Sequestration of infected red blood cells (iRBC) within the cerebral and pulmonary microvasculature is a hallmark of human cerebral malaria (hCM). The interaction between iRBC and the endothelium in hCM has been studied extensively and is linked to the severity of malaria. Experimental CM (eCM) caused by Plasmodium berghei ANKA reproduces most features of hCM, although the sequestration of RBC infected by P. berghei ANKA (PbA-iRBC) has not been completely delineated. The role of PbA-iRBC sequestration in the severity of eCM is not well characterized. Using static and flow cytoadherence assays, we provide the first direct in vitro evidence for the binding of PbA-iRBC to murine brain and lung microvascular endothelial cells (MVEC). We found that basal PbA-iRBC cytoadherence to MVECs was significantly higher than that of normal red blood cells (NRBC) and of RBC infected with P. berghei K173 (PbK173-iRBC), a strain that causes noncerebral malaria (NCM). MVEC prestimulation with tumor necrosis factor (TNF) failed to promote any further significant increase in mixed-stage iRBC adherence. Interestingly, enrichment of the blood for mature parasites significantly increased PbA-iRBC binding to the MVECs prestimulated with TNF, while blockade of VCAM-1 reduced this adhesion. Our study provides evidence for the firm, flow-resistant binding to endothelial cells of iRBC from strain ANKA-infected mice, which develop CM, and for less binding of iRBC from strain K173-infected mice, which develop NCM. An understanding of P. berghei cytoadherence may help elucidate the importance of sequestration in the development of CM and aid the development of antibinding therapies to help reduce the burden of this syndrome.
troversial, and of the few studies on strain ANKA binding in vivo (33, 34, 37–41), one suggests that sequestration is not linked to the development of CM (33), and this has challenged the relevance of the murine model to the human syndrome (42).

The interaction between iRBC and the endothelium is not passive. Parasite proteins interact with the host RBC to change its morphology, physiology, and function (43). Parasites produce mediators that trigger the release of cytokines from a range of host cells, including EC. These cytokines facilitate cytoadherence by upregulating the expression of ligands available on the surface of host cells, and this interaction activates signaling cascades that downregulate genes involved in the inflammatory response and apoptosis (44). In eCM, tumor necrosis factor (TNF) has long been known to be an important mediator in the pathogenesis of CM (45–47). A number of factors support the adhesion of the parasite to the endothelium, including host cells such as macrophages (47), lymphocytes (48), platelets (49), and plasma microparticles (50). IRBC can directly stimulate the endothelium in the absence of proinflammatory cytokines, inducing the expression of adhesion molecules and chemokines and mimicking the effects caused by cytokines such as TNF (51–55).

Sequestration of IRBC in the microvasculature has been implicated in the severity of P. falciparum infection, as reviewed in reference 56, and in eCM, with P. berghei ANKA sequestering in an analogous fashion, albeit to a less pronounced degree (15, 33, 34, 57). Postmortem studies show a strong association between the number of tightly packed RBC in vessels and hCM (2, 36) and also in eCM (58, 59). In patients dying from P. falciparum infection, sequestered IRBC are higher in CM than in necrobiotic cerebral malaria (NCM), and brain and lung are sites of this vascular pathology (2, 3, 36). Murine models of CM have provided histopathological evidence for IRBC sequestration on cerebral microvascular endothelium (34, 37, 40, 59, 60) and in the lung and adipose tissue (15, 33), as well as leukocyte sequestration in retinal vessels (61), although no direct evidence exists to delineate this phenomenon as being either IRBC accumulation or sequestration. CD36-mediated sequestration has been described in both hCM and eCM, although the corresponding ligand proteins are not the same (15). P. falciparum mediates CD36 binding in hCM (62) and the rodent equivalent has not yet been identified, although deletions studies on the corresponding ligand proteins are not the same (15). PIEMP1 mediates CD36 binding in hCM (62) and the rodent equivalent has not yet been identified, although deletions studies on the corresponding ligand proteins are not the same (15). PIEMP1 mediates CD36 binding in hCM (62) and the rodent equivalent has not yet been identified, although deletions studies on the corresponding ligand proteins are not the same (15). PIEMP1 mediates CD36 binding in hCM (62) and the rodent equivalent has not yet been identified, although deletions studies on the corresponding ligand proteins are not the same (15). PIEMP1 mediates CD36 binding in hCM (62) and the rodent equivalent has not yet been identified, although deletions studies on the corresponding ligand proteins are not the same (15). PIEMP1 mediates CD36 binding in hCM (62) and the rodent equivalent has not yet been identified, although deletions studies on the corresponding ligand proteins are not the same (15). PIEMP1 mediates CD36 binding in hCM (62) and the rodent equivalent has not yet been identified, although deletions studies on the corresponding ligand proteins are not the same (15). PIEMP1 mediates CD36 binding in hCM (62) and the rodent equivalent has not yet been identified, although deletions studies on the corresponding ligand proteins are not the same (15). PIEMP1 mediates CD36 binding in hCM (62) and the rodent equivalent has not yet been identified, although deletions studies on the corresponding ligand proteins are not the same (15). PIEMP1 mediates CD36 binding in hCM (62) and the rodent equivalent has not yet been identified, although deletions studies on the corresponding ligand proteins are not the same (15). PIEMP1 mediates CD36 binding in hCM (62) and the rodent equivalent has not yet been identified, although deletions studies on the corresponding ligand proteins are not the same (15).

No data exist on the ability of RBC infected by P. berghei ANKA (PbA-iRBC) or P. berghei K173 (PbkK173-IRBC) to firmly adhere to the microvascular endothelium, a mechanism that may underlie CM pathogenesis. A more complete understanding of the mechanisms involved in IRBC sequestration is required, so that strategies to prevent adhesion can be developed and implemented to reduce disease burden. Here, we describe an adapted assay that isolates the sequestration phenomenon in eCM. In this study, we have used an in vitro coculture assay in which freshly isolated RBC are incubated with murine MVEC from the brain and lung to quantify binding. We provide the first direct in vitro evidence for binding of PbA-iRBC to murine brain (B3) and lung (L2) MVEC. We investigated under static and/or flow conditions the cytoadherence of PbA-iRBC parasites to TNF-activated murine brain and lung MVEC and compared it to PbkK173-IRBC, with these being used to model NCM. Mice infected with large P. berghei K173 inocula develop high parasitemia levels and anemia and die without cerebral signs at least 14 days postinfection (27). Furthermore, we assessed the involvement of the endothelial molecules ICAM-1 and VCAM-1, known to mediate P. falciparum cytoadherence and present on our MVEC lines, in the observed binding.

MATERIALS AND METHODS

Research procedures were approved by the Animal Ethics Committee of the University of Sydney (K2017/006/3/4434 and K0010/2010/3/5317).

**Microvascular endothelial cells.** Primary mouse brain MVEC (B3) were isolated as described previously (63). Primary mouse lung MVEC (L2) were derived from primary cultures of peripheral lung parenchyma of CBA/Ca mice. These cells have been immortalized by transfection of a plasmid construct containing Bcl-x	extsubscript{L} protein. Cells were seeded in culture flasks and grown to confluence in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in 5% CO	extsubscript{2}.

**Surface phenotyping of B3 and L2 cells.** Monolayers of B3 and L2 cells were cultured until confluence and then either left unstimulated or incubated with recombinant murine TNF (10 ng/ml; Peprotech) for 4 h or overnight. Cells were then washed in phosphate-buffered saline (PBS) and exposed to a short trypsin-EDTA treatment to harvest a cell suspension. To identify resting and activated cellular markers, the cells were directly labeled using anti-mouse monoclonal antibodies directed against PECAM-1, ICAM-1, integrin beta-3, endoglin, VCAM-1, and E-selectin (CD31, CD54, CD61, CD105, CD106, and CD62E, respectively; Biocytex) and their respective isotype-matched immunoglobulins. Cells were then resuspended in PBS prior to flow-cytometric analysis (Beckman Coulter FC500). E-selectin was measured after a 4-h TNF treatment, while the remaining molecules were measured after an overnight treatment.

**Preparation of red blood cells.** Infection was induced in 8-week-old CBA female mice by intraperitoneal injection of 1 × 10	extsuperscript{8} P. berghei ANKA- or 2 × 10	extsuperscript{8} P. berghei K173-parasitized RBCs as described previously (26, 27). Blood was collected in citrated tubes via retro-orbital sampling from infected mice 6 days postinfection, while noninfected mice were used as controls. Parasitemia was determined by Diff-Quick-stained thin tail vein blood smears.

Red blood cells were separated from whole blood by centrifugation at 500 × g for 5 min and were washed at low speed in PBS three times. Mixed-stage RBC were used at 6 to 7% parasitemia. Mature-stage iRBC were selected and concentrated using automatic magnetically activated cell sorting (MACS) as previously described (64). Enriched RBC were used at 40% parasitemia with 70% of the parasites at the mature stage. Red blood cells were labeled with green fluorescent Calcein-acetoxymethyl (Calcein-AM) (Molecular Probes) and resuspended in medium. Briefly, 1 × 10	extsuperscript{7} RBC were washed in Dulbecco’s modified Eagle medium without phenol red (DMEM; Gibco) and loaded with 1 μg/ml Calcein-AM for 30 min at 37°C. Subsequently, cells were washed, resuspended in 10 ml DMEM, and allowed to rest for 30 min; this procedure was repeated twice. RBC were checked for optimal loading of dye on an Olympus IX71 inverted microscope.

**Static cytoadherence assays.** B3 and L2 cells were cultured to confluence on 24-well plates. Cells were either left unstimulated or were activated overnight with 50 ng/ml murine TNF. The MVEC were then washed in medium and incubated with RBC (RBC/MVEC ratio, 20:1) for 90 min at 37°C. Nonadherent RBCs were then removed by gentle washing in medium, followed by more thorough washing with PBS and plate inverter. Remaining cells were fixed by incubation with 1% (wt/vol) paraformaldehyde at 4°C overnight. Bound RBCs were counted on at least 15 fields from 3 separate experiments imaged on an Olympus IX71 inverted microscope (×400 magnification).

**Flow cytoadherence assays.** Cell adherence assays were performed using parallel-plate flow chambers (Glycotech). Confluent monolayers of B3 and L2 cells, seeded in 35-mm petri dishes, were stimulated with TNF by following the same regimen as that for the static conditions. RBC were resuspended at 1% hematocrit and perfused over endothelial monolayers for 15 min at a shear stress of 0.5 dynes/cm	extsuperscript{2}. Plates were washed and fixed prior to imaging (Olympus IX71 inverted microscope at ×400 magnification).

Downloaded from http://iai.asm.org/ on September 3, 2017 by UNIVERSITY OF TECH SYDNEY
In the magnified inset, arrows indicate bound RBC.

**RESULTS**

**Adhesion of *Plasmodium berghei* ANKA- and K173-infected red blood cells to murine brain and lung microvascular endothelial cells under static conditions.** Static cytoadherence assays were performed using fresh blood samples obtained directly from uninfected mice and mice at 6 days postinfection. We first studied the binding of mixed-stage iRBC on monolayers of B3 and L2 cells. Ninety minutes after incubation with RBC and removal of nonadherent cells, we found that PbA-iRBC bound significantly more than NRBC and PbK173-iRBC to both B3 and L2 MVECs, and that prestimulation with TNF did not affect the level of cytoadherence of any of the RBC tested (Fig. 1). Interestingly, more PbA-iRBC adhesion was observed on brain MVEC than on lung MVEC.

Both B3 and L2 cells readily adhere to plastic culture flasks and exhibit contact inhibition. Figure 1 demonstrates the clear binding of mixed-stage fluorescent-labeled iRBC from strain ANKA-infected mice to B3 monolayers. Similar binding was seen on L2 monolayers (data not shown).

**Firm, flow-resistant binding of PbA-iRBC to murine brain and lung microvascular endothelial cells.** Following the demonstration that PbA-iRBC cytoadherence to MVEC under static conditions was greater than that of NRBC and PbK173-iRBC, we evaluated the strength of the cytoadherence of PbA-iRBC under more biologically relevant conditions. Flow cytoadherence assays were conducted on B3 and L2 monolayers at a shear stress of 0.5 dynes/cm², consistent with what is measured in the microvasculature and used in *in vitro* assays (66). Under flow conditions, basal PbA-iRBC cytoadherence to B3 and L2 MVECs was significantly higher than that of NRBC (Fig. 2). As seen in the static assays, MVEC prestimulation with TNF failed to induce any further increase in RBC adherence, and fewer RBC bound to lung MVEC monolayers than brain MVEC monolayers.

**Phenotyping of B3 and L2 cells under resting and inflammatory conditions.** The surface phenotype of both cell lines in resting and inflammatory conditions was assessed by flow cytometry (Fig. 3). In resting conditions, cells expressed CD54 (ICAM-1) and CD106 (VCAM-1). Following overnight TNF stimulation, both B3 and L2 MVECs upregulated ICAM-1 and VCAM-1 to a greater extent than brain MVECs. VCAM-1 expression was significantly higher on L2 cells than on brain MVECs. Interestingly, more PbA-iRBC adherence was observed on brain MVEC than on lung MVEC.

**Enrichment for mature parasites increases the level of PbA-iRBC binding on MVECs prestimulated with TNF, and blocking VCAM-1 reduces this binding.** To investigate the effect of TNF on the binding of iRBC harboring mature stages of the parasite, blood was enriched for mature forms and incubated on B3 monolayers in resting and activated conditions. Prestimulation with TNF significantly increased the number of mature PbA-iRBC bound compared to that of resting monolayers (Fig. 4). We found no difference between the binding of mature PbA-iRBC and NRBC on resting monolayers (Fig. 1, 3, and 4).

Enrichment of adhesion molecules ICAM-1 and VCAM-1 on the surface of B3 and L2 cells represented potential...
receptors by which the RBC were binding under experimental inflammatory conditions (Fig. 4). To address this possible interaction, B3 monolayers were incubated with purified anti-mouse ICAM-1 or anti-mouse VCAM-1 (LEAF) and their respective iso-type-matched controls. These monoclonal antibodies selectively block ICAM-1 binding to LFA-1 and Mac-1 receptors, as well as VCAM-1 binding to VLA-4 (\(\alpha 4\beta 1\) integrin) and LPAM-1 (\(\alpha 4\beta 7\) integrin) receptors. A 65% reduction in the number of PbA-iRBC was observed when blocking VCAM-1, whereas no effect was seen when blocking ICAM-1 or when the isotype-matched controls were used. The addition of the blocking antibodies had no effect on the number of binding NRBC compared to that of isotype controls.

**DISCUSSION**

This study describes our findings on the cytoadherence of *P. berghei*-iRBC to murine brain (B3 cells) and lung (L2 cells) MVEC. First, our data provide the first direct *in vitro* evidence, to our knowledge, that PbA-iRBC are capable of cytoadhering to murine brain and lung MVEC. Second, the proportion of binding is higher for RBC infected with strain ANKA, the strain causing eCM, than those infected with strain K173, the strain causing NCM, and this binding is independent of endothelial cell pre-stimulation by TNF. Third, this binding is firm, shear stress resistant, and significantly higher on brain than on lung MVEC. Finally, enriched mature PbA-iRBC bind to TNF-activated MVEC to a greater extent than to resting MVEC, and this binding is reduced when VCAM-1 is blocked on the endothelial surface.

In our study, the binding of fluorescent iRBC from strain ANKA- and strain K173-infected mice to brain MVEC was clearly visible, with PbA-iRBC binding more than PbK173-iRBC. Brain sections from mice infected with the nonsequestering line *P. berghei* K173 display little to no sequestered parasites (69), similar to what is observed in severe human malaria without coma (3, 4, 6, 14, 36, 70, 71). On lung MVEC we also observed binding of PbA-
iRBC, although overall it was lower than that observed on brain MVEC. We found no difference in binding of mixed-stage PbA-iRBC in resting or TNF-activated conditions (Fig. 1). The cytoadherence-linked virulence of the parasite, as displayed by strain ANKA, may support its ability to influence the development of CM in the host. It would be advantageous for early-stage parasites to directly activate the host endothelium to support adhesion in order to enable survival and transmission in vivo (51). In our system, RBC were sampled directly from the peripheral blood of mice on the day of experimentation; thus, there is no influence of long-term culture, i.e., loss of the cytoadherent phenotype (72).

Based on these observations, a series of flow assays was performed to confirm the binding under physiological conditions, similar to what is observed inside a microvessel. PbA-iRBC binding withstood flow conditions, confirming that the majority of the interactions did not resemble stasis or weak binding but true cytoadherence, and the interaction was firm and flow resistant (Fig. 2). However, we did observe lower levels of binding under flow conditions across the brain and lung endothelium, confirming that shear stress rates significantly affect cytoadherence, and that some of the binding seen under static conditions is indeed due to stasis or weak binding.

Our data demonstrate that increased binding of mature parasites compared to mixed-stage parasites occurs following TNF stimulation. In vivo, strain ANKA schizonts disappear from the peripheral circulation and have a distinct sequestration phenotype (33, 73, 74). In our system, we hypothesized that mature parasites and schizonts would bind more than mixed-stage parasites, as seen with P. falciparum (3, 75). Surprisingly, under resting conditions these mature parasites bound no differently than NRBC. However, under inflammatory conditions (TNF-stimulated monolayers), we observed a significant increase in the number of bound mature-stage PbA-iRBC (Fig. 4).

In order to determine the possible binding mechanism present in our assay, we studied inducible receptors present on the surface of the MVEC. From the candidate receptors present on B3 and L2 cells (Fig. 3), we focused on those upregulated following TNF activation, namely, ICAM-1 and VCAM-1, mimicking the inflammatory conditions seen in CM. Previous studies have shown that ICAM-1 and VCAM-1 expression is upregulated by TNF on endothelial cells, and these molecules are receptors for P. falciparum (76). ICAM-1 becomes upregulated on the brain and lung endothelium during eCM (77–79) and is known to mediate iRBC binding to the vascular endothelium (80). Moreover, there is evidence for the colocalization of sequestration and ICAM-1 expression in the brain in CM (81). Surprisingly, in our study, inhibition of ICAM-1 had no significant effect on binding of mature-stage PbA-iRBC to brain (Fig. 4) or lung MVEC (data not shown). Further experiments using other clones of ICAM-1 are required to confirm whether this lack of effect is due to the blocking antibody or the noninvolvement of ICAM-1.

There is evidence for a role for VCAM-1 in P. falciparum sequestration (76, 82, 83). In clinical malaria, P. falciparum-infected RBC roll and adhere to VCAM-1, although the epitope involved in the interaction is not yet known (84, 85). Our study demonstrates that VCAM-1 may be important in maintaining the adherence of mature PbA-iRBC to the cerebral endothelium (Fig. 4), since binding was inhibited in the presence of an anti-VCAM-1 monoclonal antibody (Fig. 4). The binding of PbA-iRBC in cultures may be a result of the interaction between VCAM-1 and parasite ligands or VLA-4-like antigen present on the surface of RBC, although further studies are required to determine this.

In other studies, CD36 has been identified as the host cell receptor for PbA-iRBC adherence. This is also one of the major receptors of P. falciparum iRBC and could account for the differential binding observed on brain and lung MVEC (33). Orthologues of PfEMP1 are absent from P. berghei and, as yet, strain ANKA ligands for CD36 have not been identified (62). Interestingly, mice express little or no CD36 in the brain (86) and CD36-mediated sequestration of P. berghei is not necessary for eCM, since cerebral complications still develop in the absence of this molecule (33). Although the lung is a site of pathology in CM, pulmonary sequestration of iRBC is not necessary for the development of acute lung injury or CM (87). However, peripheral parasite burden induces acute lung injury in eCM (79). In mice infected with the nonencephalitic strain, K173, lung pathology develops in the absence of CM (88) and at low inoculum size can induce CM without evidence for sequestration (89, 90). Alternative adhesion pathways that may be responsible for the binding are still unknown.

Our work demonstrates that mixed-stage PbA-iRBC, containing the parasite strain that causes CM, can bind firmly to the cerebral endothelium in the absence of TNF. However, mature parasites may require TNF to bind, and VCAM-1 could be mediating this binding (Fig. 4). Our in vitro model isolates three major components of the cerebral lesion: the endothelial cell, the RBC, and the parasite. However, there are other effectors in the CM lesion; thus, our data require careful interpretation. Nevertheless, target organ-specific cell lines, such as B3 and L2, expressing cytoadherence receptors for PbA-iRBC may provide a solid in vivo system that supplements the eCM model to study molecular mechanisms of strain ANKA binding. Future work may involve the addition of platelets, MP, and leukocytes to the system in order to model closely what is seen in vivo (10, 49). Existing eCM models enable access to a large library of genetically modified mice from which MVEC from various organs could be purified. Further experiments are required to identify which receptors are recognized by P. berghei parasites and discern the specific parasite stages of the intraerythrocytic cycle that bind. Ultimately, this could reveal how parasites use cytoadherence to evade the immune system inside the host, how they contribute to the severity of disease, and, how therapies work at interfering with this binding to lessen the global burden of CM (91, 92).

ACKNOWLEDGMENTS
This work was supported by NHMRC project grant APP1028241, 571014, the Rebecca L. Cooper Medical Research Foundation, and the National Institutes of Health (1R01NS079873-01). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

F.E. designed and performed experiments, collected and analyzed data, and wrote the manuscript. J.W. performed phenotyping experiments and interpreted data. A.J.M. and N.H.H. provided reagents, technical support, and conceptual advice and interpreted data. G.E.G. and V.C. designed the study, gave conceptual advice and technical support, analyzed data, and edited the manuscript.

We declare no competing financial interests or conflicts of interest.

REFERENCES


