/icam1null

D

% CD19+ B cells

0 25 50 75 100

WT

CD2-icam1fl/icam1null

E

% Survival

0 20 40 60 80 100

5 6 7 8 9 10

Days Post Infection

WT (n=17)

CD2-icam1fl/icam1null (n=18)

F

Clinical Score

0 1 2 3 4

5 6 7 8 9 10

Days Post Infection

Wild Type (n=17)

CD2-icam1fl/icam1null (n=18)
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