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RESEARCH ARTICLE

Activated Neutrophils Are Associated with Pediatric Cerebral Malaria Vasculopathy in Malawian Children

Catherine Manix Feintuch,[a] Alex Saidi,[b] Karl Seydel,[b,c] Grace Chen,[d] Adam Goldman-Yassen,[a] Neida K. Mita-Mendoza,[a] Ryung S. Kim,[e] Paul S. Frenette,[d] Terrie Taylor,[b,c] Johanna P. Daily[a,f]

Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, USA[a]; Blantyre Malaria Project, University of Malawi College of Medicine, Blantyre, Malawi[b]; Department of Osteopathic Medical Specialties, College of Osteopathic Medicine, Michigan State University, East Lansing, Michigan, USA[c]; Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research and Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York, USA[d]; Department of Epidemiology and Population Health, Albert Einstein College of Medicine, Bronx, New York, USA[e]; Division of Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, USA[f]

ABSTRACT **Most patients with cerebral malaria (CM) sustain cerebral microvascular sequestration of *Plasmodium falciparum*-infected red blood cells (iRBCs). Although many young children are infected with *P. falciparum*, CM remains a rare outcome; thus, we hypothesized that specific host conditions facilitate iRBC cerebral sequestration. To identify these host factors, we compared the peripheral whole-blood transcriptomes of Malawian children with iRBC cerebral sequestration, identified as malarial-retinopathy-positive CM (Ret+CM), to the transcriptomes of children with CM and no cerebral iRBC sequestration, defined as malarial-retinopathy-negative CM (Ret-CM). Ret+CM was associated with upregulation of 103 gene set pathways, including cytokine, blood coagulation, and extracellular matrix (ECM) pathways ($P < 0.01$; false-discovery rate [FDR] of <0.05). Neutrophil transcripts were the most highly upregulated individual transcripts in Ret+CM patients. Activated neutrophils can modulate diverse host processes, including the ECM, inflammation, and platelet biology to potentially facilitate parasite sequestration. Therefore, we compared plasma neutrophil proteins and neutrophil chemotaxis between Ret+CM and Ret-CM patients. Plasma levels of human neutrophil elastase, myeloperoxidase, and proteinase 3, but not lactoferrin or lipocalin, were elevated in Ret+CM patients, and neutrophil chemotaxis was impaired, possibly related to increased plasma heme. Neutrophils were rarely seen in CM brain microvasculature autopsy samples, and no neutrophil extracellular traps were found, suggesting that a putative neutrophil effect on endothelial cell biology results from neutrophil soluble factors rather than direct neutrophil cellular tissue effects. Meanwhile, children with Ret-CM had lower levels of inflammation, higher levels of alpha interferon, and upregulation of Toll-like receptor pathways and other host transcriptional pathways, which may represent responses that do not favor cerebral iRBC sequestration.**

IMPORTANCE **There were approximately 198 million cases of malaria worldwide in 2013, with an estimated 584,000 deaths occurring mostly in sub-Saharan African children. CM is a severe and rare form of *Plasmodium falciparum* infection and is associated with high rates of mortality and neurological morbidity, despite antimalarial treatment. A greater understanding of the pathophysiology of CM would allow the development of adjunctive therapies to improve clinical outcomes. A hallmark of CM is cerebral microvasculature sequestration of *P. falciparum*-infected red blood cells (iRBCs), which results in vasculopathy in some patients. Our data provide a global analysis of the host pathways associated with CM and newly identify an association of activated neutrophils with brain iRBC sequestration. Products of activated neutrophils could alter endothelial cell receptors and coagulation to facilitate iRBC adherence. Future studies can now examine the role of neutrophils in CM pathogenesis to improve health outcomes.**

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Address correspondence to Johanna P. Daily, johanna.daily@einstein.yu.edu.

Cerebral malaria (CM) is associated with high case fatality rates, and a third of survivors develop epilepsy or other neurological sequelae (1, 2). Identification of novel strategies to decrease the high rates of morbidity and mortality associated with CM (3) in African children are needed (4, 5). CM is defined as the presence of coma with confirmed *Plasmodium falciparum* infection, exclusive of other identifiable etiologies of coma (6). Microvasculature sequestration of late-stage *P. falciparum*-infected red blood cells (iRBCs) occurs in all *P. falciparum* clinical syndromes; however, in patients with CM, microvascular iRBC sequestration occurs in the brain, as well as other vital organs (7–10). iRBC sequestration is accompanied by endothelial cell activation, upregulation of intracellular adhesion molecule 1 (ICAM-1) and other endothelial cell receptors, alterations in endothelial cell protein C receptor (EPCR), and deposition of platelets and fibrin in the brain microvasculature during CM (11–14). This vasculopathy is associated

TABLE 1 Characteristics of children with cerebral malaria by retinopathy status

Characteristic	Value for characteristic in the following patients[a]: RET+CM ($n = 64$)	RET-CM ($n = 33$)	P value
Age (mo)	50 (36–65)	58 (31–74)	0.855
Sex (% male)	47	39	0.482
Temp (°C)	39.0 (38.2–39.8)	39.1 (38.1–39.9)	0.775
Respirations (no. of breaths/min)	43 (39–52)	42 (36–52)	0.654
Pulse (no. of beats/min)	147 (133–170)	160 (145–183)	0.143
Parasitemia (no. of parasites $\times 10^3/\mu$l)	56 (21–331)	45 (13–84)	0.159
HRP2 (ng/ml)	6,783 (2,602–9,916)	446 (207–604)	<0.001
Hematocrit (%)	20.4 (17.5–23.9)	25.6 (22.6–31.7)	<0.001
WBC ($\times 10^3/\mu$l)[b]	8.3 (6.7–14.9)	10.0 (7.3–13.4)	0.477
Neutrophils	5.2 (3.5–7.5)	5.1 (4.2–8.2)	0.557
Lymphocytes	2.4 (1.4–4.1)	2.1 (1.7–3.1)	0.770
Monocytes	0.8 (0.2–1.6)	1.1 (0.5–1.6)	0.299
Platelets ($\times 10^3/\mu$l)	50 (33–85)	149 (46–221)	<0.001
Death (%)	22	6	0.080

[a] Patient characteristics at admission of 97 samples by retinopathy status (Ret+CM or Ret-CM). Continuous variables were compared by using the Mann-Whitney U test, and dichotomous variables were compared by the χ^2 test and Fisher's exact tests where appropriate. Values are reported as medians and interquartile ranges (25% and 75%) for continuous variables and percentages and numbers of observations for dichotomous variables.
[b] WBC, white blood cells.

with elevated inflammation, blood-brain barrier breakdown, severe brain swelling, and death in some individuals (15–18). The identification of host factors that contribute to cerebral iRBC sequestration and vasculopathy could lead to novel therapies for CM to improve clinical outcomes.

Cerebral sequestration of iRBCs during pediatric CM occurs in 75% of cases and can be identified clinically through a retinal exam (19). The presence of microvasculature abnormalities in the ocular fundus ("malarial retinopathy") is strongly associated with the cerebral iRBC sequestration identified at autopsy (10, 20). Children with malarial-retinopathy-positive CM (Ret+CM) have a higher mortality rate than children with CM without malarial retinopathy (Ret-CM) (21, 22). Specific CM-associated parasite proteins expressed on the iRBCs are associated with brain sequestration (14, 23–25). These CM-associated parasites are likely arbitrarily transmitted throughout the general population, yet only a small percentage of infections in young children result in cerebral iRBC sequestration. Therefore, we hypothesized that in addition to infection with CM-associated parasites, specific host factors modify the risk for iRBC sequestration in CM. To identify host factors associated with cerebral iRBC sequestration, we compared host whole-blood transcription profiles from Malawian children with Ret+CM to profiles from children with Ret-CM. Our data newly suggest that activated neutrophils play a role in Ret+CM.

RESULTS

We studied Malawian children with CM enrolled in the Blantyre Malaria Research Project (BMP) as part of an ongoing longitudinal study (2). We performed whole-blood transcriptional profiling on 98 of the 205 blood samples obtained from patients at enrollment in the study during the 2009 and 2011 malaria seasons. There were no significant differences between patient characteristics of the hybridized samples and the complete cohort (see Table S1 in the supplemental material).

Peripheral blood parasitemia is associated with whole-blood transcriptional profiles. To first discover patterns in the whole-blood transcriptomes, we performed unsupervised hierarchical clustering analysis of 98 samples. We identified three transcriptional clusters (see Fig. S1 in the supplemental material). Most demographic, clinical, and laboratory features, including age and white cell subsets, were similar between the transcriptional clusters (see Table S2 in the supplemental material). Histidine-rich protein 2 (HRP2), a parasite protein that can provide an estimate of the total body parasite biomass, was not significantly different between clusters (26, 27). In contrast, cluster 2 was significantly associated with high peripheral parasitemia, which represents circulating early stage parasites ($P < 0.005$ by the Mann-Whitney U test). Ret+CM and Ret-CM phenotypes were found in each cluster, though cluster 3 was significantly enriched for Ret-CM samples ($P = 0.020$ by the χ^2 test). Because of the association of peripheral parasitemia with global transcription, we adjusted each transcript by peripheral parasitemia to compare the transcriptional profiles between Ret+CM versus Ret-CM in our primary analysis.

We then compared the whole-blood transcriptomes from 64 Ret+CM patients and 33 Ret-CM patients. These children were comparable in age and manifested similar elevations in temperature and respiratory and pulse rates (Table 1). The median peripheral blood parasitemia was similar between the two groups ($P = 0.159$ by the Mann-Whitney U test). HRP2 was higher in Ret+CM patients, reflecting their large sequestered parasite biomass ($P < 0.0001$ by the Mann-Whitney U test) (26, 27). The Ret+CM patients had higher mortality (22% versus 6%; $P = 0.08$ by the χ^2 test), lower hematocrits (20.4% versus 25.6%; $P < 0.0001$ by the Mann-Whitney U test), and lower platelet counts ($50 \times 10^3/\mu$l versus $149 \times 10^3/\mu$l; $P < 0.001$ by the Mann-Whitney U test) compared to Ret-CM patients. White blood cell subsets were equivalent between Ret+CM and Ret-CM patients, including the absolute number of neutrophils.

To identify gene pathways that differed in Ret+CM and Ret-CM patients, we carried out Gene Set Enrichment Analysis (GSEA) after performing linear regression analysis with gene expression as the outcome and retinopathy status as the predictor of interest and adjusting for peripheral parasitemia for each gene.

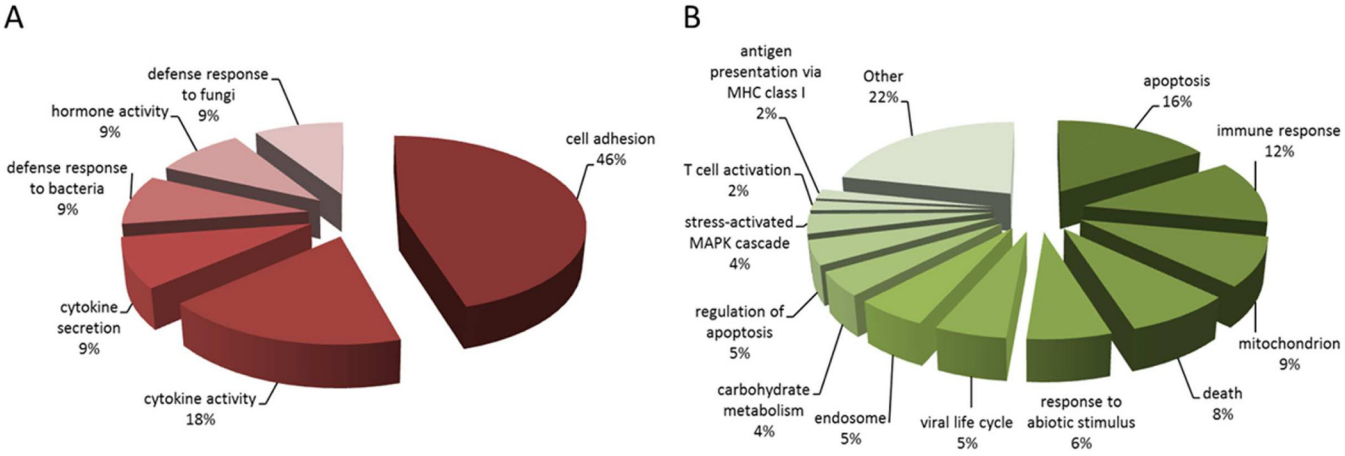

FIG 1 Gene Set Enrichment Analysis identifies distinct host responses in Ret+CM and Ret-CM patients. Ret+CM was associated with upregulation of 103 gene sets, and Ret-CM was associated with upregulation of 522 gene sets ($P < 0.05$ and a FDR of <0.20). We used GO Slim categories to summarize the top 100 gene sets for each group. (A) Ret+CM is associated with cell adhesion and cytokine pathways. (B) Ret-CM is associated with apoptosis and antigen processing pathways. MHC, major histocompatibility complex; MAPK, mitogen-activated protein kinase.

We identified 103 Gene Ontology (GO) pathways that were positively associated with Ret+CM and 522 GO gene pathways positively associated with Ret-CM ($P < 0.05$ by the Kolmogorov-Smirnov test and a false-discovery rate [FDR] of <0.20), which are summarized using GO slim categories (Fig. 1; see Table S3AB in the supplemental material for gene sets significantly enriched in Ret+CM and Ret-CM patients).

Association of cell adhesion and extracellular matrix pathways with Ret+CM. Ret+CM was associated with the GO slim category "Cell adhesion," which included cell adhesion, homophilic cell adhesion, and calcium-dependent cell adhesion GO pathways ($P < 0.01$ and FDR of ≤0.06; Fig. 1A; see Table S3A in the supplemental material). These pathways include transcripts encoding cell adhesion molecules and extracellular proteins, such as multimerin 1 (MMRN1), P-selectin (SELP), CD9, α-integrins and β-integrins, which were all higher in Ret+CM patients ($P < 0.01$ by Student's t test; Table S4A).

Despite the significantly lower platelet counts in Ret+CM patients compared to Ret-CM patients, there were higher levels of platelet-associated transcripts, such as glycoprotein Ib alpha polypeptide (GP1BA), glycoprotein IX (GP9), and platelet glycoprotein IIIa (GP1BA) ($P < 0.01$; see Table S4A in the supplemental material). In addition, there was a positive association of platelet alpha granule lumen and platelet degranulation GO pathways in Ret+CM patients ($P < 0.01$ and FDR of <0.05; Table S3A). The coagulation cascade was also positively associated with Ret+CM by Ingenuity Pathway Analysis (IPA) pathway analysis ($P = 2.6 \times 10^{-6}$ by Fisher's exact test; Fig. S2A), which is consistent with the microvasculopathy marked by fibrin deposition seen at autopsy and the procoagulant state reported during CM (11, 28, 29).

The Ret+CM samples demonstrated higher markers of inflammation, including an upregulation of GO slim categories "Cytokine activity" and "Cytokine secretion" and higher transcript levels of monocyte chemotactic protein 1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α) ($P < 0.05$; see Table S4A in the supplemental material). To confirm higher inflammatory cytokine levels and further examine additional cytokines associated with CM, we measured plasma tumor necrosis factor alpha (TNF-α), MCP-1, and interleukin 10 (IL-10) and found higher concentrations in Ret+CM patients compared to Ret-CM patients ($P < 0.003$ by the Mann-Whitney U test; Fig. 2) (30). In contrast, alpha interferon 2 (IFN-α2), a type I IFN, was higher in Ret-CM patients compared to Ret+CM patients ($P = 0.021$). There was no difference in the levels of plasma IFN-γ, IL-1 receptor alpha (IL-1Ra), IL-8, and RANTES (regulated upon activation, normal T cell expressed and secreted) between Ret+CM and Ret-CM groups (Fig. S3).

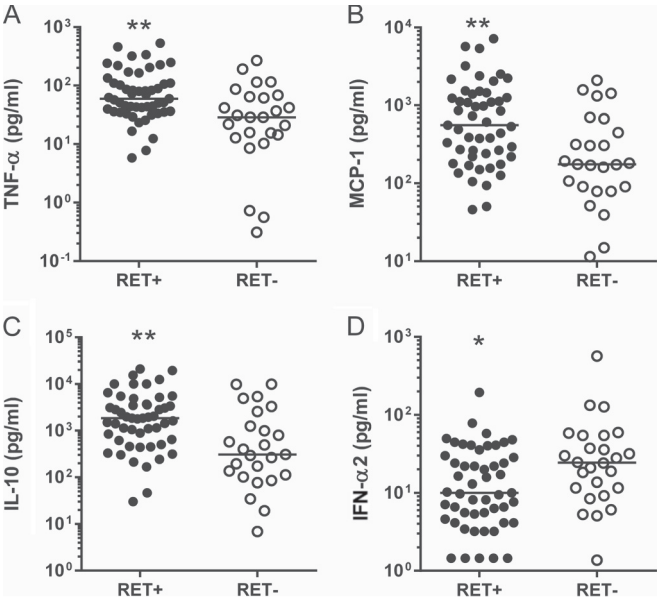

FIG 2 Cytokines associated with inflammation are higher in Ret+CM patients, whereas type I interferon is higher in Ret-CM patients. Selected plasma cytokine levels are shown using a logarithmic scale. (A-C) Higher TNF-α, IL-10, and MCP-1 concentrations are found in Ret+CM patients than in Ret-CM patients. (D) IFN-α2 levels in contrast are greater in plasma samples from Ret-CM patients than in Ret+CM patients. The Mann-Whitney U test was used for all comparisons. Each symbol represents the value for an individual patient (51 Ret+CM patients and 25 Ret-CM patients). Black bars denote median values. Median values that are statistically significantly different by the Mann-Whitney U test are indicated by asterisks as follows: *, $P = 0.021$; **, $P < 0.003$.

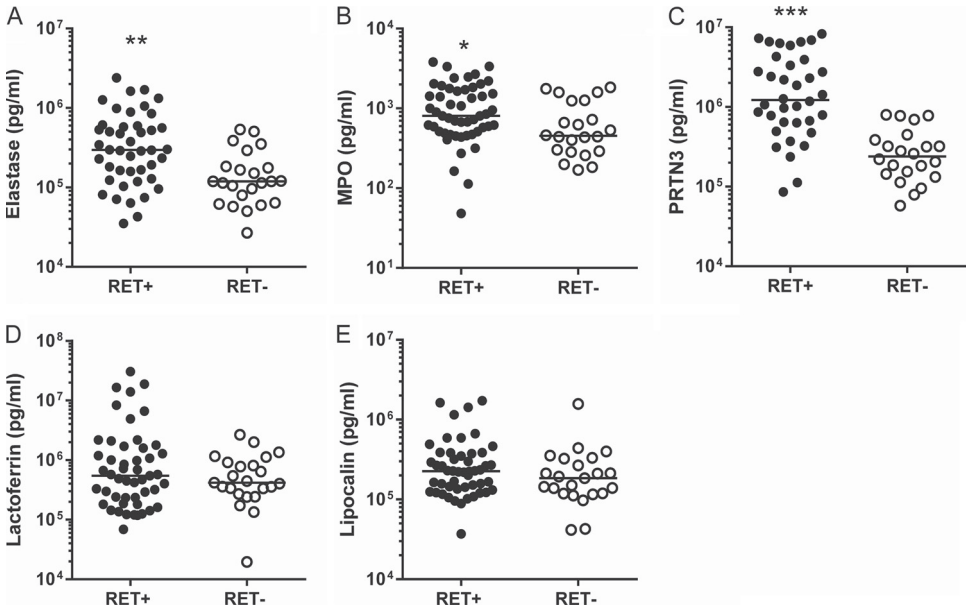

FIG 3 Plasma neutrophil primary granule protein concentrations are significantly higher in Ret+CM patients than in Ret-CM patients. Plasma levels of neutrophil primary granule proteins are shown using a logarithmic scale. (A) Human neutrophil elastase, (B) myeloperoxidase (MPO), and (C) proteinase 3 (PRTN3) are significantly higher in Ret+CM patients than in Ret-CM patients. No significant difference in neutrophil secondary granule protein (D) lactoferrin or (E) neutrophil gelatinase-associated lipocalin was found. Each symbol represents the value for an individual patient (51 Ret+CM patients and 25 Ret-CM patients). The Mann-Whitney U test was used for all comparisons. Black bars denote median values. Median values that are statistically significantly different by the Mann-Whitney U test are indicated by asterisks as follows: *, $P < 0.010$; **, $P < 0.001$; ***, $P < 0.0001$.

Extracellular matrix (ECM) pathways were upregulated in Ret+CM patients, including the proteinaceous extracellular matrix, extracellular matrix disassembly, and ECM GO pathways ($P < 0.01$ and FDR of <0.05; see Table S3A in the supplemental material). Upregulation of cell adhesion and cell matrix pathways has many potential downstream effects, including platelet activation, upregulation of cell-surface adhesion molecules, and cytokine signaling, all of which are hallmarks of Ret+CM (31). The ECM pathways included higher levels of neutrophil transcripts involved in ECM degradation and inflammation, such as neutrophil collagenase (matrix metalloproteinase 8 [MMP8]), skin-derived peptidase inhibitor 3 (SKALP), human neutrophil elastase (HNE), cathepsin G (CTSG), and secretory leukocyte peptidase inhibitor (SLPI) in Ret+CM patients ($P < 0.05$; see Table S4A in the supplemental material).

Neutrophil activation and dysfunction in Ret+CM patients. Activated neutrophils can mediate changes in endothelial cell receptors and have broad effects on innate and adaptive immunity though their role in CM has not previously been explored (32–36); thus, we chose to further examine the role of neutrophils in Ret+CM. We measured plasma concentrations of neutrophil primary granule proteins HNE, myeloperoxidase (MPO), and proteinase 3 (PRTN3). These three proteins (HNE, MPO, and PRTN3) were significantly higher in Ret+CM patients, supporting a higher neutrophil activation state in Ret+CM patients ($P = 0.001$, $P = 0.026$, and $P < 0.001$ by the Mann-Whitney U test, respectively, Fig. 3A to C). There was no significant difference in the amount of neutrophil secondary granule protein lactoferrin or neutrophil gelatinase-associated lipocalin (Fig. 3D and E).

To examine functional neutrophil differences in Ret+CM and Ret-CM patients, we examined the migratory capacity of neutrophils isolated from blood samples from patients enrolled in the study in 2013. Neutrophils isolated from Ret+CM patients had decreased chemotaxis toward IL-8 and *N*-formyl-methionyl-leucyl-phenylalanine [fMLP] compared to neutrophils from Ret-CM patients ($P = 0.002$ by the Mann-Whitney U test; Fig. 4A). Elevated levels of cell-free heme have been shown to decrease neutrophil migratory capacity. Therefore, we examined plasma heme levels and found significantly higher levels in the Ret+CM patients than in Ret-CM patients ($P = 0.002$ by the Mann-Whitney U test; Fig. 4B) (37).

Activated neutrophils can mediate changes in microvascular endothelial cells at the site of sequestration through soluble factors and/or direct contact. Therefore, to determine whether neutrophils were present at the site of the infected endothelial tissue, we examined formalin-fixed, paraffin-embedded brain sections from Ret+CM and Ret-CM patients (four sections from Ret+CM patients and five sections from Ret-CM patients) for the presence of neutrophils. In hematoxylin-and-eosin (H&E)-stained sections, we found only low levels of intravascular neutrophils in all of the Ret+CM sections and in four out of the five Ret-CM sections (Table 2). The Ret+CM sections had more endothelial cells that appeared to be reactive, and some sections also had acute vascular necrosis with hemorrhage and edema and fibrin thrombi compared to Ret-CM sections, which is consistent with previous autopsy studies (11). We then examined the tissue for the presence of neutrophil extracellular traps (NETS), which are released by activated neutrophils and are composed of granular proteins, histones, and DNA (38). To detect NETS, we carried out confocal microscopy on the brain sections using antibodies to neutrophil elastase and citrullinated histone H3 and DNA staining and found no evidence of NETS (data not shown).

In view of the association of neutrophil biology with Ret+CM, we examined the Duffy null polymorphism which is associated

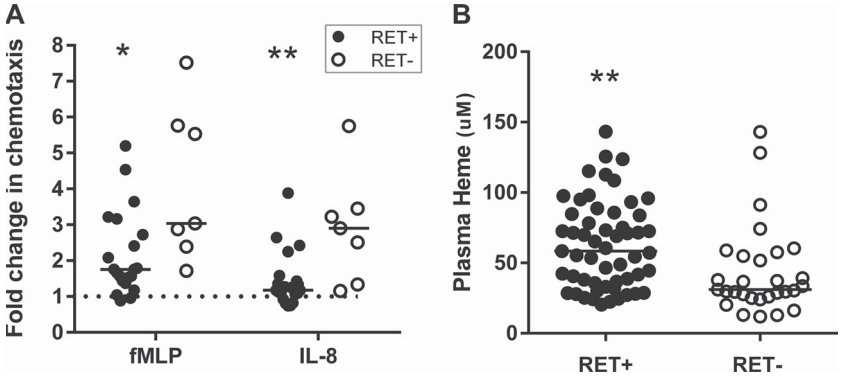

FIG 4 Ret+CM samples demonstrate impaired neutrophil chemotaxis and higher plasma heme compared to Ret-CM samples. (A) Fold change in neutrophil chemotaxis to fMLP or IL-8 compared to medium alone was compared for samples from Ret+CM ($n = 21$) and Ret-CM ($n = 7$) patients. Neutrophils from Ret+CM patients showed decreased chemotaxis toward IL-8 and fMLP compared to neutrophils from Ret-CM patients. (B) Plasma heme, an inhibitor of neutrophil chemotaxis, was higher in Ret+CM patients ($n = 56$) than in Ret-CM patients ($n = 29$). Bars represents median values. Median values that are statistically significantly different by the Mann-Whitney U test are indicated by asterisks as follows: *, $P = 0.005$; **, $P = 0.002$.

with low circulating neutrophil numbers commonly found in individuals with African ancestry (39). This benign ethnic neutropenia results in a state of persistently fewer absolute neutrophils compared to individuals of European descent. We determined whether allelic differences existed in samples from 15 Ret+CM patients (8 of these patients died)and 7 Ret-CM samples. All samples were homozygous for the Duffy null polymorphism.

Association of Toll-like receptor signaling in Ret-CM patients. We then examined the transcriptional pathways and transcripts associated with Ret-CM. Ret-CM was associated with stress response GO slim categories such as "Apoptosis," "Response to abiotic stimuli," "Endosomal formation" and "Stress-activated MAPK (mitogen-activated protein kinase) cascade" (Fig. 1B). The GO slim category "Immune response," which is associated with Ret-CM, included the Toll-like receptor 1 (TLR1), the TLR4 signaling pathways, the myeloid differentiation factor 88 (MyD88)-dependent Toll-like receptor signaling pathways, and the Toll/IL-1R domain-containing adaptor-inducing beta interferon (TRIF)-dependent Toll-like receptor signaling pathway ($P < 0.001$ and a FDR of <0.001; see Table S3B in the supplemental material). Details of the upregulated transcripts within the TLR signaling pathway are shown in the IPA pathway analysis ($P = 4.2 \times 10^{-3}$ by Fisher's exact text; see Fig. S2B in the supplemental material). Ret-CM was also associated with other stress response pathways, including upregulation of DNA repair, proteosome complex, and ubiquitin protein ligase activity ($P < 0.001$ and a FDR of <0.001; Table S3B).

The heme biosynthetic process pathway was associated with Ret-CM. GATA-1, a transcription factor necessary for erythroid development that regulates fetal and adult hemoglobin production under both neonatal development and anemia (40), was also upregulated in Ret-CM patients (see Table S4B in the supplemental material). We found higher gamma globin transcript levels, a component of fetal hemoglobin, in Ret-CM patients and confirmed the higher levels by quantitative reverse transcription-PCR (qRT-PCR) ($P = 0.013$ by the Mann-Whitney U test; see Fig. S4 in the supplemental material). The sickle cell allele is associated with increased gamma globin; however, none of the samples ($n = 73$) tested from Ret+CM and Ret-CM patients were carriers of the sickle cell allele (41, 42).

DISCUSSION

CM is a potentially devastating complication of *P. falciparum* infection. A greater understanding of the molecular mechanisms leading to cerebral iRBC sequestration and vasculopathy may lead to the development of adjunctive therapies to improve clinical outcomes. We compared whole-blood transcriptional profiles and plasma protein levels in children with Ret+CM and Ret-CM

TABLE 2 Brain microvasculature histopathology findings by retinopathy status[a]

	Score for histopathological feature in the following patients[b]:								
	RET+CM patients				RET-CM patients				
Histopathological feature	P1	P2	P3	P4	P5	P6	P7	P8	P9
Malaria pigment, intravascular	P	P	P	P	0	0	0	0	0
Intravascular neutrophils	2	2	1	1	1–2	1–2	1	1	0
Vascular necrosis and hemorrhage, acute	0	3	2	2	0	0	0	0	0
Fibrin thrombi, acute	0	2	2	2	0	0	0	0	0
Endothelial cell hypertrophy (reactive)	2	3	3	3	2	1	1	1	1–2
White matter rarefaction (edema)	0	1	1	2	1	1	1	0	1

[a] Neutrophils were rarely found in Ret+CM and Ret-CM brain histopathology microvasculature. All Ret+CM brain sections had malaria pigment within the cerebral vasculature. The vasculature in Ret+CM patients was more congested than in Ret-CM patients and typically had more endothelial cells that appeared to be reactive. Three of the four Ret+CM patients had acute vascular necrosis with hemorrhage and edema, as well as widespread vascular fibrin thrombi.

[b] The scores for four Ret+CM patients (patients 1 to 4 [P1 to P4]) and five Ret-CM patients (patients 5 to 9 [P5 to P9]) are indicated as follows: P, present; 0, no finding; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe.

and identified higher levels of activated neutrophils and inflammation and upregulation of ECM and coagulation pathways in Ret+CM patients. In contrast, the Ret-CM patients demonstrated lower concentrations of inflammatory cytokines and higher IFN-α2 levels and upregulation of alternative stress response pathways and TLR pathways. We speculate that multiple dysregulated pathways and higher levels of activated neutrophils and inflammation mediate critical changes in endothelial cells and platelets and/or coagulation to favor cerebral sequestration.

We employed whole-blood transcriptional profiling to obtain a comprehensive analysis of host physiology during CM by retinopathy status. This approach has provided insights into various infectious and vascular disease mechanisms (43–46). We first evaluated the transcriptional profiles by unsupervised clustering and identified three transcriptional clusters that did not fully segregate by retinopathy status. Cluster 2 was associated with high peripheral parasitemia, lactate levels, respiratory rates, and mortality. This constellation of clinical features has been identified in previous studies (47). Our association of a distinct transcriptional pattern with these clinical features may further suggest a pathophysiologic subtype of CM. The peripheral parasite load contribution to transcriptional variation was also observed in a mild malaria blood transcriptome study in Benin children, which suggests that the peripheral parasite load plays a dominant role on host responses irrespective of the severity of disease (48). HRP2 levels were not associated with the clusters. This suggests that the circulating parasites have an important effect on host blood transcriptional responses, perhaps due to rupture and release of parasite antigens.

Our Ret+CM cohort had features known to be associated with cerebral iRBC sequestration, including lower platelet counts and hematocrits and higher HRP2 levels and mortality compared to Ret-CM patients (21, 27). The Ret+CM and Ret-CM cohorts had similar peripheral blood parasitemias, suggesting that the development of vasculopathy is unrelated to control of peripheral parasitemia. This is consistent with other studies in regions where malaria is endemic where the severity of malaria is not associated with peripheral blood parasitemia (49).

The samples from Ret+CM patients were associated with alterations in ECM, dysregulation of the coagulation pathway, and heightened inflammatory responses, which are consistent with prior studies of CM (11, 50–53). Furthermore, we now demonstrate that these changes are associated with Ret+CM compared to Ret-CM. We also identified upregulation of platelet transcripts and platelet degranulation pathways in Ret+CM. Platelets are found in the microvasculature at autopsy in pediatric CM patients, and iRBCs can adhere to platelet/endothelial cell adhesion molecules PECAM-1/CD31, suggesting that platelets can facilitate cerebral iRBC sequestration (17, 54, 55). However, platelets have also been found to have antimalarial activity (3, 56). The harmful or protective role of platelets in Ret+CM remains to be determined. Overall, prior autopsy studies demonstrating fibrin thrombi and changes in microvascular endothelial cells reflect the pathways we found associated with Ret+CM (11).

In addition to the characterization of multiple pathways associated with Ret+CM, our novel finding is that higher levels of activated neutrophils are associated with cerebral iRBC sequestration and vasculopathy in Ret+CM patients. We focused on neutrophil biology, as activated neutrophils can mediate changes in endothelial cell receptors, platelets, and the coagulation cascade, which may be relevant to CM iRBC sequestration and pathology. There is scant information on the role of neutrophils in severe malaria. Prior studies have found an association of elevated serum lipocalin and HNE concentrations in severe malaria compared to mild malaria (57). Neutrophil elastase was 2.9-fold higher in plasma samples from Ret+CM patients, and it can mediate endothelial cell disruption and damage (32–34). Neutrophils produce superoxide anions in response to *P. falciparum*, which can decrease vascular integrity and endothelial cell function, upregulate endothelial cell receptors to facilitate iRBC sequestration, and enhance platelet activation (58–60). Inhibition of neutrophil-derived reactive oxygen species by superoxide dismutase or inhibition of neutrophil elastase with ONO-5046 Na could be examined to determine whether this inhibits iRBC sequestration to endothelial cells (61).

Plasma PRTN3, a neutrophil product, was fivefold higher in Ret+CM patients, and it cleaves surface EPCR (35). PRTN3 could account for the reduced detection of EPCR and increased cerebrospinal fluid (CSF) soluble EPCR (sEPCR) reported in CM patients and contribute to the procoagulant state in Ret+CM patients (24, 51). It is unknown whether neutrophils are an essential component in the development of Ret+CM. Neutrophil depletion studies in the animal model of malaria have shown protection against experimental cerebral malaria (ECM), though the interpretation of these data may be limited due to the concomitant potential depletion of non-neutrophil cell types (62–64). Studies on how activated neutrophils alter endothelial cells and may enhance iRBC sequestration are under way.

Our study, like others, found neutrophils in the brain microvasculature only rarely, and furthermore, we did not detect any NETS (11). The paucity of neutrophils at the site of malaria infection could be due to their generalized chemotactic dysfunction to both IL-8 and fMLP. The chemotactic dysfunction may result from the elevation in heme, which was greater in Ret+CM patients; however, the factors that mediate neutrophil recruitment into the microvasculature are complex (37, 65). Neutrophil chemotactic dysfunction has been previously reported in malaria, and neutrophils exposed to iRBC microparticles have been shown to migrate more slowly (66, 67). Neutrophil dysfunction has also been reported in sepsis and has been linked to inducible nitric oxide synthase, which can inhibit neutrophil migration (68, 69). Postmortem analysis cannot completely exclude the possibility that neutrophils are present locally in the microvasculature *in vivo* due to their short cellular life spans, and interestingly, neutrophil recruitment in the microvasculature has been detected in the *Plasmodium berghei* ANKA ECM model during intravital microscopy (70).

Why neutrophil activation and dysfunction are more elevated in children with Ret+CM is unclear. Children with Ret+CM had higher levels of TNF-α, a known inducer of neutrophil activation (36). Differences in TNF-α promoter polymorphisms or other host mutations mediating inflammatory responses could be driving the higher neutrophil activation state in Ret+CM patients (71–73). We examined only a limited number of host polymorphisms that are associated with reductions in risk of severe malaria; none of our patients with CM had α-thalassemia or the sickle cell trait, which is prevalent in the general population in Malawi (74, 75). All of the patients in our cohort had the Duffy null polymorphism, which is associated with benign ethnic neutropenia. Why this allele is enriched in individuals with African ancestry is

unknown, and it is intriguing to consider whether *P. falciparum* played a role in its selection (39). The examination of other host mutations that control neutrophil activation or other aspects of neutrophil biology, such as the single-nucleotide polymorphisms in neutrophil-derived α-defensin and neutrophil elastase, could be examined (76, 77).

The Ret-CM patients provide a valid comparison group, because they also have severe illness and were infected with *P. falciparum*. These children also manifested an encephalopathy with similar clinical and laboratory features, but they lacked cerebral iRBC sequestration, and this is reflected in the lower levels of HRP2 (27). Ret-CM patients had lower levels of inflammatory cytokines and displayed a very distinct set of host transcriptional responses. They exhibited higher induction of TLR pathways which has been shown in other studies of malaria (78, 79). We also found that a subset of patients with Ret-CM had higher levels of fetal hemoglobin transcripts. iRBCs with fetal hemoglobin exhibit decreased adherence to endothelial cells *ex vivo*, and further studies are needed to determine whether iRBCs from Ret-CM patients have higher fetal hemoglobin protein and are less adherent to endothelial cells (80).

Ret-CM was associated with higher concentrations of plasma type I IFN. The type I IFN pathway appears to play an important role in the host response to malaria, as mutations in the type I IFN receptor gene are associated with protection to severe malaria, and type I interferons may modulate changes in the endothelium to protect against iRBC sequestration (81, 82). In the ECM model, mice treated with type I interferons have enhanced survival, reduced ICAM-1 expression in brain endothelial cells, and reduction in serum TNF-α concentrations (83, 84). The pathways associated with Ret-CM may provide insights into host protective mechanisms against brain sequestration in CM. Specific parasite *var* genes are also associated with brain sequestration (85). Thus, targeted host response studies combined with parasite *var* gene analysis could identify novel mechanisms of protection from cerebral iRBC sequestration.

A limitation of this study is that we examined the patients after their illness was under way, which restricts our ability to identify the pathological processes that mediate cerebral iRBC sequestration. Differences in the number of episodes of malaria prior to their presentation with CM could underlie the variation in host responses in Ret+CM and Ret-CM patients, and this information is unknown in our cohort (49, 86). Longitudinal studies of immune responses and clinical presentation in infant cohorts could characterize the effect of the timing and number of infections on the development of protective host responses in CM-related vasculopathy.

In conclusion, we have identified host response pathways and heightened neutrophil activation in Ret+CM patients compared to Ret-CM patients. A threshold of heightened neutrophil activation may trigger downstream events to alter endothelial cell receptors and coagulation to facilitate cerebral iRBC sequestration (Fig. 5). In contrast, children without vasculopathy have very distinct host responses which may be protective from cerebral iRBC sequestration. Further studies on the role of activated neutrophils in vasculopathy and the potentially protective host responses in Ret-CM children are needed to identify rational targets for adjunctive therapy.

MATERIALS AND METHODS

Patient population. This study was conducted in Malawian children between the ages of 6 months and 12 years with *P. falciparum* infection and a Blantyre coma score of <3 enrolled in the Blantyre Malaria Research Project (BMP) as part of an ongoing longitudinal study of CM (2). Upon enrollment in the study, a whole-blood sample aliquot was collected for this substudy, and a fundoscopic exam was performed to determine the presence of malarial retinopathy (Ret+CM) or absence of malarial retinopathy (Ret-CM) (87). Children were excluded if they had a positive blood or CSF bacterial culture. Clinical characteristics and laboratory data were extracted from the study database. During the 2009 transmission season, 122 patients with CM were enrolled into the BMP. High-quality RNA from 63 samples was isolated for hybridization and included 38 randomly selected Ret+CM samples and 24 randomly selected Ret-CM samples. In addition, we collected 83 samples in 2011 and hybridized 26 randomly selected Ret+CM samples and all 9 Ret-CM samples. A total of 98 samples were used for unsupervised hierarchical clustering. One sample did not have a confirmed retinopathy status and was not included in the Ret+CM and Ret-CM comparisons. For the neutrophil chemotaxis experiments, neutrophils were isolated from patients and analyzed on the day of collection during the 2013 transmission season. Institutional review board (IRB) approvals were obtained from the Albert Einstein College of Medicine and Michigan State University and from the University of Malawi College of Medicine Research and Ethics Committee.

RNA sample collection and microarray data analysis. For whole-blood transcriptional analysis, 3 ml of whole blood in EDTA was added to Tri Reagent BD (Molecular Research Center) and frozen at −80°C. The RNA was isolated as previously described and hybridized to Affymetrix GeneChip standard 1.0 ST arrays (Affymetrix) (44).

Expression profiles were generated using the robust multi-array average (RMA) algorithm implemented by GenePattern (88). The data were normalized using quantile normalization and background corrected. We collapsed the 32,322 probes to 24,891 genes and removed unannotated genes. Unsupervised hierarchical clustering analysis was performed in dChip on 2,000 transcripts with the highest coefficient of variation. To determine which genes and gene sets associated with Ret+CM and Ret-CM, we used the full 24,891-gene list and performed linear regression analysis with gene expression as the outcome and retinopathy status as a predictor of interest and adjusted for peripheral parasitemia.

Differential gene expression between Ret+CM and Ret-CM samples was determined by calculating the *P* value by Student's *t* test and the fold change between samples by using R. We identified significantly differentially expressed genes with a *P* value less than 0.05. For pathway analysis, we employed Gene Set Enrichment Analysis (GSEA) (89, 90), the online CateGOrizer tool (91), and Ingenuity Pathway Analysis (IPA) (Qiagen, Redwood City, CA). For GSEA, we used an FDR of <0.20 to report a large number of pathways associated with each clinical phenotype and discuss pathways with a FDR of ≤ 0.06. For the GO slim analysis, we used the top 100 GO gene sets significantly enriched in either Ret+CM or Ret-CM identified by GSEA and summarized the result with the CateGOrizer online tool.

Plasma protein measurement. Plasma samples were obtained by centrifugation of whole-blood samples collected in EDTA and stored at −80°C on the day of collection. Plasma protein levels were assessed by Luminex using the human sepsis magnetic bead panel 3 (lactoferrin, lipocalin-2/neutrophil gelatinase-associated lipocalin [NGAL], and neutrophil elastase-2/ELA2), the human cardiovascular disease (CVD) panel 2 (MPO, P-selectin, soluble ICAM-1 [sICAM-1], soluble vascular cell adhesion molecule 1 [sVCAM-1]) and the human cytokine panel (IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1Ra, IL-8, IL-10, IL12p40, MCP-1, MIP-1α, RANTES, TNF-α, and vascular endothelial growth factor [VEGF]) according to the manufacturer's instructions (Millipore). Luminex panels were read on a Magpix multiplex reader (Luminex). Cell-free plasma heme levels were measured by using an enzyme-linked immunosorbent

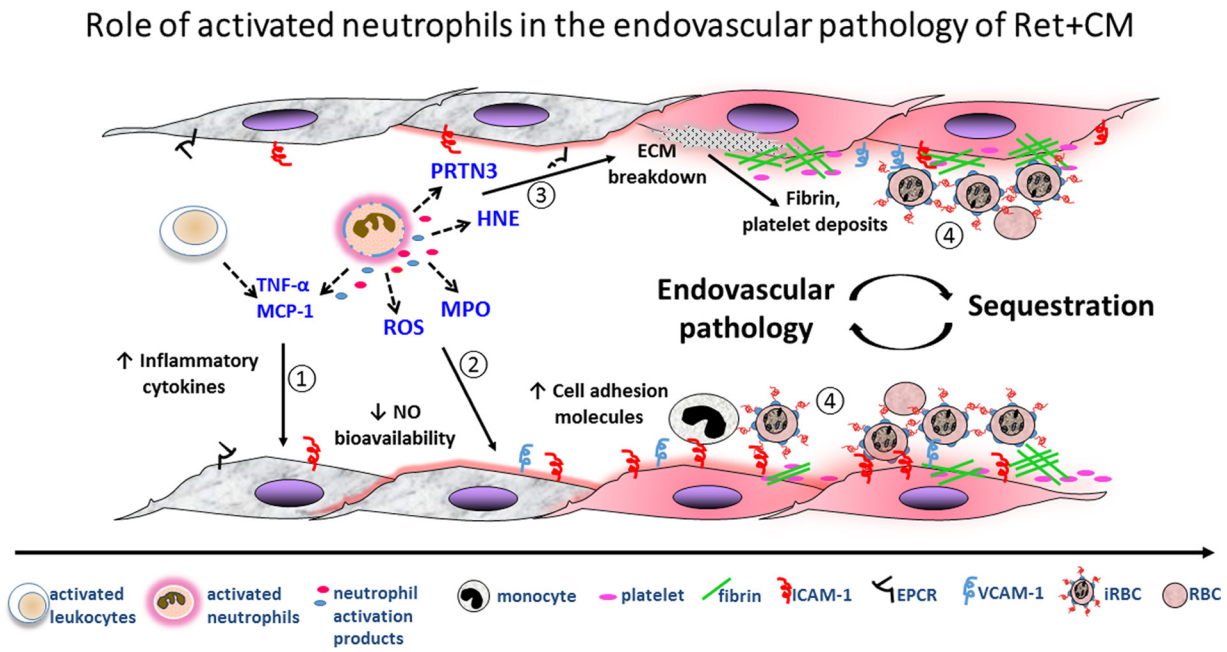

FIG 5 A model of cerebral iRBC sequestration in pediatric Ret+CM patients. A model for the role of activated neutrophils in the endovascular pathology of Ret+CM is shown. Our model proposes that multiple systemic factors modulate endothelial cell biology to result in the widespread endothelial iRBC sequestration seen in Ret+CM patients. Our data suggest a novel role of activated neutrophils in this process, and under conditions of inflammation and coagulopathy, the vascular endothelium is modified to facilitate iRBC adherence. Soluble neutrophil factors include the following. (1) Inflammatory cytokines and chemokines (e.g., TNF-α, MCP-1) (93–96) that stimulate the endothelium to increase cell adhesion molecule expression (e.g., ICAM-1, VCAM-1). (2) Reactive oxygen species (ROS) and myeloperoxidase (MPO) that impair endothelial nitric oxide (NO) bioavailability (97–99) to promote a pro-inflammatory response, increase cell adhesion molecule expression, disrupt the endothelial blood-brain barrier, and cause endothelial cell dysfunction. (3) Proteinase 3 (PRTN3) and neutrophil elastase (HNE) can modify the endothelial extracellular matrix (ECM) (100, 101), and PRTN3 cleaves endothelial protein C receptor (EPCR), an endothelial cytoprotective and anti-coagulation mediator (102), promoting coagulation cascade activation, fibrin formation, and activated platelet deposition (35) on brain vascular endothelium. (4) This systemic pro-coagulant and adhesive endothelium state leads to increased recruitment of immune host cell (e.g., monocytes, white blood cells) recruitment and widespread iRBC sequestration in the microvasculature of the brain and other vital organs, where local and systemic stimulation of endothelial and white blood cells causes an exacerbated host response leading to and perpetuating the vasculopathy of Ret+CM.

assay (ELISA) kit from Cayman Chemical. Plasma PRTN3 was measured by using an ELISA kit from R&D Systems.

Neutrophil chemotaxis assay. Whole blood was collected in heparin tubes. Neutrophils were enriched by gradient centrifugation over Ficoll-Paque^{plus} (GE Healthcare) within 6 h of blood collection. Red blood cells were lysed with ACK (ammonium-chloride-potassium) lysis buffer, and the remaining neutrophils were washed with Hanks balanced salt solution (HBSS) supplemented with 0.05% heat-inactivated fetal bovine serum (FBS), and resuspended at a concentration of 10^7 cells/ml in RPMI 1640 supplemented with 10% FBS.

Neutrophil chemotaxis was assessed using a 48-well chemotaxis chamber (NeuroProbe). RPMI 1640, fMLP (100 nM), or IL-8 (100 ng/ml) was loaded into the bottom of the chamber, a polycarbonate filter with 5-μm pores was laid down, and 10^5 neutrophils in 50 μl of RPMI 1640 was loaded into the top of the chamber. After 1 h of incubation at 37°C, the filter was removed, fixed, and stained. Densitometry analysis was performed to determine the relative amount of neutrophil chemotaxis across the membrane toward the bottom chamber.

Fetal hemoglobin and sickle cell assessment. β-Globin and γ-globin transcripts were quantified by qRT-PCR using methods previously described (92). The sickle cell trait was identified by restriction fragment length polymorphism (RFLP) using BsuI36 restriction enzyme (NEB) on qRT-PCR products. Genomic DNA (gDNA) from a patient homozygous for the sickle cell trait was used as a positive control.

Duffy null polymorphism assessment. DNA was extracted from dried blood spots using a DNeasy blood and tissue kit (Qiagen). The Duffy null polymorphism was genotyped using the TaqMan single-nucleotide polymorphism (SNP) genotyping assay and the 7300 fast system (Applied Biosystems).

Histology. To examine for the presence of tissue neutrophils or NETS, we examined four histological sections from the brains of Ret+CM patients and five histological sections from the brains of Ret-CM patients obtained at autopsy. The sections were fixed in 10% formalin and embedded in paraffin. Sections were cut and provided unstained and stained with hematoxylin and eosin (H&E) (19). Images were captured using an Axioskop2 microscope (Zeiss) equipped with a 40× objective and AxioCam high-resolution microscope camera (HRc) (Zeiss).

To examine for NETS, antigen-unmasking solution (Vector Laboratories) was used to reveal antigens in unstained formalin-fixed paraffin-embedded brain tissue sections. The antigen retrieval process was performed according to the manufacturer's instructions. Following antigen retrieval, the slides were blocked and stained with anti-neutrophil elastase (C-17; Santa Cruz Biotechnology), or rabbit citrullinated anti-histone H3 (anti-H3Cit) (citrulline 2, 8, and 17; Abcam) followed by species-specific secondary antibodies coupled with Alexa Fluor dyes (Invitrogen). DNA was stained using Hoechst 33342 (Sigma). Images were captured using an Axio Examiner D1 microscope (Zeiss) equipped with a Yokogawa CSU-X1 confocal scan head with a four-stack laser system (405-nm, 488-nm, 561-nm, and 642-nm wavelengths) and a 40× water immersion objective. Images were obtained using Slidebook software (Intelligent Imaging Innovations). Thirty random fields were imaged and analyzed. A tonsillar abscess similarly processed and stained was used as a positive control.

Microarray data accession number. Microarray data have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE72058.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01300-15/-/DCSupplemental.

Figure S1, PDF file, 0.3 MB.
Figure S2A, TIF file, 2.4 MB.
Figure S2B, TIF file, 0.8 MB.
Figure S3, TIF file, 0.2 MB.
Figure S4, TIF file, 0.02 MB.
Table S1, DOCX file, 0.02 MB.
Table S2, DOCX file, 0.01 MB.
Table S3AB, DOCX file, 0.05 MB.
Table S4AB, DOCX file, 0.1 MB.

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