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Functional Roles for C5a and C5aR but Not C5L2 in the Pathogenesis of Human and Experimental Cerebral Malaria

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The host immune response plays an important role in the onset and progression of cerebral malaria (CM). The complement system is an essential component of the innate immune response to malaria, and its activation generates the anaphylatoxin C5a. To test the hypothesis that C5a signaling contributes to the pathogenesis of CM, we investigated a causal role for the C5a receptors C5aR and C5L2 in a mouse model of experimental CM (ECM) induced by *Plasmodium berghei* ANKA infection, and using a case-control design, we examined levels of C5a in plasma samples from Ugandan children presenting with CM or uncomplicated malaria (UM). In the ECM model, $C5aR^{-/-}$ mice displayed significantly improved survival compared to their wild-type (WT) counterparts ($P = 0.004$), whereas $C5L2^{-/-}$ mice showed no difference in survival from WT mice. Improved survival in $C5aR^{-/-}$ mice was associated with reduced levels of the proinflammatory cytokines tumor necrosis factor (TNF) and gamma interferon (IFN-γ) and the chemokine, monocyte chemoattractant protein 1 (MCP-1) (CCL2). Furthermore, endothelial integrity was enhanced, as demonstrated by increased levels of angiopoietin-1, decreased levels of angiopoietin-2 and soluble ICAM-1, and decreased Evans blue extravasation into brain parenchyma. In the case-control study, the median levels of C5a at presentation were significantly higher in children with CM versus those in children with UM (43.7 versus 22.4 ng/ml; $P < 0.001$). These findings demonstrate that C5a is dysregulated in human CM and contributes to the pathogenesis of ECM via C5aR-dependent inflammation and endothelial dysfunction.

Malaria is a leading cause of global morbidity and mortality, with an estimated 243 million cases and 1.24 million deaths occurring worldwide annually (1, 2). The majority of malaria deaths are due to severe and cerebral malaria (CM) caused by *Plasmodium falciparum* infection (2). Currently, the first-line therapy for CM is intravenous artesunate, based on the superiority of artesunate over quinine in large randomized trials in adults and children with severe malaria (3, 4). However, despite treatment with parenteral artesunate, the case fatality rate of CM remains high, indicating that antimalarial therapy alone may be insufficient to prevent fatality and long-term neurocognitive deficits in survivors (3, 4).

The pathogenesis of CM is multifactorial and involves both host and pathogen determinants. These include dysregulated host immune responses to infection and the sequestration of parasitized erythrocytes (PE) in cerebral microvasculature contributing to vessel occlusion, hypoxia, endothelial activation, and blood-brain barrier (BBB) dysfunction (5–7). Elevated levels of inflammatory cytokines, such as gamma interferon (IFN-γ) and tumor necrosis factor (TNF), are often observed in human CM compared to uncomplicated malaria (8, 9). While it is unclear whether inflammatory cytokines have a causal role in human CM, it is postulated that these cytokines contribute to endothelial activation and sequestration of PEs in the brain microvasculature by upregulating the expression of cell adhesion molecules such as ICAM-1 on cerebral endothelium to which PEs bind (7, 10, 11). This hypothesis is supported, at least in part, by observations in the murine model of experimental CM (7, 12, 13). A detailed understanding of the molecular basis of pathological host responses in CM may facilitate the identification of novel interventions to improve clinical outcome over that achievable with antimalarial therapy alone.

The complement system represents a key component of the innate immune response and consists of three major pathways—classical, lectin, and alternative—which all converge at the cleavage of C5 into C5a and C5b (14). Additional pathways can generate C5a independently of C3 via thrombin or serine proteases (15, 16). C5a is a potent inducer of inflammatory mediators and anti-angiogenic factors (17–20). C5a signaling primarily through its major receptor, C5aR, has been shown to contribute to a variety of pathological processes, including sepsis, ischemia-reperfusion injuries, age-related macular degeneration, autoimmune disorders, altered angiogenesis, and neurodegenerative disorders (21, 22).

Increased complement activation has been consistently observed in human malaria infections (23). However, in human studies, it has been difficult to establish a causal role for complement activation in the pathogenesis of CM due to the challenges of performing mechanistic studies in human populations. Using murine models of experimental CM (ECM) to investigate causal-

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ity, previous studies have reported that genetic deficiency of C5 (24, 25) or antibody blockade of C5a or C5a receptor (C5aR) (25) significantly protects against ECM. C5a has an alternative receptor, C5L2, whose role in malaria has not been previously reported. C5L2 is similar to C5aR in structure, with 60% sequence homology. However, it lacks the key amino acid motif (known as the DRY motif) required for binding G proteins and is, therefore, unable to induce classic G protein-dependent signaling functions, such as the release of intracellular Ca^{2+} (26, 27). C5a and its degradation product, $C5a_{desArg}$, display high affinity for both C5aR and C5L2 (28). Because C5L2 is not coupled to G proteins, it was hypothesized that C5L2 may be a nonsignaling decoy receptor for C5a or that it may have an anti-inflammatory role (26, 29). In contrast, other studies have implicated a proinflammatory role for C5a-C5L2 signaling in different disease contexts, such as sepsis (30).

In this study, we investigated the roles of C5a, C5aR, and C5L2 in the pathogenesis of CM. Here we show that circulating levels of C5a are elevated in children with CM compared to the levels in children with uncomplicated malaria (UM). Moreover, C5a contributed to the pathogenesis of ECM via C5aR, as evidenced by decreased inflammation, decreased endothelial activation, and improved blood-brain barrier integrity and survival in *C5aR*$^{-/-}$ but not in *C5L2*$^{-/-}$ mice. These results support a role for C5a and C5aR but not C5L2 in the pathogenesis of ECM.

MATERIALS AND METHODS

Murine model of ECM. Wild-type (WT) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *C5aR*$^{-/-}$ and *C5L2*$^{-/-}$ mice (both on a C57BL/6 background) were bred from the founders kindly donated by Craig Gerard (Boston Children's Hospital, Boston, MA). In all experiments, male or female mice aged 7 to 11 weeks were used. *P. berghei* ANKA was obtained from the Malaria Research and Reference Reagent Resource Centre (MR4, Bethesda, MD) and maintained by passage in naive mice. The University Health Network Animal Use Committee approved all experiments. Infection was initiated by an intraperitoneal injection of freshly isolated 1×10^6 *P. berghei* ANKA-parasitized erythrocytes (PEs)/mouse and was monitored daily for up to 14 days by determining weight and parasitemia on thin blood smears stained with Diff Quik (American Scientific Products, Mississauga, Ontario, Canada). Mice were evaluated for signs of CM, including limb paralysis, seizures, and coma, and were euthanized when moribund.

Measurement of cytokines and endothelial regulators. Serum was collected from mice infected with *P. berghei* ANKA on days 5 and 6 postinfection via the saphenous vein and frozen at −80°C. Serum cytokine levels were assessed using the mouse inflammation cytokine bead array (BD Bioscience, Mississauga, Ontario, Canada). Serum levels of Ang-1 and soluble ICAM-1 (sICAM-1) were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN).

Assessment of blood-brain barrier integrity. A solution of 1% Evans blue dye was prepared using Evans blue powder (Sigma-Aldrich Canada, Ltd., Oakville, Ontario, Canada) and phosphate-buffered saline (PBS), and the solution was filter sterilized. On day 6 postinfection, 100 μl of 1% Evans blue was injected intravenously and left to circulate for 1 h. Mice were euthanized using isoflurane and perfused with 20 ml of PBS. Brains were collected, photographed, and placed in formamide for 48 h to extract Evans blue. Representative photographs were chosen based on animals with the most similar parasitemia. Evans blue was quantified using a spectrophotometer at 605 nm and compared to a standard curve.

Quantitative real-time PCR. RNA was extracted from the snap-frozen brain tissue after homogenization in TRIzol (1 ml/100 mg tissue; Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's protocol. Extracted RNA (1 μg/sample) was treated with DNase I (Fermentas, Burlington, Ontario, Canada) and reverse transcribed to cDNA (Bio-Rad, Mississauga, Ontario, Canada). cDNA was amplified in triplicate with SYBR green master mix (Roche, Laval, Quebec, Canada) in the presence of 1 μM each forward and reverse primers in a Light Cycler 480 (Roche, Laval, Quebec, Canada). Transcript number was calculated based on threshold cycle (C_T) compared to a standard curve of mouse genomic DNA included on each plate by Light Cycler 480 software (Roche, Laval, Quebec, Canada) and normalized by geometric averaging of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin expression levels, as previously described (31). The following primer sequences (5′→3′) were used: *Gapdh*, forward, TCAACAGCAACTCCCACTCTT CCA, and reverse, TTGTCATTGAGAGCAATGCCAGCC; *Angpt2*, forward, AGAGTACTGGCTGGGCAATGAGTT, and reverse, TTCCCAGTCCTTC AGCTGGATCTT; *Icam*, forward, TGGCTGAAAGATGAGCTCGAGAGT, and reverse, GCTCAGCTCAAACAGCTTCCAGTT; and β-*actin*, forward, CTGAATGGCCCAGGTCTGA, and reverse, CCCTGGCTGCCTCAACAC.

HMGB-1 Western blot. Plasma was collected via the saphenous vein in heparanized tubes (Sarstaedt, Montreal, Quebec, Canada) and stored at −80°C. There is no standard loading control for measuring plasma proteins; therefore, we measured the levels of high-mobility group protein B-1 (HMGB-1) from an equal volume of plasma for all samples according to a previously published protocol (32). Two microliters of plasma was diluted with 18 μl of cell lysis buffer (Cell Signaling Technology, Billerica, MA). Twenty microliters of diluted plasma was mixed with an equivalent volume of 2× Laemmli buffer containing 100 mM dithiothreitol and boiled for 5 min. Forty microliters of the diluted plasma was separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane for immunoblotting. The blots were probed with rabbit HMGB-1 monoclonal antibody (clone EPR3507; 1:1,000) (Abcam, Cambridge, MA). For quantification, blots were scanned and band densities were determined by using the NIH Image-J software.

Case-control study participants. Between 15 October 2007 and 30 October 2009, children aged 0.6 to 10.6 years living in an area in Uganda where malaria transmission is holoendemic and presenting to the Acute Care Unit of Mulago Hospital in Kampala with fever and *Plasmodium falciparum* malaria were eligible for enrollment in a nested case-control study, as described previously (33, 34). Briefly, children were diagnosed with uncomplicated (UM) or cerebral (CM) malaria according to World Health Organization criteria (35). Clinical and demographic data and a venous blood sample were collected at enrollment for measurement of hemoglobin, platelet count, and citrate-anticoagulated plasma for the analysis of C5a. Plasma samples were frozen immediately and stored at −80C until analysis to preserve analyte integrity. Thick and thin blood smears were obtained at presentation for determination of parasitemia, reported as the arithmetic mean of two independent readings by expert microscopists. The study was approved by the Mulago Hospital Research Ethics Committee, Makerere University Faculty of Medicine Research Ethics, Uganda National Council on Science and Technology, and Toronto Academic Health Sciences Network Research. Written informed consent was obtained from the parents/guardians of all participants.

Analysis of C5a levels. C5a levels were analyzed in citrate-anticoagulated plasma samples by ELISA according to the manufacturers' instructions (R&D Systems, Minneapolis, MN).

Statistical analysis. Statistical analyses were performed using the GraphPad Prism software (LaJolla, CA). Statistical significance for survival studies was assessed by the log rank test. For all data, nonparametric analyses were performed. Specifically, comparison of two groups was performed by Mann-Whitney test. Whenever three or more groups were compared, the Kruskal-Wallis test was performed. If a statistically significant difference was found among the groups, the Mann-Whitney test was performed to make comparisons between two groups. The paired *t* test was used to compare the levels of HMGB-1 expression between day 0 and day 5 postinfection. In all cases, a *P* value of <0.05 was considered significant.

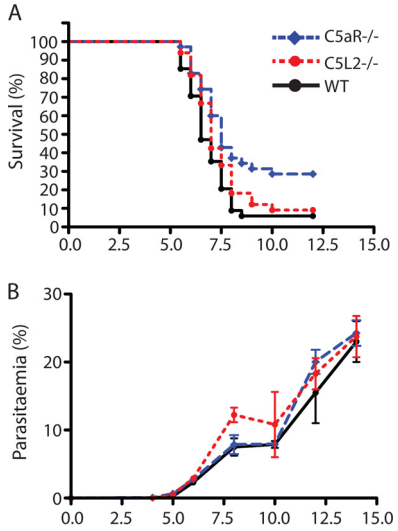

FIG 1 Deletion of C5aR, but not C5L2, significantly improves survival in ECM. Wild type (WT) (black circles, solid line), C5aR$^{-/-}$ (blue diamonds, dashed lines), and C5L2$^{-/-}$ (red circles, dotted lines) mice were infected with 1×10^6 *P. berghei* ANKA-parasitized erythrocytes (PEs) and were monitored for survival (A) (pooled result from four independent experiments; $n = 30$ to 35/group) and peripheral parasitemia (B) (representative of four independent experiments; $n = 8$ to 10 in each experiment). Survival differences were assessed by the log rank test. Differences between the WT and C5aR$^{-/-}$ mice were significant ($P = 0.004$) (A).

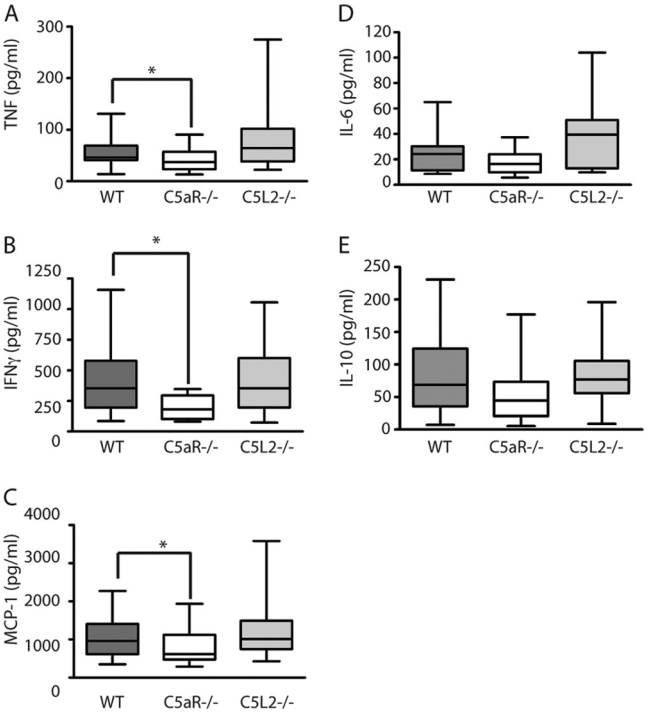

FIG 2 Deletion of C5aR but not C5L2 reduces systemic inflammation in ECM. (A to E) Levels of TNF, IFN-γ, MCP-1 (CCL2), IL-6, and IL-10 were measured from serum collected on day 5 postinfection by using the mouse inflammation cytometric bead array. Box plots represent median and interquartile range (IQR), and whiskers denote range. *, $P < 0.05$, Kruskal-Wallis test for comparing all groups, followed by Mann-Whitney test to compare WT and C5aR$^{-/-}$. Data are representative of 3 independent experiments ($n = 8$ to 10/group).

RESULTS

Deletion of C5aR but not C5L2 improves survival in a mouse model of ECM. We investigated whether C5a and its receptors, C5aR and C5L2, play causal roles in ECM by examining survival following *P. berghei* ANKA infection of *C5aR*$^{-/-}$ and *C5L2*$^{-/-}$ mice compared to their wild-type (WT) counterparts. In these experiments, ~90% of the WT mice succumb to ECM, displaying various degrees of difficulty in righting, ataxia, seizures, and coma, as described previously (36). Importantly, survival was significantly improved in *C5aR*$^{-/-}$ mice (Fig. 1A) (log rank test, $P = 0.004$). In contrast, *C5L2*$^{-/-}$ mice were fully susceptible to ECM, with survival similar to that of WT mice. No significant difference was observed in levels of parasitemia among the three groups (Fig. 1B). To confirm that our findings were robust, we repeated survival studies in 8 additional independent experiments (totaling $n = 79$ to 90 mice per group), and in each experiment, significantly improved survival was observed in *C5aR*$^{-/-}$ mice compared to *C5L2*$^{-/-}$ and WT mice (log rank test for the combined results, $P < 0.0001$) (data not shown).

Deletion of C5aR but not C5L2 reduces systemic inflammation. Dysregulated systemic inflammation, characterized by high circulating levels of proinflammatory cytokines, such as tumor necrosis factor (TNF), IFN-γ, and the chemokine, monocyte chemoattractant protein 1 (MCP-1) (CCL2), has been associated with poor clinical outcomes in human infections and in mouse models of malaria (7, 9, 37, 38). Mice susceptible to *P. berghei* ANKA develop a marked systemic proinflammatory response during infection (7, 37, 39). To determine if improved survival in *C5aR*$^{-/-}$ mice was associated with decreased levels of malaria-induced inflammation, serum levels of proinflammatory cytokines were assessed on day 5 postinfection (Fig. 2). Baseline levels of all cytokines measured were below the limit of detection in uninfected mice from the three groups (data not shown). Compared to *P. berghei* ANKA-infected WT mice, infected *C5aR*$^{-/-}$ mice had significantly lower serum levels of TNF, IFN-γ, and MCP-1 (Fig. 2A to C) ($P < 0.05$). These data suggest that a reduced inflammatory response can contribute to the improved survival observed in *C5aR*$^{-/-}$ mice and are in agreement with previous studies showing that C5a-C5aR signaling contributes to the synergistic induction of proinflammatory cytokines and chemokines in response to malaria parasite products (17).

HMGB-1 is associated with progression of ECM. High-mobility group protein B-1 (HMGB-1) is an inflammatory mediator associated with tissue injury and sepsis (40). In a previous study examining the role of C5a and its receptors in the pathogenesis of sepsis, Rittirsch et al. reported that the plasma level of HMGB-1 was significantly increased in experimental sepsis and that the release of HMGB-1 required C5L2, suggesting that HMGB-1 is a key mediator of C5a downstream of C5L2 in a murine model of sepsis (19). We investigated whether the same relationship existed between C5L2 and HMGB-1 in ECM by determining circulating HMGB-1 levels on day 5 postinfection in WT, *C5aR*$^{-/-}$, and *C5L2*$^{-/-}$ mice. Due to the lack of reliable mouse HMGB-1 ELISA reagents, Western blotting was employed to measure the expression of HMGB-1, as described previously (32). In order to account for the semiquantitative nature of Western blotting, quantitative comparisons of the HMGB-1 band densities were made only among the bands blotted on the same membrane (Fig. 3). All blots

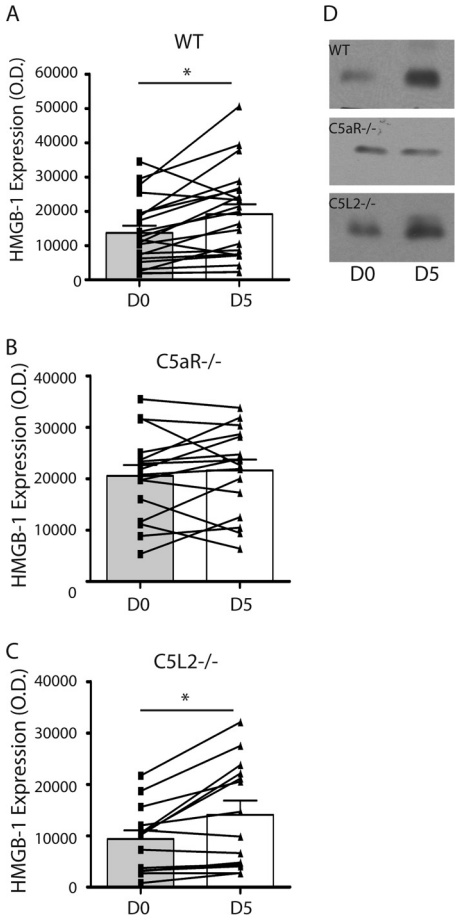

FIG 3 HMGB-1 is associated with progression of ECM. Expression levels of HMGB-1 were measured by Western blotting from plasma collected on day 5 (D5) postinfection. (A to C) Band intensities for day 0 (gray bars) and day 5 (white bars) in each group were compared on the same blot by using the NIH Image J software. *, $P < 0.05$ (paired *t* test). Histograms represent means ± standard errors of the means (SEM) obtained from pooled results from two independent Western blot analyses from two independent *P. berghei* ANKA infection studies ($n = 15$ to 20/group). Representative Western blots are shown in panel D.

were repeated twice using plasma samples collected from at least two independent infection studies. No significant difference was found at the basal level of HMGB-1 expression among three groups (data not shown). In agreement with a previous study, the plasma level of HMGB-1 significantly increased after *P. berghei* ANKA challenge in WT mice (Fig. 3A and D) (41). Of note, C5L2-deficient mice showed a similar pattern of induction of HMGB1 upon *P. berghei* ANKA infection (Fig. 3C) ($P < 0.05$), suggesting that C5L2 is not required for HMGB1 release in the ECM model. In contrast, induction of HMGB1 after *P. berghei* ANKA challenge was not observed in *C5aR*$^{-/-}$ mice (Fig. 3B).

Deletion of C5aR but not C5L2 reduces endothelial cell activation. In addition to enhanced production of proinflammatory cytokines, endothelial activation is a central feature of CM (10, 42, 43). The angiopoietin (Ang)-Tie2 pathway is a key regulator of endothelial activation and vascular integrity (44). Constitutive interaction between angiopoietin 1 (Ang-1) and Tie2 functions to maintain endothelium quiescence, while Ang-2 antagonizes the Ang-1–Tie2 interaction, promoting vascular permeability (44). Several studies have previously reported that elevated levels of Ang-2 and sICAM-1 and reduced levels of Ang-1 are correlated with severe disease and fatal outcome in patients with CM (5, 34, 45, 46). Similar observations have been reported in ECM after *P. berghei* ANKA challenge (47, 48). Consistent with these studies, we observed that *P. berghei* ANKA infection was associated with decreased levels of Ang-1 and increased levels of sICAM-1 in all three groups (Fig. 4). Importantly, endothelial activation was significantly attenuated in the C5aR-deficient mice infected with *P. berghei* ANKA, which displayed higher levels of Ang-1 and lower levels of sICAM than their WT counterparts ($P < 0.05$). Due to a lack of ELISA reagents to measure circulating mouse Ang-2, we assessed the changes in Ang-2 by measuring Ang-2 mRNA using the quantitative real-time PCR in brain homogenates of infected mice (Fig. 4C and D). Transcript levels of Ang-2 and ICAM-1 were significantly lower in the *C5aR*$^{-/-}$ mice on day 5 postinfection than those in WT mice ($P < 0.05$). Collectively, these data support the hypothesis that C5aR deficiency can contribute to the improved survival in ECM by promoting endothelial quiescence.

Deletion of C5aR promotes blood-brain barrier integrity. One of the hallmarks of CM is the loss of BBB integrity (6, 10, 43, 49). Based on our observation that C5aR deletion can improve endothelial quiescence, we hypothesized that BBB integrity would be better preserved in *C5aR*$^{-/-}$ mice after *P. berghei* ANKA infection. Therefore, we compared Evans blue extravasation in parasitemia-matched WT versus *C5aR*$^{-/-}$ mice following *P. berghei* ANKA infection. Consistent with our hypothesis, Evans blue leakage into brain parenchyma was significantly reduced in *C5aR*$^{-/-}$ mice compared to that in WT mice with similar parasitemia (Fig. 5) ($P < 0.05$).

C5a is elevated in children with CM. In order to extend these murine observations to an informative patient population, we investigated a role for C5a in the pathobiology of human CM by comparing plasma levels of C5a in Ugandan children with CM to those in matched children with UM in a nested case-control design (Table 1). Circulating C5a levels were significantly higher in children with CM versus those in children with UM as assessed by the Mann-Whitney test (median [interquartile range] for CM, 43.7 [22.9 to 59.5], versus UM, 22.4 [15.7 to 32.2]; $P = 0.0004$) (Fig. 6). All other patient characteristics were comparable between the two groups as assessed by the Mann-Whitney test. The median parasitemia was higher among the children with CM, but this difference did not reach statistical significance.

DISCUSSION

The complement system plays an important role in the host response to infectious processes. However, complement activation is tightly regulated, and the loss of this control can contribute mechanistically to the pathogenesis of multiple conditions, including sepsis, asthma, transplant rejection, and neurodegenerative disorders (18, 19, 21, 22, 50). Although previous studies have reported activation of complement via several pathways during malaria infection (23), it has been difficult to determine whether excessive complement activation plays a causal role in the pathogenesis of severe disease in humans. In this study, we present several lines of evidence supporting a causal role for C5a-C5aR signaling in the pathogenesis of CM. Using an experimental model of CM, we extend our previous findings that deficiency of C5 confers protection as does antibody blockade of C5a or C5aR in CM-susceptible wild-type mice (25). Providing further mechanistic

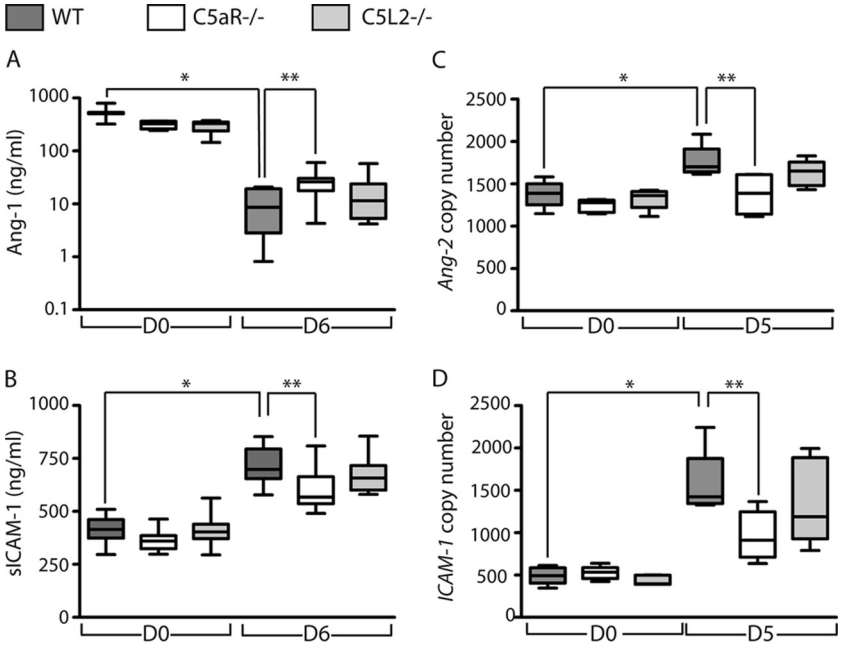

FIG 4 C5aR deletion but not C5L2 is associated with reduced endothelial activation in ECM. (A and B) Serum samples collected from on days 0 and 6 postinfection (D0 and D6, respectively) were analyzed for the levels of Ang-1 and sICAM-1 by ELISA. (C and D) Quantitative real-time PCR was performed on RNA extracted from the brains collected on days 0 and 5 postinfection to measure the mRNA levels of Ang-2 and ICAM. Copy numbers of Ang-2 and ICAM were normalized by the copy numbers of GAPDH and β-actin. Box plots represent the median and IQR, and whiskers denote range. The Mann-Whitney test was performed to compare WT mice on D0 and D5/D6 postinfection as a control (denoted as "*") and to compare WT and C5aR$^{-/-}$ mice on D5/D6 (denoted as "**"). * and **; $P < 0.05$. Data are representative of two independent experiments ($n = 8$ to 10/group).

insights, our data suggest that the protective mechanisms by which C5aR deficiency improves outcome are likely to involve reduced systemic inflammation, decreased endothelial activation, reduced PE sequestration in the brain, and improved vascular integrity: features previously associated with improved clinical outcomes in human malaria (5, 34, 43, 45, 51, 52). Importantly, our analysis revealed divergent roles for C5aR and C5L2 in the context of malaria infection. Deletion of C5L2 failed to reduce inflammation or endothelial activation or improve survival. We extended these observations to human CM and show in a nested case-control study that C5a levels are associated with disease severity and are significantly higher in African children presenting with CM than in children with uncomplicated malaria, which is consistent with our previous finding in ECM (25). Collectively these data implicate C5a in human CM and provide direct evidence for a role for C5a acting through C5aR in the pathogenesis of ECM.

Reduced levels of proinflammatory mediators (TNF and IFN-γ) and markers of improved endothelial stabilization (decreased levels of sICAM-1 and increased levels of Ang-1) were observed in *C5aR*$^{-/-}$ mice following *P. berghei* ANKA infection. This is consistent with the role of C5a as a potent inducer of inflammation and with our previous *in vitro* findings that C5a and *Plasmodium falciparum* bioactive product, *pf*GPI, can synergistically induce cytokines (interleukin-6 [IL-6], TNF, IL-1β, and IL-10), chemokines (IL-8, MCP-1, macrophage inflammatory protein 1α [MIP-1α], and MIP-1β) and the anti-angiogenic factor sFlt-1 (17). Dysregulation of proinflammatory cytokines and chemokines is likely to contribute to aberrant accumulation of mononuclear cells and PEs in the cerebral vasculature in the CM-susceptible mice, as previously reported (25). Importantly, mice that are naturally resistant to ECM do not express C5a, underscoring the involvement of C5a-C5aR in leukocyte trafficking to the brain, and are consistent with the known role of C5a-C5aR-mediated induction of inflammatory cytokines and chemokines (25).

In addition, our observations of endothelial activation and dysfunction following *P. berghei* ANKA infection are in agreement with accumulating evidence that increased levels of sICAM-1 and Ang-2, and decreased levels of Ang-1 are associated with both adverse clinical outcomes in human malaria infections and progression of ECM in mice (5, 34, 45–48, 53). Of note, in the same cohort of Ugandan children with CM we studied with increased C5a levels, we also observed significantly increased levels of sICAM-1 and Ang-2 compared to those in children with uncomplicated malaria (34). The Ang-Tie2 pathway plays a pivotal role in the transition from a quiescent endothelium to an activated endothelial phenotype, which in addition to severe malaria is associated with a number of prothrombotic states and inflammatory disorders (44). Infection of *C5aR*$^{-/-}$ mice was associated with decreased endothelial activation and the preservation of BBB integrity, supporting the hypothesis that C5a-C5aR signaling contributes to endothelial dysfunction and the loss of BBB integrity that characterize ECM (54, 55). Interestingly, C5a and C5aR have been shown to mediate BBB dysfunction in experimental lupus, and blockade of C5a or C5aR significantly improved BBB dysfunction in this model, suggesting that the role of C5a in vascular leakage in the brain may extend to a variety of disease states (56, 57). Taken together, these data support a mechanistic model whereby C5a-C5aR contributes

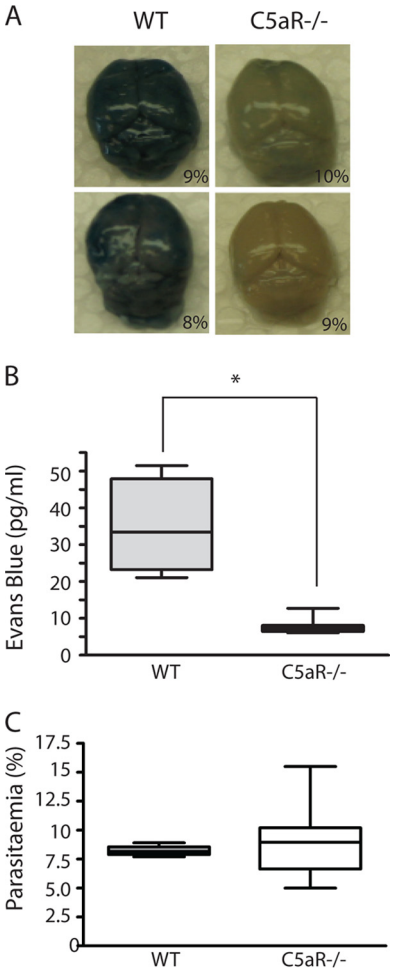

FIG 5 Deletion of C5aR improves blood-brain barrier integrity in ECM. (A) Representative photographs of brains from WT mice (left panels) and C5aR$^{-/-}$ mice (right panels) after Evans blue infusion on day 6 postinfection. Peripheral parasitemia is indicated in the bottom right corner of each panel. (B) Quantification of total Evans blue extravasation in brain as an indication of vascular leak. (C) Peripheral parasitemia of WT mice and C5aR$^{-/-}$ mice used for Evans blue extravasation. Box plots represent the median and IQR, and whiskers denote range. *, $P < 0.01$, Mann-Whitney test ($n = 6$ to 8/group). Data are representative of two independent experiments.

to the ECM pathogenesis by multiple mechanisms, including endothelial activation and cytokine and chemokine induction, resulting in aberrant leukocyte accumulation in the brain and breakdown of the BBB.

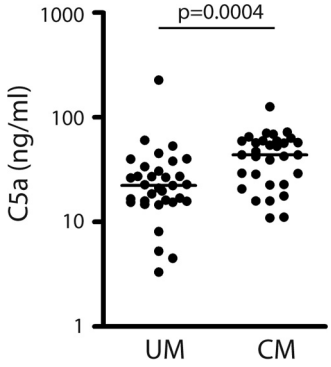

FIG 6 C5a levels at presentation are significantly increased in children with cerebral malaria. C5a levels were measured in plasma of Ugandan children with uncomplicated malaria (UM; $n = 33$) and cerebral malaria (CM; $n = 31$) at clinical presentation. The Mann-Whitney test was performed for the comparison of the groups.

In contrast to *C5aR*$^{-/-}$ mice, levels of proinflammatory cytokines did not differ significantly between WT and *C5L2*$^{-/-}$ mice after *P. berghei* ANKA infection, suggesting that inflammation induced by C5a is mediated primarily via C5aR in this model. The role of C5L2 has been examined in animal models of several human diseases, including allergic asthma, lung injury, sepsis, and peritonitis (19, 29, 30, 58). Data from these studies suggest both a positive and a negative role of C5L2 in complement-mediated inflammation, depending on the disease context and the animal models used, and the precise role of C5a-C5L2 signaling in the pathogenesis of complement-mediated diseases remains controversial. In ovalbumin (OVA)-induced allergic asthma, C5L2 plays a positive regulatory role much like C5aR, and C5L2 deficiency is associated with reduced levels of airway hyperresponsiveness, serum IgE, and mucus production (30, 58, 59). Similarly, in the midgrade cecal ligation puncture model of sepsis, deficiency or blockade of C5aR or C5L2 was associated with improved survival (19). In contrast, C5L2 was shown to have an anti-inflammatory role in the lipopolysaccharide (LPS)-induced shock (58) and in an immune complex-mediated lung injury model (29). In the LPS-induced shock model, C5L2 deficiency was associated with increased susceptibility to lethality, and it was associated with increased levels of neutrophil infiltration, serum TNF, and IL-6 in the immune complex-mediated lung injury model (29, 58).

In our hands, C5L2 deficiency did not accelerate disease progression as would be predicted if this receptor played an anti-inflammatory role as suggested by others (26, 29). Alternatively,

TABLE 1 Patient characteristics[a]

Characteristic	Value for patient group	
	UM	CM
No. of patients	33	31
Age (yr)	3.0 (1.7–6.8)	3.1 (1.6–4.4)
Parasitemia (no. of parasites/μl)	3.9×10^4 ($6{,}120$–1.4×10^5)	1.1×10^5 (1.8×10^4–2.8×10^5)
Wt (kg)	13.0 (10.0–21.0)	13.0 (10.0–15.0)
No. of days reported sick prior to presentation	3.0 (2.0–4.0)	3.0 (2.0–3.0)
C5a level (ng/ml)	22.4 (15.7–32.2)	43.7 (22.9–59.5)[b]

[a] All variables except the number of patients are presented as medians (interquartile ranges). A Mann-Whitney test was performed to assess a difference between the UM and CM groups for each variable.
[b] $P = 0.0004$.

we also did not observe a proinflammatory role for C5L2 as reported in the cecal-ligation puncture model of sepsis or in an experimental model of allergic asthma (19, 30). Instead, both the median survival and the changes in the inflammatory mediators (including HMGB-1) were similar in WT and *C5L2*$^{-/-}$ mice. Lack of a functional role for C5L2 in ECM is consistent with observations in models of experimental placental malaria in which C5aR deficiency and antibody-mediated blockade of C5aR but not C5L2 significantly reduce fetal growth restriction and improve fetal viability (60). Collectively, these data support the view that, in the context of malaria pathogenesis, C5a appears to function predominantly through C5aR rather than C5L2.

We examined whether the C5L2-dependent regulation of HMGB-1, which has been previously observed in experimental sepsis, can be extended to ECM (19). Our data suggest that it does not occur in ECM and that C5L2-dependent regulation of HMGB-1 is context specific. Higgins et al. recently reported that the elevated plasma HMGB-1 levels at presentation were significantly associated with severe malaria and a subsequent fatal outcome in pediatric patients with *P. falciparum* infection (41). However, antibody-mediated neutralization of HMGB-1 in ECM failed to improve survival (41). These findings suggest that HMGB-1 may be a marker of severe malaria but is unlikely to be a critical mediator of the ECM pathogenesis. Lack of a significant induction observed in the *C5aR*$^{-/-}$ mice in our study is consistent with this hypothesis.

Our findings would appear to differ somewhat from those of Ramos et al. who reported that while C5 deletion conferred protection from ECM, the mechanism was via inhibition of C5b and the membrane attack complex (MAC) rather than C5aR (24). In a subsequent study, the authors demonstrated that the terminal complement pathway is activated independently of C5 convertase and that it is likely to be activated by coagulation enzymes of the extrinsic protease pathway (61). Coagulopathy has been consistently associated with falciparum malaria, with patients displaying prolonged bleeding times, prolonged prothrombin and partial prothrombin times, and impaired aggregation responses to defined stimuli (62–64). Moreover, the complement and coagulation pathways are intimately linked. The coagulation pathway can activate complement pathway and vice versa (15, 65), and both pathways can be activated by parasite components (66).

Nonetheless, if C5 is cleaved, both C5a and C5b would be generated as well as the MAC. In eight independent *P. berghei* ANKA challenge experiments, we consistently observed a significant survival advantage in *C5aR*$^{-/-}$ mice. While our data do not preclude a role for C5b as a potential contributor to ECM, our data indicate that C5a-C5aR may contribute to the pathogenesis of CM via multiple pathways, including dysregulated cytokine induction, endothelial activation, aberrant accumulation of leukocytes in the brain, and ultimately BBB dysfunction. This hypothesis is supported by our data from the present and previous *in vitro* and *in vivo* studies (17, 25) and by observations in human CM (5, 8–10, 17, 34, 43, 45, 46, 52). Furthermore, our observations are consistent with a growing body of compelling evidence implicating a causal role for C5a-C5aR in the induction of proinflammatory mediators and antiangiogenic factors in multiple disease conditions that share features with cerebral malaria, including sepsis, ischemia-reperfusion injuries, pathological angiogenesis, and neurocognitive and neurodegenerative disorders and most recently in studies of human and experimental placental malaria demonstrating a causal role for C5a-C5aR in altering placental angiogenesis and mediating adverse birth outcomes associated with malaria infection (19, 22, 60).

In summary, we provide the first evidence of elevated C5a in human CM and using an experimental model demonstrate a causal role for C5aR but not C5L2 in the enhanced cytokine (e.g., TNF and IFN-γ) and endothelial (e.g., decreased Ang-1, increased Ang-2) activation and BBB dysfunction associated with poor clinical outcomes in human malaria infections.

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