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Dysregulation of angiopoietin-1 plays a mechanistic role in the pathogenesis of cerebral malaria

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Abstract

Cerebral malaria is a leading cause of global morbidity and mortality. Interventions targeting the underlying pathophysiology of cerebral malaria may improve outcomes compared to treatment with antimalarials alone. Microvascular leak plays an important role in the pathogenesis of cerebral malaria. The angiopoietin (Ang)–Tie-2 system is a critical regulator of vascular function. We show that Ang-1 expression and soluble Tie-2 expression were associated with disease severity and outcome in a prospective study of Ugandan children with severe malaria and in a preclinical murine model of experimental cerebral malaria. Ang-1 was necessary for maintenance of vascular

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Competing interests: S.E.Q. is an advisor to Aerpio Pharmaceuticals. S.J.H., K.C.K., and L.A.P. are listed as inventors on a patent entitled “Angiopoietin-based interventions for treating cerebral malaria” (application no. 17289177) held by Regeneron Pharmaceuticals Inc./University Health Network. K.C.K. and W.C.L. are inventors on patents related to the use of angiopoietin markers, entitled “Angiopoietin-1 and -2 biomarkers for infectious diseases that compromise endothelial integrity” (application no. WO2009059404) and “Biomarkers for early determination of a critical or life threatening response to illness and monitoring response to treatment” (application no. CA2769433). L.A.P. and G.T. are employees of Regeneron and hold a patent entitled “Methods for treating malaria by administering an antibody that specifically binds angiopoietin-2 (ang-2)” (publication no. US9371379 B2). The other authors declare that they have no competing interests.

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integrity and survival in a mouse model of cerebral malaria. Therapeutic administration of Ang-1 preserved blood-brain barrier integrity and, in combination with artesunate treatment, improved survival beyond that with artesunate alone. These data define a role for dysregulation of the Ang–Tie-2 axis in the pathogenesis of cerebral malaria and support the evaluation of Ang–Tie-2–based interventions as potential adjunctive therapies for treating severe malaria.

INTRODUCTION

The majority of *Plasmodium falciparum*–infected individuals can be effectively managed with prompt diagnosis and oral antimalarial treatment. However, a proportion of patients will present with, or progress to, severe malaria, including cerebral malaria, a potentially fatal neurological complication of *P. falciparum* infection (1, 2). Parenteral artesunate is the current first-line treatment for cerebral malaria (3, 4). Despite treatment with this potent antiparasitic agent, neurological sequelae and death occur in a subset of patients. Reported fatality rates for children and adults with cerebral malaria treated in large clinical trials with intravenous artesunate were 18 and 30%, respectively (3, 4). Overall, an estimated 652,000 lives are lost each year to severe malaria and cerebral malaria (1). For children surviving cerebral malaria, up to one-third may develop neurocognitive deficits including epilepsy, behavioral disorders, and/or motor, sensory, or language deficits (5). Together, these data suggest that strategies targeting the parasite alone are insufficient to prevent neurological complications and death in all individuals with severe infection.

A complementary approach to antimalarial agents includes the use of adjunctive therapies that target mechanistic pathways mediating severe disease. The pathogenesis of cerebral malaria is complex and incompletely understood, but several lines of evidence support an important role for host response in determining disease severity and outcome (6, 7). The ability to maintain microvascular function in the face of systemic inflammation is critical to host survival, particularly at the level of the blood-brain barrier (BBB) (8). Fatal outcome in cerebral malaria is associated with endothelial activation and up-regulation of endothelial adhesion molecules [for example, intercellular adhesion molecule-1 (ICAM-1)], culminating in increased microvascular permeability (6, 7, 9, 10).

The angiopoietin (Ang)–Tie-2 system is a critical regulator of endothelial cell function (11). Ang-1 binds to and induces phosphorylation and translocation of Tie-2, a receptor tyrosine kinase class protein (also known as *Tek*) on endothelial cells (12), to control vascular quiescence and stability, in part by means of phosphatidylinositol 3-kinase/Akt activation and maintenance of endothelial cell junction integrity (13–18). Conversely, Ang-2, which is released from endothelial cells in response to inflammatory and vasoactive stimuli (19, 20), generally functions as an antagonist of Tie-2 to promote vascular permeability and a proinflammatory vascular phenotype (21, 22). Altered angiopoietin concentrations are associated with disease severity and fatality in cerebral malaria (23). However, it is unknown whether alterations in the Ang–Tie-2 axis play a mechanistic role in cerebral malaria pathogenesis.

Here, we examined the kinetics of circulating Ang-1 and the soluble form of its cognate receptor, Tie-2 (sTie-2), over the course of severe malaria and cerebral malaria and

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investigated the mechanism and causality of the Ang–Tie-2 axis in an experimental model of cerebral malaria. We show that Ang-1 concentrations were associated with clinical recovery in a prospective cohort of *P. falciparum*–infected Ugandan children. Using the experimental cerebral malaria model, we established that Ang-1 is required for maintenance of vascular integrity and survival. Pharmacological strategies to supplement Ang-1 improved vascular integrity and survival in experimental cerebral malaria, suggesting their potential as adjunctive therapies for treating cerebral malaria.

RESULTS

Longitudinal kinetics of Ang-1 and sTie-2 are associated with severe malaria

To examine the relevance of the Ang–Tie-2 pathway to the pathobiology of malaria, we investigated Ang-1 kinetics in a cohort of children presenting with cerebral malaria who were enrolled in a prospective study at the Jinja Regional Referral Hospital in Uganda (ClinicalTrials.gov identifier: NCT01255215). Tie-2 can be cleaved into soluble ectodomain fragments during states of endothelial activation (24). Hence, circulating concentrations of sTie-2 were measured as an additional indicator of alterations in the Ang–Tie-2 signaling pathway. Population characteristics of these children are provided in Table 1.

Ang-1 concentrations were decreased at admission in children with severe malaria and increased during the course of clinical recovery in survivors ($P < 0.0001$, $n = 82$; Fig. 1A). Ang-1 concentrations were higher at the time of convalescence in survivors (day 14 follow-up; $P < 0.001$) compared to Ang-1 concentrations at admission (Fig. 1B).

For sTie-2, median plasma concentrations were elevated at presentation and gradually declined with recovery in survivors ($P < 0.0001$; Fig. 1C). sTie-2 concentrations were lower at convalescence compared to admission ($P < 0.001$; Fig. 1, C and D). Collectively, these data suggest that the circulating concentrations of Ang-1 available to activate Tie-2 were decreased in children presenting with severe malaria. Moreover, higher circulating concentrations of sTie-2 support the hypothesis that Tie-2 signaling may be impaired during infection.

Disease severity in a murine model of experimental cerebral malaria is associated with decreased Ang-1 and increased Ang-2

These clinical data, along with previous reports showing high Ang-2 concentrations in severe malaria (23), suggest that altered Ang–Tie-2 signaling may contribute to malaria-associated vascular dysfunction; however, causality is difficult to establish in human observational studies. Therefore, we used the experimental cerebral malaria model to investigate a potential mechanistic role for this pathway. To validate this model for the investigation of the Ang–Tie-2 axis, we assessed the kinetics of Ang-1 and Ang-2 expression over the course of infection with *Plasmodium berghei* ANKA (PbA), a strain of *Plasmodium* parasite isolated from rodents in Central Africa often used to model human cerebral malaria. In this model, C57BL/6 mice developed marked behavioral and neurological impairments, as assessed by the rapid murine coma and behavioral score (RMCBS) (25), and became moribund with signs of limb paralysis, ataxia, and/or convulsions 6 to 10 days after PbA

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inoculation (Fig. 2, A and B). BALB/c mice receiving the same inoculum developed fewer neurological manifestations and showed prolonged survival (Fig. 2, A and B). Neurological impairment and death in both strains occurred when parasite levels were relatively low (5 to 15%) and comparable between strains (Fig. 2C). Collectively, these findings support the hypothesis that host determinants influence progression to severe and fatal disease, consistent with data for human cerebral malaria (6, 7).

BBB integrity in PbA-infected mice was assessed with the Evan's blue (EB) assay. C57BL/6 mice had increased EB extravasation into the brain parenchyma compared to BALB/c mice on the same day of infection, despite comparable parasite burdens ($P = 0.005$; Fig. 2, D and E). Longitudinal evaluation showed that Ang-1 concentrations decreased ($P < 0.0001$; Fig. 2F) and Ang-2 concentrations increased over the course of infection in both mouse strains ($P < 0.0001$; fig. S1, A and B). However, mice with prolonged survival (that is, BALB/c mice) maintained higher Ang-1 and lower Ang-2 concentrations compared to the more susceptible C57BL/6 mice ($P < 0.001$; Fig. 2F and fig. S1, A and B). PbA-infected C57BL/6 mice also displayed increased concentrations of Ang-2 in brain tissue compared to BALB/c mice (fig. S1, C and D). The decrease in Ang-1 was temporally associated with the onset of neurological impairment and experimental cerebral malaria (Fig. 2G). Overall, there was a significant relationship between circulating Ang-1 concentrations and the time to death ($P = 0.0004$; Fig. 2H), suggesting that Ang-1 may be a critical determinant of survival.

The appropriate matrix (that is, serum versus plasma) for analysis of platelet-stored angiogenic factors, such as Ang-1, has previously been investigated. In human *P. falciparum* malaria biomarker studies, platelet-poor plasma is recommended to minimize artifacts due to ex vivo platelet activation (26). To address this experimentally, longitudinal platelet-poor plasma samples were collected from experimental cerebral malaria–susceptible mice using a recommended protocol to minimize ex vivo platelet activation. Median plasma Ang-1 values in malaria-infected mice (fig. S2) were comparable to plasma concentrations observed in the human malaria patient cohort (Fig. 1A). In murine experimental cerebral malaria, plasma concentrations (fig. S2) were about 100-fold lower than serum concentrations (Fig. 2F) (that is, median concentration in PbA-naïve C57BL/6 mice: plasma = 617.7 ng/ml, serum = 5.16 ng/ml), confirming previous reports that platelets are a major source of Ang-1 (27). A decrease in Ang-1 concentrations was observed in both matrices (Fig. 2F and fig. S2).

Ang-2–specific inhibitors do not alter disease outcome in experimental cerebral malaria

Given that increased Ang-2 has been implicated in cerebral malaria pathogenesis (23), we evaluated whether blocking circulating Ang-2 would be sufficient to preserve endothelial integrity and improve outcome in the experimental cerebral malaria model. Two independent therapeutic strategies were used: an anti–Ang-2 monoclonal antibody (anti–Ang-2 monoclonal antibody, REGN910) and a peptibody (L1–10) designed to specifically inhibit Ang-2–Tie-2 interactions, using doses that have previously been used in vivo (28, 29). Unexpectedly, neither intervention improved survival in experimental cerebral malaria–susceptible C57BL/6 mice, as compared to mice receiving isotype control antibodies (fig. S3, A and B), or improved neurological impairment (fig. S3, C and D). Circulating concentrations of soluble adhesion molecules (sICAM-1 and sVCAM-1), which serve as

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indicators of endothelial activation, were increased with PbA infection in both anti–Ang-2 antibody–treated and isotype control antibody–treated mice (fig. S3E). This indicated that Ang-2 blockade did not suppress endothelial activation in experimental cerebral malaria. There was also no evidence of improved BBB function in anti–Ang-2 antibody–treated mice versus isotype control antibody–treated mice (fig. S3, F and G).

Genetic deletion of Ang-1 increases susceptibility to experimental cerebral malaria

We hypothesized that the lack of a benefit with anti–Ang-2 interventions in this model could be due to the decrease in circulating Ang-1 observed in PbA-infected experimental cerebral malaria–susceptible mice (Fig. 2F) (that is, no receptor ligand was present to induce Tie-2 activation). To test the importance of Ang-1 in this context, we used a genetic approach to investigate the role of Ang-1 in mediating protection in experimental cerebral malaria. We postulated that if Ang-1 contributes to protection in BALB/c mice, then genetic disruption of Ang-1 should render these mice more susceptible to experimental cerebral malaria. Ang-1 is necessary for embryonic development, and Ang-1–deficient mice generated by conventional knockout strategies develop vascular malformations and die by embryonic day 12.5 (30). Therefore, we used a conditional knockout system (tetracycline-inducible Cre/*lox*P system) (17), whereby the deletion of Ang-1 could be temporally controlled after embryonic development (BALB/c.*Angpt1*$^{flox/del}$, herein referred to as BALB/c.*Angpt1*$^{-/-}$). Doxycycline-induced gene deletion resulted in the loss of Ang-1 production, as assessed by circulating concentrations of Ang-1, with significantly lower Ang-1 in BALB/c.*Angpt1*$^{-/-}$ mice compared to BALB/c.*Angpt1*$^{+/+}$ controls ($P < 0.001$; Fig. 3A). Concentrations of Ang-1 in BALB/c.*Angpt1*$^{-/-}$ mice were comparable to those observed in susceptible C57BL/6 at the time of onset of experimental cerebral malaria (Fig. 3A).

Genetic deletion of *Angpt1* in BALB/c mice resulted in increased mortality after PbA challenge compared to BALB/c.*Angpt1*$^{+/+}$controls ($P = 0.02$; Fig. 3B). Survival in PbA-infected BALB/c.*Angpt1*$^{-/-}$ mice was comparable to that in experimental cerebral malaria–susceptible C57BL/6 mice (Fig. 3B). Collectively, these data indicate that genetic disruption of *Angpt1* resulted in enhanced susceptibility to experimental cerebral malaria and suggest a causal mechanistic role for Ang-1 in mediating protection in this model.

Ang-1 is necessary to maintain BBB integrity in experimental cerebral malaria

EB dye was used to assess whether loss of Ang-1 would alter microvascular function in BALB/c mice (Fig. 2D and E). On day 8 after PbA-inoculation, wild-type BALB/c mice showed little evidence of dye extravasation consistent with an intact BBB (Fig. 3C). In contrast, there was increased EB accumulation in the brain parenchyma of BALB/c.*Angpt1*$^{-/-}$ mice ($P < 0.05$; Fig. 3D). The extent of EB dye extravasation in the brain parenchyma of BALB/c.*Angpt1*$^{-/-}$ mice was comparable to that of experimental cerebral malaria–susceptible C57BL/6 mice (Fig. 3D). These results indicate that Ang-1 is required for preservation of BBB integrity in BALB/c mice during PbA infection. Changes in vascular function were only evident in animals challenged with PbA. There was no evidence of altered microvascular integrity at baseline in BALB/c.*Angpt1*$^{-/-}$ mice (Fig. 3, C and D).

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Systemic delivery of Ang-1 improves survival in experimental cerebral malaria

As a complementary approach to define a mechanistic role for Ang-1, we examined whether systemic supplementation could modulate outcome in experimental cerebral malaria–susceptible C57BL/6 mice using an adenoviral vector encoding human *Angpt1*, termed AdAng1.

Intravenous delivery of AdAng1 yielded high systemic Ang-1 concentrations in C57BL/6 mice (Fig. 4A and fig. S4) and resulted in improved survival, with 61% of AdAng1-treated mice surviving until the end of the study compared to 35% of animals receiving CtlAdV ($P = 0.027$, $n = 20$ to 25 per group; Fig. 4B). Improved neurological performance in AdAng1-treated mice was also observed, with significantly fewer AdAng1-treated mice developing experimental cerebral malaria by day 7 after inoculation ($P = 0.015$; Fig. 4C), despite having parasite burdens comparable to control-treated animals (Fig. 4D).

In these studies, the empty AdV vector yielded partial protection compared to control mice receiving saline alone (35% survival versus 3.7% survival, $P = 0.011$; Fig. 4B). Improved protection with the adenoviral vector alone is consistent with observations that intravenous viral delivery may induce a type I interferon (IFN) response, which could modify experimental cerebral malaria disease outcomes (31).

Therapeutic administration of recombinant Ang-1 improves survival

To overcome constraints associated with adenoviral delivery and confirm that protection with AdAng1 was the result of increased circulating Ang-1 and not another attribute of this reagent, we assessed the effect of therapeutic administration of a recombinant human Ang-1 protein, termed BowAng1 (32). BowAng1 contains the C-terminal fibrinogen-like domain of the angiopoietin protein fused to human immunoglobulin G1 (IgG1) Fc fragments and engineered into a tetramer conformation (Fig. 5A) for optimal Tie-2 binding (32). The ability of BowAng1 to induce phosphorylation of mouse Tie-2 in vivo has been previously demonstrated (32).

Treatment allocation to either BowAng1 or Fc control was masked. Therapeutic subcutaneous administration of BowAng1 (25 mg/kg) in mice with established PbA infection significantly improved survival compared to Fc control–treated mice ($P < 0.05$; Fig. 5B). The improved survival conferred by BowAng1 was independent of direct effects on parasitemia (Fig. 5C). Mice receiving BowAng1 had improved clinical scores compared to parasitemia-matched control-treated mice (Fig. 5, D and E). These effects were independent of body weight (Fig. 5F), used as an indirect measure to monitor food and water intake.

Ang-1 maintains a quiescent endothelial cell phenotype and preserves BBB integrity in the presence of systemic inflammation

BBB dysfunction is a hallmark of pediatric cerebral malaria and experimental cerebral malaria (6, 7, 9, 10, 33). BowAng1-treated mice had significantly reduced microvascular permeability compared to PbA-infected Fc controls ($P < 0.05$; Fig. 6, A and B), despite

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similar parasitemia. These data indicate that exogenous Ang-1 is sufficient to preserve BBB function after a lethal malaria challenge.

An enhanced proinflammatory response to infection, including increases in IFN-γ and tumor necrosis factor (TNF), has been implicated as a mediator of disease pathogenesis in malaria (34). To determine whether vascular protection with BowAng1 occurred secondary to a reduction in inflammatory cytokines or whether protection was due to direct maintenance of endothelial cell quiescence and stability in the presence of a systemic inflammatory response, we assayed plasma samples for key proinflammatory cytokines. PbA-infected C57BL/6 mice displayed a significant increase in the plasma concentration of TNF and IFN-γ, on day 6 after inoculation (Fig. 6, C and D), with a 3-fold increase in TNF and 60-fold increase in circulating IFN-γ compared to baseline concentrations (that is, plasma concentrations before PbA infection) ($P < 0.01$).BowAng1treatmentdidnot affect TNF or IFN-γ concentrations (Fig. 6, C and D).

Up-regulation of receptors on activated endothelial cells due to systemic inflammation and/or direct endothelial parasite interactions may facilitate parasite cytoadhesion (7, 11). Conversely, down-regulation of cellular adhesion molecules may reduce the potential for parasite sequestration and vascular leak. We investigated whether BowAng1 treatment would reduce circulating concentrations of cellular adhesion molecules, used as an indicator of endothelial activation and a pro-adhesive endothelial cell phenotype.

PbA-infected C57BL/6mice showed an increase in plasma sICAM-1and sVCAM-1 concentrations compared to baseline controls (that is, before infection) ($P < 0.001$; Fig. 6, E and F). These concentrations were reduced in PbA-infected, BowAng1 treated mice compared to PbA-infected controls ($P < 0.05$; Fig. 6, E and F). These data indicate that Ang-1 may contribute to vascular stability in the context of systemic inflammatory responses, in part through preservation of the endothelium in a quiescent, anti-adhesive state.

Adjunctive Ang-1 treatment improves survival compared to antimalarials alone

To determine whether adjunctive treatment with BowAng1 confers a survival benefit over antimalarial therapy alone, we evaluated the outcome in PbA-infected C57BL/6 mice treated with artesunate plus BowAng1 compared to mice receiving artesunate alone (fig. S5A). Parasitemia was significantly reduced within 24 hours of artesunate treatment in both groups (fig. S5B), indicating that Ang-1 treatment did not interfere with artesunate efficacy in this model. Despite reducing the parasite burden to preexperimental cerebral malaria levels (that is, <2% parasitemia), about 40% of artesunate-treated mice died of experimental cerebral malaria (fig. S5, C and D). However, adjunctive treatment with BowAng1 or AdAng1 on day 4 of established infection significantly improved survival over artesunate therapy alone ($P < 0.05$, log-rank test), with 100% of the mice receiving artesunate plus Ang-1 treatment surviving until the end of the study (fig. S5, C and D).

To more closely mimic the clinical scenario where children present with neurological impairment, we also evaluated the efficacy of artesunate administered with and without BowAng1 delivered after the onset of BBB leak/dysfunction. To determine the most appropriate day for intervention, we evaluated the kinetics of BBB dysfunction. Significant

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BBB leak was evident by day 5 after PbA, with 60% (three of five) of the mice in this study showing increased EB dye in the brain (Fig. 7, A and B). PbA-infected mice receiving a single-dose late-stage treatment with BowAng1 (that is, no artesunate) administered at the onset of BBB leak (that is, day 5; Fig. 7C) had significantly less BBB disruption, as determined by EB dye extravasation, compared to their parasitemia-matched Fc-treated controls [$P = 0.0059$, 90% (9 of 10); Fig. 7D], despite having comparable parasite burdens (Fig. 7E). Finally, mice receiving adjunctive Ang-1 therapy (that is, BowAng1 plus artesunate) administered after the onset of BBB leak and experimental cerebral malaria (that is, day 5 after PbA) had improved survival ($P = 0.0068$; Fig. 7F) and significantly decreased progression to severe cerebral malaria (Fig. 7G) compared to mice receiving artesunate alone.

DISCUSSION

Adjunctive therapies targeting the host response may represent a strategy to improve survival and prevent neurological injury in severe malaria (7). Here, we investigated the role of the Ang–Tie-2 axis in the pathogenesis of severe malaria and generated several lines of evidence supporting this pathway as a potential therapeutic target. First, we demonstrated in a prospective study that concentrations of Ang-1 and soluble Tie-2 are dysregulated in children with severe and cerebral malaria. Altered angiopoietin concentrations observed in cerebral malaria support the hypothesis that decreased Ang-1 and increased sTie-2 may contribute to the impaired Tie-2 activation and endothelial dysfunction associated with cerebral malaria pathogenesis. However, it is challenging to establish whether altered angiopoietin concentrations are a cause or consequence of severe disease in human studies.

Therefore, to explore mechanism and intervention strategies, we validated the involvement of the Ang–Tie-2 axis in an experimental model of cerebral malaria. In this model, we corroborated human clinical data and demonstrated that altered angiopoietin concentrations correlated with malaria disease severity and outcome. Employing both genetic and pharmacological strategies, we established that Ang-1 is required to maintain BBB integrity and, when delivered as an adjunctive therapeutic with artesunate, can improve survival above that with antimalarial therapy alone.

Previous studies have established Ang-1–Tie-2 activation as an essential pathway in developmental biology (30) and in endothelial barrier stability, in part by preventing inter-endothelial gap junction formation, stabilizing vascular endothelial–cadherin at cell junctions and preventing cytoskeletal rearrangements (11, 15–17, 35). Endothelial activation and BBB dysfunction is a central feature of human cerebral malaria and experimental cerebral malaria (7, 10, 33). Here, we show that Ang-1 preserves BBB integrity during experimental cerebral malaria. Mice with genetically disrupted Ang-1 display increased BBB dysfunction and increased mortality after PbA challenge. We observed no evidence of disrupted microvascular integrity in uninfected mice lacking Ang-1 (that is, a mature, quiescent microvasculature). These findings are consistent with previous data suggesting that Ang-1 may be redundant in a nonstressed vasculature but required during an activated or stressed state (17).

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A primary objective of this work was to address whether interventions to modulate the Ang–Tie-2 pathway could maintain vascular integrity and improve survival after a lethal malaria challenge. Adenoviral vectors to drive Ang-1 expression in vivo improved outcome in experimental cerebral malaria (Fig. 4). These findings are consistent with reports that Ang-1 can prevent vascular dysfunction (15, 16) and increase survival in murine models of sepsis (35). Although useful to establish proof of principle, issues associated with adenoviral delivery, therapeutic timing, immune activation, and potential toxicity may limit widespread clinical adoption of AdAng1-based interventions. To overcome these constraints, we used an engineered human Ang-1 protein, BowAng1 (32). Therapeutic delivery of BowAng1 preserved BBB integrity and improved survival in susceptible mice receiving no antimalarial therapy as well as in mice receiving BowAng1 in combination with artesunate, the current first-line therapy for human severe malaria. This was also the case when BowAng1 intervention was administered in a clinically relevant treatment strategy after the onset of neurological signs and BBB leak at day 5 after infection.

Infection-induced inflammation has been considered an important target for intervention to improve clinical outcome in severe malaria and other life-threatening infections (36). However, inflammation also plays critical roles in microbial control and in the development of acquired immunity (37). Therefore, it is of interest that Ang-1–based therapeutics preserved BBB integrity and improved survival, despite a robust systemic inflammatory response. These data suggest that antiinflammatory strategies may not be required to preserve microvascular integrity and improve outcome in life-threatening infections.

Increased circulating Ang-2 concentrations are associated with disease severity and poor outcome in *P. falciparum* infection (23). Here, we extended these observations to the PbA experimental cerebral malaria mouse model (fig. S2). However, it is unknown whether Ang-2 is causally involved in the pathobiology of cerebral malaria and whether inhibiting Ang-2 would improve the outcome in severe malaria. Our findings suggest that anti–Ang-2 strategies alone are insufficient to improve outcome, at least in the experimental cerebral malaria model. A potential explanation for the lack of efficacy with anti–Ang-2 approaches is that strategies to block inhibitors (that is, Ang-2) in the context of experimental cerebral malaria may be insufficient to improve outcome in the absence of a Tie-2 stimulatory ligand such as Ang-1. These observations suggest that direct activation of Tie-2 (that is, pro–Ang-1 strategies) may be a more effective intervention under conditions associated with low Ang-1, such as severe malaria.

The role of Ang-2 in experimental cerebral malaria remains unresolved; however, studies have suggested a context-specific role for this mediator (29, 38). Ang-2 expression may be induced as a compensatory response to the loss of Ang-1–induced endothelial cell survival signaling [for example, low Akt activity (38)], which could explain why blocking Ang-2 in the context of experimental cerebral malaria (that is, a condition with low circulating Ang-1) does not confer a survival advantage. However, additional studies are required to address this question.

Recently, it was reported that differential expression of *Tie-2* (also known as *Tek*) was a key determinant of resistance or susceptibility in other models of infectious diseases, such as

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sepsis (39) and Ebola virus infection (40), consistent with the hypothesis that disruptions in this regulatory pathway may contribute to microvascular leak and fatal outcome in other serious infections (35, 41). Hence, Tie-2–promoting Ang-1–based interventions may have broad therapeutic implications for a number of severe infections including sepsis, anthrax, toxic shock syndrome, dengue shock syndrome, and viral hemorrhagic fevers, for which no specific adjunctive therapies currently exist (35). However, additional investigation in both preclinical models and human studies will be required to test this hypothesis.

The underlying basis of Ang-1 suppression and sTie-2 release during malaria infection requires further study. A number of physiological processes that occur in severe malaria, including inflammation and hypoxia, have been shown to suppress Ang-1 expression. This may explain, at least partly, why experimental cerebral malaria–susceptible C57BL/6 mice, with a greater proinflammatory response to infection, have lower circulating Ang-1 concentrations. Genetic differences may also dictate the responsiveness of the endothelium to vascular challenge, as has been previously described to explain differential responses to ischemia and remodeling in inbred mouse strains (42). Decreased concentrations of Ang-1 may also be due to a loss of cellular sources of Ang-1, such as platelets (27), because thrombocytopenia and platelet aggregation are common features of severe malaria. Increased soluble Tie-2 fragments may be due to ectodomain cleavage regulated by factors such as vascular endothelial growth factor and proteolytic enzymes (that is, matrix metalloproteinases) (24).

Potential limitations of our study include the use of preclinical models to study human malaria. To mitigate these issues, we first established the relevance of the Ang–Tie-2 axis to human disease in a population with the highest burden of severe and fatal malaria—children in sub-Saharan Africa. Only after confirming that this pathway was dysregulated in the experimental model in a manner similar to that observed in human disease did we examine the mechanism and causality in this model. To help ensure the validity and utility of our preclinical studies, we used investigative principles such as (i) the use of randomized and blinded treatment allocation; (ii) the delivery of therapeutic agents after established infection; and (iii) the administration of investigational agents in combination with antiparasitic drugs, to test adjunct benefit and ensure no interference with artesunate drug efficacy. In accordance with our animal care guidelines, experimental mice were euthanized when they showed signs of neurological impairment, and therefore, we were not able to directly model the scenario where children present with coma. Nonetheless, we showed that BowAng1 delivered after the onset of BBB dysfunction and leak can improve outcome above that of antimalarials alone in our mouse model. This study provides an experimental system to establish proof of principle for the mechanistic involvement of the Ang–Tie-2 axis and to investigate interventions based on this pathway. Any promising adjunctive therapeutic intervention identified using the experimental models will ultimately require rigorous clinical trials before any conclusions about human efficacy can be reached.

The data presented in this study provide additional evidence that circulating markers of underlying pathophysiology could be used to risk-stratify those patients most likely to benefit from endothelial-based interventions such as Ang-1. Here, we show that changes in Ang-1 and Ang-2 concentrations occur before the onset of marked neurological disease and

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that Ang-1 therapeutics delivered in these atrisk animals can improve outcome beyond that achieved with artesunate alone. Ultimately, using circulating markers to enable risk stratification and to inform evidence-based therapeutic decisions (for example, pro–Ang-1 therapies) may help to close the mortality gap in human severe malaria (23).

Collectively, our findings support further investigation of Ang–Tie-2 interventions as potential adjunctive therapeutics for severe malaria and other life-threatening infections associated with endothelial dysfunction and multiple-organ failure. However, it remains to be proven whether targeting this pathway will be sufficient to reverse endothelial dysfunction and microvascular compromise in established human disease.

MATERIALS AND METHODS

Human clinical study

Study design, population, and ethics—The objective of this study was to assess Ang-1 and sTie-2 kinetics in severe malaria patients from hospital presentation to recovery. Plasma samples were obtained from children with confirmed *P. falciparum* severe malaria (that is, cerebral malaria and/or respiratory distress; Table 1) enrolled in the placebo arm of a prospective, randomized control trial (ClinicalTrials.gov identifier: NCT01255215). Patients received intravenous artesunate at the recommended dose and frequency (4). Only plasma samples from patients that survived infection ($n = 82$) were included in the analysis. Four patient samples were excluded from analysis because of missing longitudinal data. The study was approved by the Makerere University School of Medicine Research Ethics Committee, the Uganda National Council on Science and Technology, the National Drug Authority of Uganda, and the University Health Network Research Ethics Committee.

Human clinical sample collection and biomarker assessment—Blood samples were collected in K2EDTA Microtainer tubes (BD Biosciences) at admission (day 0), daily for the first 72 hours (days 1, 2, and 3) of hospital admission, and at follow-up (day 14). Plasma was stored at −80° C until Ang-1 and sTie-2 ELISA assays were performed in duplicate according to the manufacturer's instructions (R&D Systems), by investigators blinded to the clinical data.

Murine studies

Study design and ethics approval—Preclinical studies were designed to investigate whether angiopoietins were causally involved in cerebral malaria pathogenesis with survival as the primary end point. Investigators were blinded to treatment allocation (that is, treatment type was labeled "A" or "B"). The masking code was not revealed until completion and analysis of three independent studies. Experiments involving animals were approved by the University Health Network and Toronto General Hospital Animal Care Committee.

Experimental animals—Female 8-week-old C57BL/6 and BALB/c mice were obtained from the Jackson Laboratory. Angpt1 *flox* (*floxed*), Angpt1 *del*, *ROSA-rtTA (ROSA*), and *tet-O Cre* (*Cre*) mice (17) were backcrossed with BALB/c mice from the Jackson Laboratory

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for 10 generations. Mice hemizygous for the ROSA-rtTA and tetO-Cre transgene but not the Ang-1^{flox} or del allele were used as wild-type controls (*Angpt1*$^{+/+}$).

Transgene mice were treated with doxycycline via drinking water ad libitum for 14 days to induce floxed exon gene excision, as assessed by Ang-1 ELISA (R&D Systems). Mice were kept on normal drinking water for 2 weeks before initiation of PbA experiments to clear doxycycline, which has antimalarial activity. Both male and female *Angpt1*$^{-/-}$ mice were used for experimental analysis (with littermate, sex, and age-matched *Angpt1*$^{+/+}$ controls).

Murine experimental cerebral malaria model—Infection was initiated by intraperitoneal injection of 1×10^6 PbA (MR4)–parasitized erythrocytes (PEs) (considered day 0 of infection), unless otherwise stated. Peripheral parasitemia was determined by modified Giemsa-stained thin-blood smears (Protocol Hema 3 Stain Set, Sigma). Quantitative assessment of experimental cerebral malaria–associated neurological impairment was performed using a modified version of the RMCBS (25). In these studies, a conservative cutoff score (<30%) was used to classify mice with experimental cerebral malaria.

Drug treatment regimens—Initial Ang-based interventions were initiated on day 4 after inoculation when C57BL/6 mice have detectable peripheral parasitemia, unless otherwise specified. The replication-deficient human type 5 adenoviral vector coding for human *Angpt1* (AdAng1) and the E1 region-deleted AdEasy-1 empty vector control (CtlAdV) were generated as described (43). AdAng1 and CtlAdV were propagated in human embryonic kidney–293 cells (American Type Culture Collection) and purified using the Adeno-X Maxi Purification Kit (Clontech).

For survival studies, experimental mice were randomized to receive either AdAng1 (10^9 pfu) or CtlAdV (10^9 pfu) in sterile saline via intravenous injection. A pilot study demonstrated that increased circulating Ang-1 concentrations are detectable 24 hours after AdAng1 delivery and remain detectable for at least 3 to 5 days after injection (fig. S4).

BowAng1 (REGN108), an engineered construct consisting of the fibrinogen-like binding domain of Ang-1 fused to the Fc fragments of human IgG (32), was provided by Regeneron Pharmaceuticals. A schematic comparison of native Ang-1 with BowAng1 is shown in Fig. 5A [modified from (32)]. BowAng1 has been shown to induce phosphorylation of Tie-2 in endothelial cells in vitro and in vivo (32).

PbA-infected mice were randomized to receive either (i) BowAng1 (25 mg/kg) in injectable saline solution (0.9% sodium chloride), (ii) human IgG1 Fc control (2G8 α-*Candida* glucan, 25 mg/kg), or (iii) an equal volume of saline via subcutaneous injection. Treatment was continued every 48 hours. In these survival studies, the treatment type was masked, and the code was not revealed until after data analysis.

Adjunctive treatment in combination with artesunate—For assessment of adjunctive benefit in mice with experimental cerebral malaria and onset of BBB dysfunction, mice received a subcurative dose of the antiparasitic drug artesunate (7.5 mg/kg; Sigma-

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Aldrich) delivered by a single intraperitoneal injection alone or with a single dose of BowAng1 (25 mg/kg) on day 5 after PbA inoculation with 5×10^5 PEs (Fig. 7C).

Statistical analysis—Statistical analysis was performed using Graph-Pad Prism version 4.0. Survival was analyzed by log-rank test. Independent survival studies were conducted, and data were pooled unless otherwise specified. Differences between groups were analyzed by Mann-Whitney test or Kruskal-Wallis test followed by Dunn's post hoc. Two-way ANOVA was used to compare groups over multiple times. For plasma marker testing, the Friedman test was used to compare sequential patient samples with Dunn's multiple comparison test to examine differences at specified times (for example, admission versus convalescence). Normally distributed data are presented as means ± SEM. A P value of <0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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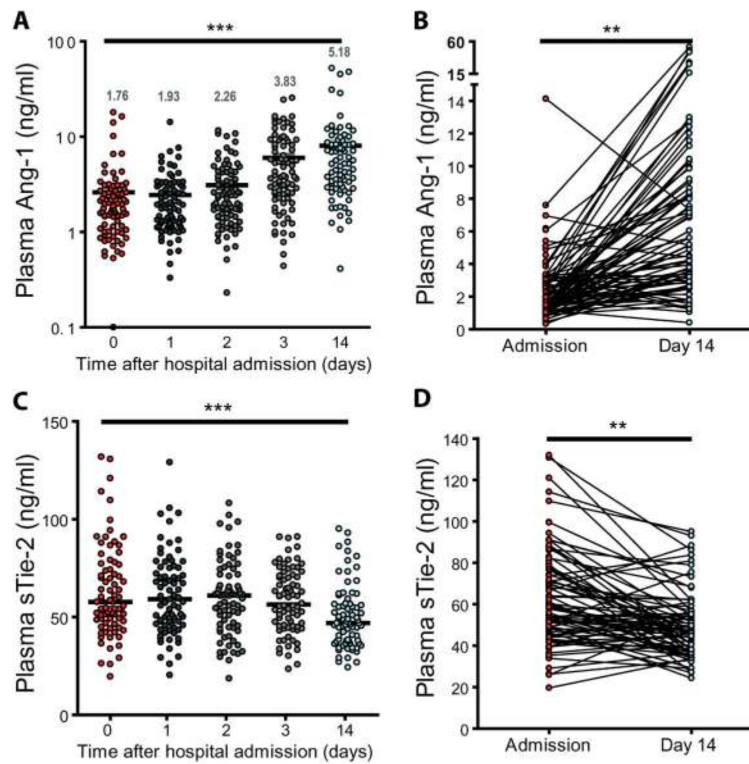

Fig. 1. Longitudinal Ang-1 and sTie-2 concentrations are associated with clinical recovery in Ugandan children presenting with severe malaria

(**A** to **D**) Plasma Ang-1 (A and B) and sTie-2 (C and D) concentrations were measured by enzyme-linked immunosorbent assay (ELISA) in a cohort of Ugandan children with confirmed *P. falciparum* severe malaria at presentation, during in-hospital treatment (days 1 to 3 after admission) and at day 14 follow-up after hospital discharge (representing clinical recovery). Graphs show median and scatters of (A) $\log_{10}$-transformed Ang-1 (nanogram per milliliter), with median concentration (nanogram per milliliter) numerically represented above each plot, and (C) sTie-2 (nanogram per milliliter) values. ***$P < 0.0001$ Friedman test ($n = 82$ plasma samples per time, except day 14 where $n = 78$ plasma samples). A representation of the change in protein concentrations from admission (red circle) to recovery (day 14 follow-up; blue circle) in the same patient is shown for Ang-1 (B) and sTie-2 (D). **$P < 0.001$, Friedman test with Dunn's multiple comparison ($n = 78$ plasma samples).

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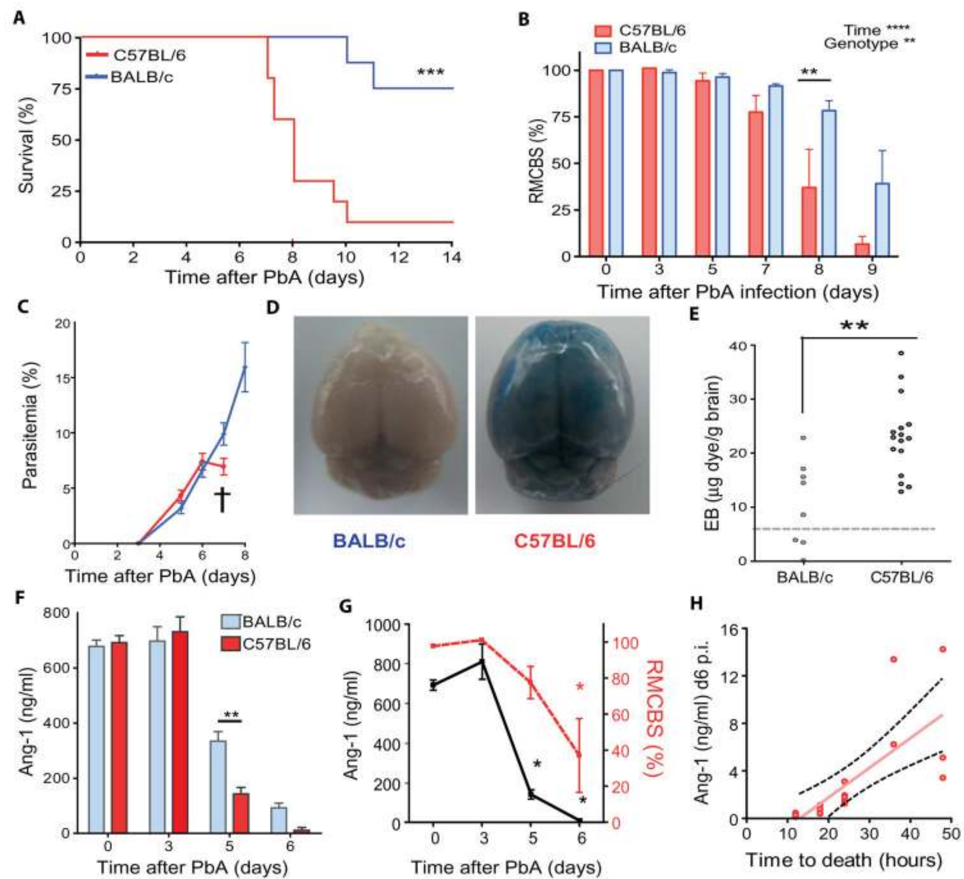

Fig. 2. Ang-1 concentrations are associated with disease severity and outcome in a murine model of experimental cerebral malaria

(**A**) Kaplan-Meier survival analysis of C57BL/6 mice (red line) and BALB/c mice (blue line) infected with 1×10^6 PbA-parasitized erythrocytes (****P* = 0.001, log-rank test; *n* = 10 mice per group). (**B**) RMCBS for C57BL/6 (red) and BALB/c mice (blue) over the course of infection (***P* < 0.01, two-way analysis of variance (ANOVA) with Bonferroni's posttest; *n* = 5 mice per group per time point from a representative study, *****P* < 0.0001). (**C**) Mean peripheral parasitemia for C57BL/6 (red line) and BALB/c mice (blue line) after PbA infection. Error bars represent SEM. *n* = 10 mice per group; † indicates all C57BL/6 mice succumbed by day 7 after inoculation. (**D**) Representative photographs of brains collected from EB dye–injected PbA-infected BALB/c and C57BL/6 mice on day 6 after inoculation. (**E**) Quantification of EB dye extravasation in brain normalized to brain weight of each mouse. Dots are individual values; bars represent the means ± SEM for each group. Data are combined from two independent experiments (***P* = 0.0054, Mann Whitney; *n* = 10 to 15 mice per group). (**F**) Ang-1 serum concentrations from BALB/c mice (blue) and C57BL/6 mice (experimental cerebral malaria–susceptible mice; red) over the course of PbA infection (1×10^6 parasitized erythrocytes). ***P* < 0.001 two-way ANOVA with Bonferroni's posttest for the indicated comparison [*P* < 0.0001 (time), *P* < 0.05 (strain); *n* = 10 mice per time]. (**G**) RMCBS (%) and serum Ang-1 concentrations (nanogram per milliliter) plotted over the course of PbA infection (**P* < 0.05 compared to naïve, one-way ANOVA). (**H**) Scatter plots showing linear regression analysis between serum Ang-1 (nanogram per milliliter)

concentrations and time to death (hours) from the time of sample collection ($r^2 = 0.5492$, $P = 0.0004$; $n = 18$). d6 p.i., day 6 after infection.

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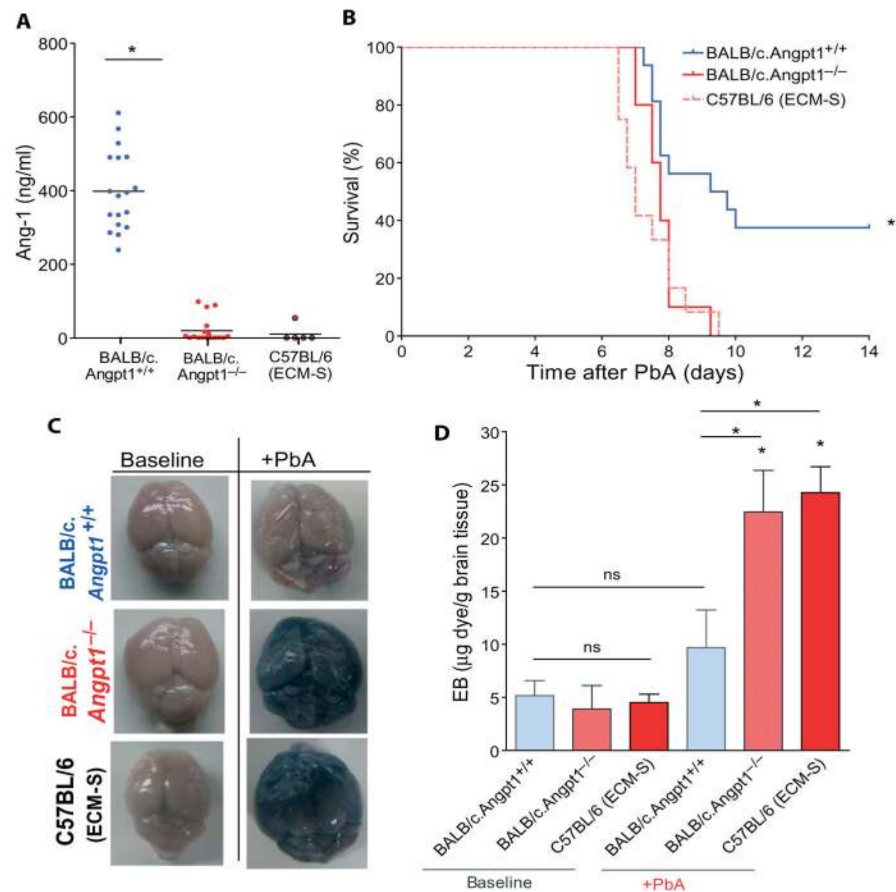

Fig. 3. Ang-1 is required for resistance to PbA infection

(**A**) Serum Ang-1 concentrations in BALB/ c.*Angpt1*$^{-/-}$ and BALB/c.*Angpt1*$^{+/+}$ mice after doxycycline to induce floxed exon gene excision and C57BL/6 experimental cerebral malaria–susceptible (ECM-S) comparator mice (*$P < 0.05$, Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison test; $n = 20$). (**B**) Kaplan-Meier survival analysis after PbA infection in BALB/c.*Angpt1*$^{-/-}$ and BALB/c.*Angpt1*$^{+/+}$ mice (*$P < 0.01$, log-rank test; $n = 20$ per group, and C57BL/6 ECM-S mice from a representative study). (**C**) Photographs of brains collected from representative control BALB/c.*Angpt1*$^{+/+}$ mice (top), Ang-1–deficient mice (BALB/c.*Angpt1*$^{-/-}$; center) and comparator experimental cerebral malaria–susceptible mice (C57BL/6; bottom) injected intravenously with EB dye at baseline (left column) or after PbA infection (right column; + PbA) with parasitemia-matched control collected at day 8 after inoculation (comparator ECM-S samples were collected at day 6 after PbA infection). (**D**) Quantification of EB dye normalized to brain weight. Bars represent the means ± SEM (*$P < 0.05$, Kruskal-Wallis with Dunn's multiple comparison test; $n = 4$ to 8 per group).

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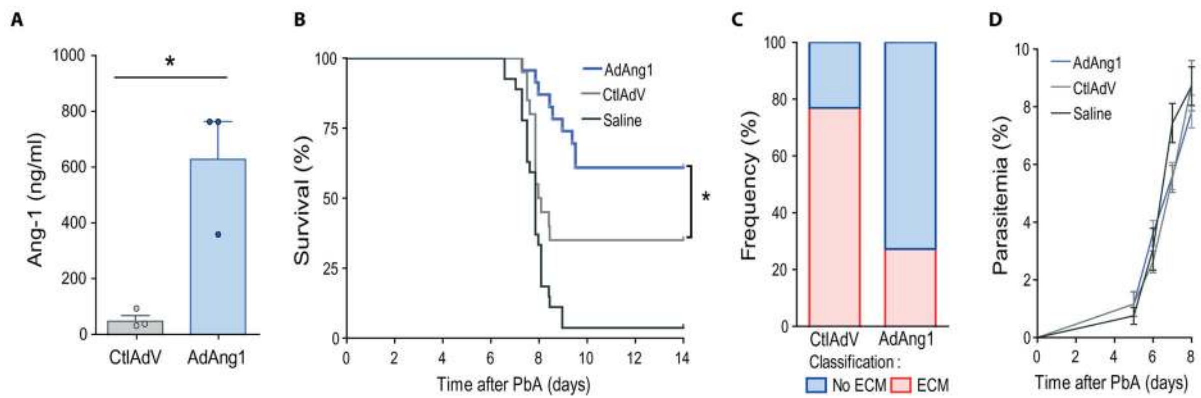

Fig. 4. Adenoviral vector expression of Ang-1 during PbA infection improves survival

(**A**) Serum Ang-1 concentrations in experimental cerebral malaria–susceptible C57BL/6 mice receiving AdAng1 or CtlAdV via intravenous injection on day 4 after PbA infection. Serum was collected 24 hours after AdV delivery (for example, day 5 after PbA infection) and analyzed by ELISA (*$P = 0.014$, *t* test; $n = 3$ per group). (**B**) Kaplan-Meier survival analysis for PbA-infected C57BL/6 mice receiving either AdAng1 [10^9 plaque-forming units (pfu)], CtlAdV (10^9 pfu), or saline on day 4 after PbA inoculation (*$P = 0.027$, log-rank test comparing AdAng1 to CtlAdV; $n = 20$ mice per group. Data combined from two independent studies). (**C**) Frequency of mice developing experimental cerebral malaria as determined by RMCBS in parasitemia-matched AdAng1- and CtlAdV-treated mice ($P < 0.05$, Fischer's exact test for categorical data). (**D**) Mean peripheral parasitemia for AdAng1 (10^9 pfu), CtlAdV (10^9 pfu), and vehicle control–treated mice after PbA infection.

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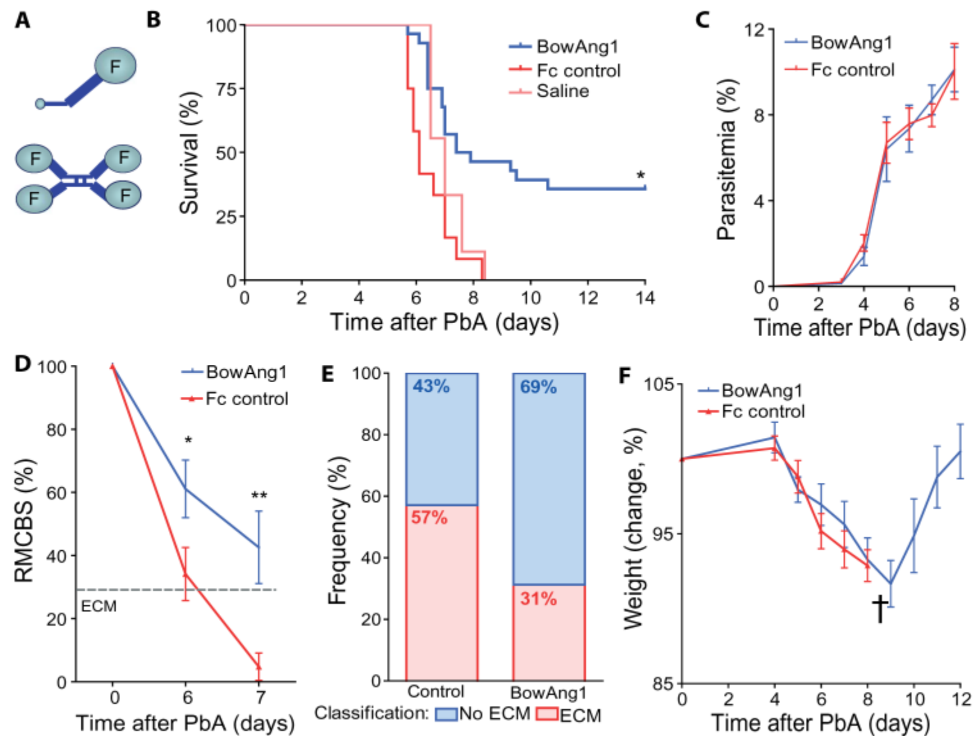

Fig. 5. Therapeutic Ang-1 supplementation with BowAng1 improves outcome in experimental cerebral malaria–susceptible mice after lethal *Plasmodium* challenge

(**A**) Schematic comparison of native Ang-1 (top) and the defined structure and multimerization state of BowAng1 (bottom). “F” refers to the fibrinogen-like domain that mediates angiopoietin receptor binding and ligand activity. (**B**) Kaplan-Meier survival analysis of PbA-infected C57BL/6 experimental cerebral malaria–susceptible mice treated with BowAng1 (25 mg/kg; blue line), Fc isotype control (25 mg/kg), or saline beginning on day 4 after inoculation and every 48 hours thereafter (*$P < 0.05$, log-rank test comparing BowAng1 treatment group to isotype control; $n = 30$ per group). (**C**) Mean parasitemia [$P =$ not significant (ns), two-way ANOVA]. ECM, extracellular matrix. (**D**) Temporal assessment of disease progression comparing BowAng1-treated versus control-treated mice over time (*$P < 0.05$; **$P < 0.01$, two-way ANOVA with Bonferroni’s posttest for multiple comparisons). (**E**) Frequency of experimental cerebral malaria as defined by RMCBS in parasitemia-matched BowAng1- and control-treated mice ($P < 0.05$, Fischer’s exact test for categorical data). (**F**) Weight loss data for BowAng1- and control-treated mice after PbA infection ($P =$ ns, two-way ANOVA). Data are presented as means ± SEM.

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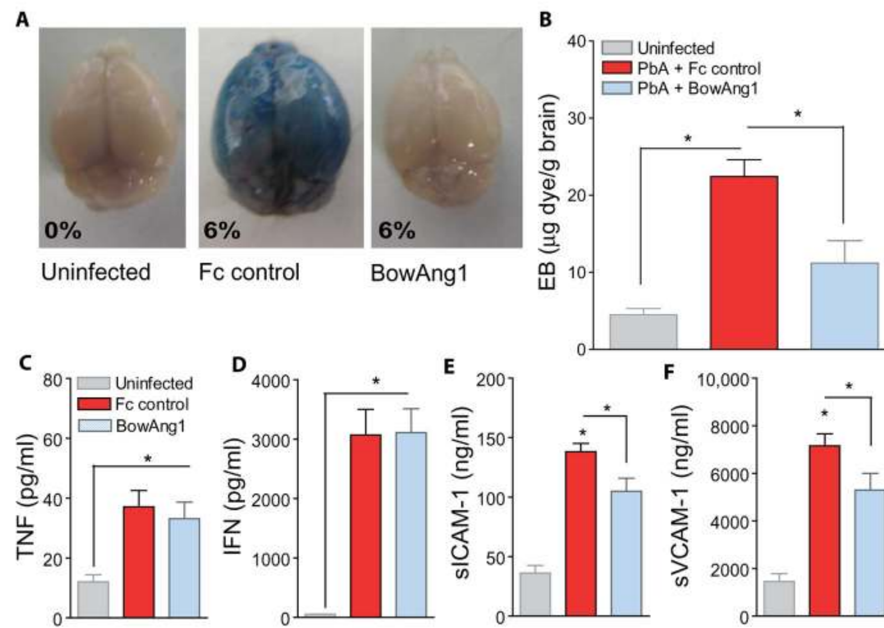

Fig. 6. Ang-1 preserves BBB integrity and decreases circulating concentrations of soluble endothelial adhesion molecules during PbA infection

(**A**) Representative brain images from EB dye–injected malaria naïve or PbA-infected C57BL/6 mice treated with either BowAng1 (25 mg/kg) or Fc isotype control (25 mg/kg). Parasitemia for each mouse is provided in the lower left corner of each image. (**B**) Quantification of EB dye in brain from uninfected and PbA-infected BowAng1- or Fc isotype control–treated mice (*$P < 0.05$, Kruskal-Wallis with Dunn's multiple comparison test; $n = 6$ to 12 mice per group). (**C** to **F**) Concentrations of TNF (C), IFN-γ (D), soluble vascular adhesion molecules, sICAM-1 (E), and sVCAM-1 (F) in plasma of malaria naïve and PbA-infected BowAng1- or control-treated C57BL/6 mice on day 6 after inoculation. *$P < 0.05$, one-way ANOVA with multiple comparison test as indicated; $n = 10$ mice per group. Data are presented as means ± SEM.

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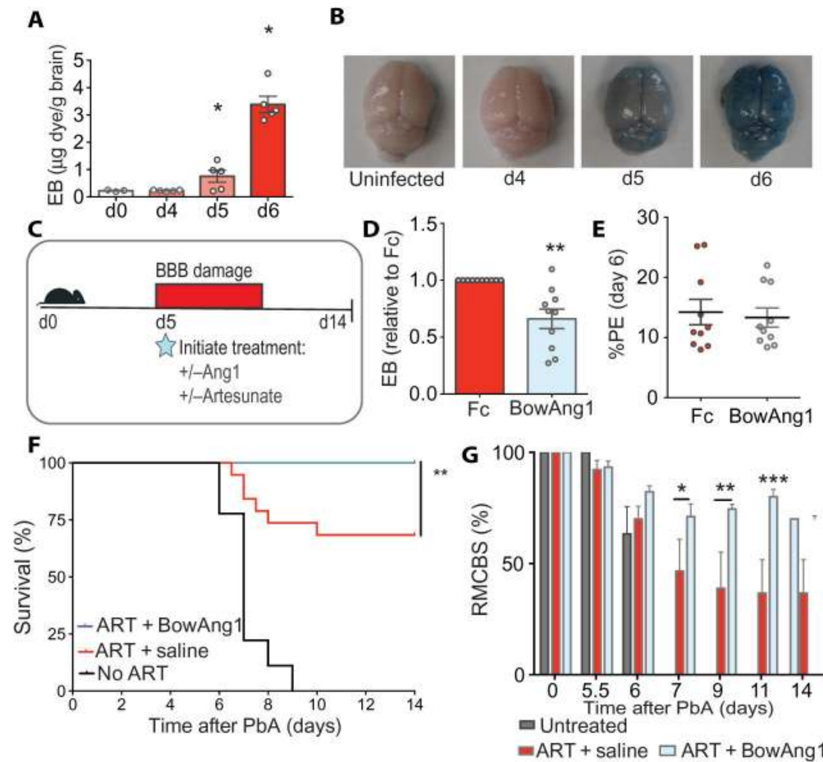

Fig. 7. Adjunctive Ang-1 treatment improves survival compared to artesunate alone

(**A**) The kinetics of BBB leak in untreated experimental cerebral malaria–susceptible C57BL/6 mice was determined by quantifying the amount of EB dye in the brain of PbA-infected mice on days 4, 5, and 6 (d4, d5, and d6, respectively) after inoculation and compared to that in uninfected controls (d0). (**B**) Representative brain images from EB dye–injected naïve and PbA-infected mice. (**C**) Schematic overview of the experimental design to examine the effect of late-stage BowAng1 treatment on rescuing the BBB leak phenotype in experimental cerebral malaria–susceptible C57BL/6 mice. (**D**) Quantification of EB dye in brains from PbA-infected BowAng1- or Fc isotype control–treated mice (** $P = 0.0059$, Wilcoxon signed-rank test; $n = 10$ mice per group). (**E**) Parasitemia of BowAng1- and control-treated mice on day 6 of PbA infection. (**F**) Kaplan-Meier survival analysis of PbA-infected C57BL/6 mice treated on day 5 after PbA inoculation with (i) artesunate (ART) (7.5 mg/kg; red line) and saline vehicle control, (ii) artesunate (7.5 mg/kg) plus BowAng1 (25 mg/kg; blue line), or (iii) control untreated mice (black line) (** $P = 0.0068$, log-rank test comparing artesunate treatment groups; $n = 19$, 20, and 9 mice per group, respectively). (**G**) Longitudinal disease score assessment (RMCBS) for PbA-infected C57BL/6 mice treated on day 5 with artesunate, artesunate plus BowAng1, or untreated (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, two-way ANOVA for artesunate versus artesunate plus BowAng-1 with Sidak's multiple comparison test; $n = 5$ mice per group).

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Table 1

Characteristics of children with severe *P. falciparum* malaria enrolled in a prospective study at the Jinja Regional Referral Hospital in Uganda (ClinicalTrials.gov identifier: NCT01255215; *n* = 92).

Characteristic	Variable[*]
Sex, *n* (% female)	43 (46.7%)
Age (years)	2.0 (1.0–3.0)
Estimated time ill before presentation (days)	3 (2–4)
Blantyre coma score	2 (2–3)
Temperature (°C)	37.8 (37.0–38.9)
Parasitemia (parasites per microliter)	29,460 (2720–73,860)
Hemoglobin (gram per deciliter)	6.9 (5.6–8.0)
Platelet count ($\times 10^9$/liter)	73.5 (39.8–126.3)
WHO-defined (44) **cerebral malaria, *n* (%)**	40 (43.5%)

[*] All variables are presented as median (interquartile range) unless otherwise indicated. No parasite recrudescence or reinfection was detected at day 14 follow-up.