Pharmacologic Inhibition of CXCL10 in Combination with Anti-malarial Therapy Eliminates Mortality Associated with Murine Model of Cerebral Malaria

Nana O. Wilson¹, Wesley Solomon¹, Leonard Anderson², John Patrickson³, Sidney Pitts³, Vincent Bond¹, Mingli Liu¹, Jonathan K. Stiles¹*

1 Department of Microbiology, Biochemistry, and Immunology, Morehouse School of Medicine, Atlanta, Georgia, United States of America, 2 Cardiovascular Research Institute, Morehouse School of Medicine, Atlanta, Georgia, United States of America, 3 Department of Pathology, Morehouse School of Medicine, Atlanta, Georgia, United States of America

Abstract

Despite appropriate anti-malarial treatment, cerebral malaria (CM)-associated mortalities remain as high as 30%. Thus, adjunctive therapies are urgently needed to prevent or reduce such mortalities. Overproduction of CXCL10 in a subset of CM patients has been shown to be tightly associated with fatal human CM. Mice with deleted CXCL10 gene are partially protected against experimental cerebral malaria (ECM) mortality indicating the importance of CXCL10 in the pathogenesis of CM. However, the direct effect of increased CXCL10 production on brain cells is unknown. We assessed apoptotic effects of CXCL10 on human brain microvascular endothelial cells (HBVECs) and neuroglia cells in vitro. We tested the hypothesis that reducing overexpression of CXCL10 with a synthetic drug during CM pathogenesis will increase survival and reduce mortality. We utilized atorvastatin, a widely used synthetic blood cholesterol-lowering drug that specifically targets and reduces plasma CXCL10 levels in humans, to determine the effects of atorvastatin and artemether combination therapy on murine ECM outcome. We assessed effects of atorvastatin treatment on immune determinants of severity, survival, and parasitemia in ECM mice receiving a combination therapy from onset of ECM (day 6 through 9 post-infection) and compared results with controls. The results indicate that CXCL10 induces apoptosis in HBVECs and neuroglia cells in a dose-dependent manner suggesting that increased levels of CXCL10 in CM patients may play a role in vasculopathy, neuropathogenesis, and brain injury during CM pathogenesis. Treatment of ECM in mice with atorvastatin significantly reduced systemic and brain inflammation by reducing the levels of the anti-angiogenic and apoptotic factor (CXCL10) and increasing angiogenic factor (VEGF) production. Treatment with a combination of atorvastatin and artemether improved survival (100%) when compared with artemether monotherapy (70%), p<0.05. Thus, adjunctively reducing CXCL10 levels and inflammation by atorvastatin treatment during anti-malarial therapy may represent a novel approach to treating CM patients.

Introduction

Malaria caused by Plasmodium falciparum infection remains a leading cause of global morbidity and mortality. An estimated 216 million cases of malaria occurred in 2010 globally, most of which were reported in sub-Saharan African children less than five years of age and resulted in 655,000 deaths [1]. Plasmodium falciparum in a subset of patients can lead to a diffuse encephalopathy known as cerebral malaria (CM). Cerebral malaria is a neurological complication of falciparum infections associated with high mortality rates and long-term morbidity. Despite appropriate anti-malarial treatment using quinine or artesinin derivatives, CM mortalities remains high (approximately 30% in adults and 18% in children) while 25% of survivors experience long-term neurological and cognitive impairment [2–6]. These observations suggest that strategies targeting the elimination of parasites during CM pathogenesis may be insufficient to prevent post treatment neurological complication and death [7]. Plasmodium berghei ANKA infection in mice is utilized as an experimental model of CM (ECM) that exhibits several of the neurological manifestations observed in human CM [8–15]. Similar to human CM, P. berghei ANKA-infected mice develop neurological signs including hemi- and paraplegia, ataxia, and convulsions [14–19]. Inflammatory cytokines are up-regulated in both human CM and murine ECM brains. In addition, reduced blood flow and increased lactate production are found in the brains of both humans and mice [9,20]. Plasmodium falciparum and P. berghei ANKA induce endothelial activation with expression of
adhesion molecules such as ICAM-1 and E-selectin which are upregulated in both human CM and murine ECM [12]. Additionally, human CM and murine ECM are characterized by severe platelet activation and accumulation within the cerebral microvasculature, coagulopathy, vascular leakage, edema, and microhemorrhages [21].

However, *P. berghei* ANKA-infected red blood cells may accumulate in the murine brain under certain circumstances, but whether infected red blood cells arrest is required for murine CM development and whether the underlying mechanism qualifies as true sequestration has been a matter of debate [9–13]. Studies have shown little or no accumulation of infected red blood cells in the murine brain and failed to demonstrate a correlation between sequestration of infected red blood cells and ECM [13,15,22]. Due to this discrepancy, the value of the *P. berghei* ANKA model for screening therapeutics against human CM has been questioned [13]. Nevertheless, *P. berghei* ANKA-infected mice do exhibit substantial platelet sequestration and leukocyte marginalization [21] processes that can contribute to severe malaria [23]. While differences between murine ECM and the human CM are often emphasized [8,24], there are also important similarities [9,11,12,25,26]. Thus, this model is suitable for studying certain aspects of the pathogenesis of human CM [12,23,27–31]. Nevertheless, we acknowledge that caution is required when translating experimental findings to clinical pathology.

Clinical studies in humans and murine models of ECM have shown that dysregulated inflammatory responses to infection and adverse effects on vascular endothelium play a central role in CM progression and outcome [20,32]. Furthermore, accumulating evidence indicate that host immune responses modulate pathogenesis and adverse clinical outcomes of CM. These observations have led to several clinical trials that utilize adjunctive therapies to modulate host immune responses as a means of managing human CM. Unfortunately, none of these adjunctive therapies investigated to date in randomized control trials have proven to be efficacious [33–35]. In addition, most adjunctive therapies tried so far in murine models have been administered as prophylactics to prevent development of neurological signs. The prophylactic strategy may not be clinically relevant since most CM patients develop neurological symptoms or present at late stages of the disease at clinics. The failure of adjunctive therapies in randomized control trials suggest an urgent need for development of novel adjunctive immunomodulatory therapeutics that target and ameliorate late stages of CM in order to improve clinical outcomes and prevent or reduce neurological complication in patients who survive treatment.

Our recent studies in West Africa and South Asia demonstrated for the first time that increased plasma and cerebrospinal fluid levels of interferon gamma induced protein 10 (CXCL10) secreted by several cell types in response to IFN-γ predict fatal human CM [36–38]. CXCL10 is a chemokine that performs several roles, including chemo-attraction for monocytes and T cells, promotion of T cell adhesion to endothelial cells, antitumor activity, inhibition of bone marrow colony formation and angiogenesis [39]. There is a growing appreciation of the role of CXCL10 in both infectious and non-infectious causes of central nervous system (CNS) neuronal injury, dementia, and inhibition of angiogenesis [40–43]. Following the first report of CXCL10 association with fatal human CM, Campanella et al. [44] observed that CXCL10 was the most highly induced chemokine during murine ECM and that mice deficient in CXCL10 gene were partially protected against CM when infected with *P. berghei* ANKA [44]. Furthermore, the study revealed that CXCL10 was induced in the brain during the onset of the disease and was highly expressed in the brain of mice infected with *P. berghei* ANKA at the late stage of ECM [44]. However, the effects of CXCL10 on the microvascular endothelial or brain cells are unknown. Both human and murine studies indicate that CXCL10 is linked to the pathogenesis of CM and justifies further evaluation of its effects on brain cells since the disease is associated with the brain. Determining the effect of CXCL10 on brain cells and developing a therapeutic strategy to target its production during human CM could be a novel approach to ameliorating or reversing CM pathogenesis to improve clinical outcomes while preventing neurological complications in survivors.

Atorvastatin (ATV), a Food and Drug Administration (FDA) approved drug that inhibits 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase significantly inhibits the expression of CXCL10 in humans [45]. However, the mechanism ATV utilizes to inhibit CXCL10 expression is unknown. Atorvastatin primarily inhibits HMG-CoA reductase to decrease cholesterol synthesis as well as increase the synthesis of low-density lipoprotein (LDL) receptors that result in an increased clearance of cholesterol and LDL from the bloodstream [46]. Furthermore, ATV has other beneficial effects of preventing thrombosis, protecting the vascular endothelium, stabilizing atheromatous plaque, neutralizing reactive oxygen radicals and exerting an anti-inflammatory effect [47]. In this study, we determined the effect of CXCL10 on cellular components of blood-brain barrier (human brain vascular endothelial cells [HBVECs] and neuroglia cells) by measuring apoptotic activities in vitro. Based on ATV’s potent inhibitory effect on CXCL10 expression [45] and the fact that ATV is an FDA approved drug, we evaluated ATV as adjunctive therapy by repurposing it to target CXCL10 as a strategy to ameliorate CM in murine ECM. We demonstrate that CXCL10 induces apoptosis in HBVECs and neuroglia cells. We also show that ATV (25 mg/kg) significantly reduces serum CXCL10 concentration and brain inflammation and improves survival in mice with ECM. Therefore, blocking the production of CXCL10 by ATV-like agents may represent a candidate approach to the treatment and management of human CM in the future.

**Results**

**CXCL10 induces caspase-mediated apoptosis in HBVECs and neuroglia cells**

To determine the effect of CXCL10 on brain cells, we examined the apoptotic effect of CXCL10 on HBVECs and neuroglia cells after 24 hours of exposure with different concentrations of recombinant human CXCL10 protein (rhCXCL10) by in situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Figure 1A) and Homogenous Caspases Assay (Figure 1B). Recombinant human CXCL10 protein caused dose-dependent apoptosis in HBVECs (Pearson’s *r* = 0.914, *p* = 0.029) and neuroglia cells (Pearson’s *r* = 0.965, *p* = 0.006) which was evident 24 hours after exposure (Figure 1A). The percentage of apoptotic cells increased from 8.9% to 26% in HBVECs after treatment with 0.002 μg/ml and 0.020 μg/ml respectively of rhCXCL10. Apoptosis in neuroglia cells increased from 12% to 40.6% with CXCL10 treatment of 0.002 μg/ml and 0.020 μg/ml respectively. Figure 1B shows rhCXCL10 induced caspase activation in HBVECs (Pearson’s *r* = 0.970, *p* = 0.006) and neuroglia cells (Pearson’s *r* = 0.935, *p* = 0.019) treated with different concentrations of rhCXCL10. The assay detects caspases 3 and 7 and indicates a role for these caspases in CXCL10-mediated cell death. Apoptosis in both cell lines were accompanied by a significant increase in the activation of caspases.
Combination of Artemether with Atorvastatin increases survival of mice with ECM

The control group developed murine ECM between days 5 and 12 post-infection with mortality between 30%–100% (Figure 2A). Artemether (ARM) has been successfully used to treat *P. berghei* ANKA-infected mice [48]. Using ARM treatment alone (25 mg/kg/day from day 6 to day 9 post-infection), clinical recovery began after initiating the treatment with significant reduction in parasitemia (Figure 2B). Mortality following ARM treatment was between 15% and 25% when compared to controls (p<0.001, Kaplan-Meier, log rank) (Figure 2A). Mice treated with ATV alone (25 mg/kg/day from day 6 to day 9 post-infection) showed reduced mortality at 50% when compared to the controls although parasitemia remained high (Figure 2A and B) suggesting that increased survival was not attributed to anti-parasitic effects but more likely modulation of host immune system.

Based on our hypothesis that the mortalities associated with late stage treatment of murine ECM was linked to the dysfunction and weakened stability of the blood-brain barrier, we have continued to search for adjunctive treatments that will stabilize the BBB at late stages of murine ECM while eliminating parasitemia and increasing survival to 100%. In this study, we have used ATV as an example of adjunctive treatment used in combination with ARM. To approximate the clinical settings, where treatment occurs after admission, mice were treated with a combination of ATV (25 mg/kg/day) and ARM (25 mg/kg/day) from day 6 to day 9 post-infection. Protection from late-stage murine ECM was significantly increased to 100% by adjunctive treatment with ATV together with ARM when compared to controls (p<0.0001), ARM alone (p<0.005), and ATV alone (p=0.025) groups (Figure 2A).

In order to assess effects of ATV on parasitemia, we assessed parasitemia before and after treatment. There were significant differences among treatment groups when parasitemia levels were compared with saline-treated mice on day 11 post-infection (p<0.05). Levels of parasitemia for saline-treated mice increase rapidly from day 5 to day 11 post-infection by which time all the control mice had been euthanized (Figure 2A and B). Although there was steady increase in parasitemia in the ATV treatment group, the parasitemia was significantly lower (p<0.05) when compared to the control group (Figure 2B) suggesting direct ATV effects on parasitemia. Artemether treatment efficacy was demonstrated by decreased parasitemia from 21% (day 5 post-infection) to<5% (day 11 post-infection) (Figure 2B). This parasitemia was increased to 8% on day 15 post-infection. The ATV/ARM treatment group showed a decrease in parasitemia from 20% on day 5 to 0% on day 15 post-infection (Figure 2B).

Survival of ECM mice treated with Atorvastatin is associated with decreased expression of CXCL10

We have previously established that elevated levels of CXCL10 are associated with fatal CM in human [36–38]. Thus, we attempted to reduce mortality in mice with ECM by testing the hypothesis that modulating CXCL10 during murine ECM will improve survival. The strategy involves repurposing ATV to modulate CXCL10 levels during murine ECM. Atorvastatin alone and ATV/ARM as well as ARM alone significantly decreased serum CXCL10 levels (p<0.001) and improved survival in ECM mice when compared with controls, p<0.05 (Figure 2A and G). The control group showed significant increase in CXCL10 on day 11, as previously reported, compared to day 5 post-infection, p<0.001 (Figure 2C).

Atorvastatin decreases leukocyte accumulation in brain vessels

Leukocyte adherence and accumulation in brain vessels correlate with brain inflammation and are key features of murine ECM [49]. To determine whether drug treatment was effective in reducing brain inflammation, we quantified the number of adherent leukocytes after treatment with ATV on day 11 post-infection. The numbers of leukocytes per vessels and per mm² decreased after treatment when compared with controls (Figure 3). Although ATV-treated mice had moderate-to-high levels of parasitemia compared to ARM-treated mice, there was a significant difference in number of leukocytes per vessels between ATV- and ARM-treated mice (Figure 3). However, ATV treatment in combination with ARM reduced the number of leukocytes adherent in the brain of the mice.
Atorvastatin and Artemether combination treatment decreases activation of vascular endothelium and enhances blood-brain barrier integrity in murine ECM

To evaluate the effects of the treatments on endothelium activation and blood-brain barrier integrity, mRNA transcription of specific protein markers (angiopoietin-1 and 2, perforin, Fas and IFN-γ) involved in vascular endothelium activation and breakdown of BBB were assessed. Brain mRNA expression was analyzed to contrast regulated changes with those occurring at day 5 and day 11 post infection in each group.

Angiopoietin-1 and angiopoietin-2 are antagonistic factors shown to trigger endothelial cell activation [50–53]. Angiopoietin-1, a marker of vascular endothelial quiescence and stability, was increased in mRNA expression in brain tissue of mice treated with ATV (2.5-fold) or ATV/ARM (2-fold) at late stage murine ECM compared with saline-treated mice, p<0.001 (Figure 4A). However, there were no significant changes in angiopoietin-1 mRNA expression in saline-treated and ARM-treated mice on day 11 when compared to untreated mice (Figure 4A). Angiopoietin-2, a marker that directly reflects vascular barrier breakdown was significantly reduced in expression in the brains of ATV- or ATV/ARM-treated mice when compared with saline-treated mice, p<0.001 (Figure 4B). There was a significant increase in angiopoietin-2 expression in the brain tissues of mice in saline-treated (1.9-fold) and ARM-treated (1.8-fold) mice at day 11 when compared to untreated mice at day 5, p<0.001 (Figure 4B). Conversely, ICAM-1, a marker of endothelial activation [47], showed about 6-fold induction in saline-treated and 4-fold in ARM-treated mice, p<0.001 (Figure 4C). While there was moderate increase in the level of ICAM-1 mRNA expression in the brain of ATV-treated mice (1.8-fold), there was a decrease in expression in ATV/ARM-treated mice (0.5-fold) when compared to saline-treated mice, p<0.001 (Figure 4C).

Perforin mRNA expression was significantly increased in brain tissue in saline-treated (3.5-fold), ARM-treated (2.5-fold) and ATV-treated (2.2-fold) mice compared with untreated mice on day 5 post-infection p<0.001 (Figure 4D). However, there were no significant changes in expression comparing ATV/ARM-treated mice with untreated mice on day 5 post-infection (Figure 4D). Compared to saline-treated mice there was a significant decrease in expression of perforin in the other treatment groups; p<0.001 (Figure 4D).

Both Fas and IFN-γ have been implicated in the pathogenesis of CM [34,55]. There were significant increases in the transcription of Fas in saline-treated (6.7-fold), ARM-treated (5-fold) and ATV-treated (4-fold) mice compared with untreated mice on day 5 post-infection, p<0.001. There was a decrease in expression of IFN-γ in ATV/ARM-treated mice (0.5-fold) compared with saline-treated mice, p<0.001 (Figure 4D).
Figure 3. Inhibition of inflammation in the brain of P. berghei-infected mice with ECM after treatment. (A) The number of intravascular leukocytes per mm² of brain area was markedly decreased after treatment. Parenchymal vessel of (B) untreated mice with ECM on day 5 and (C) saline-treated mice on day 11, plugged with leukocytes (black arrows). Parenchymal vessels of (D) ARM-treated mice, (E) ATV-treated mice and (F) ATV/ARM-treated mice showing remnant adherent leukocytes (black arrows) on day 11. The leukocyte counts are mean ± standard error. A p value of <0.05 was considered significant. Asterisks (*) denote statistically significant change compared with ctrl D5 and section sign ($) denote statistically significant change compared with ctrl D11. Ctrl = control; D = day; ARM = Artemether; ATV = Atorvastatin.

doi:10.1371/journal.pone.0060898.g003

Figure 4. Assessment of transcriptome associated with endothelium activation and blood-brain barrier integrity. mRNA expression of angiopoietin-1 (A), angiopoietin-2 (B), ICAM-1 (C), perforin (D), IFN-γ (E), and FasL (F) in whole brain homogenates of control, ARM, ATV, and ATV/ARM-treated P. berghei-infected mice (n = 5 per group). Expression, measured by real-time PCR on day 11 post-infection, is relative to the mean expression value in control mice day 5 post-infection brain samples and normalized with HPRT values. Treatment with ATV or ATV/ARM decreases endothelium activation and improves blood-brain barrier integrity. Columns and bars represent means ± standard error. Mean expression values were determined to be significantly different using one-way ANOVA with Holm-Sidak post-tests method for all pairwise multiple comparison. A p value of <0.05 was considered significant. Asterisks (*) denote statistically significant change compared with ctrl D5 and section sign ($) denote statistically significant change compared with ctrl D11. Note the different scales used in each graph. Ctrl = control; D = day; ARM = Artemether; ATV = Atorvastatin.
of IFN-γ mRNA in the brains of control, ARM-treated and ATV-treated mice, p<0.001 (Figure 4E). However, there were no significant changes in expression of IFN-γ mRNA among ATV/ARM-treated mice on day 11 when compared with untreated mice at day 5, (Figure 4E). Nevertheless, the expression of IFN-γ in ATV/ARM-treated mice was significantly lower when compared with saline-treated mice, p<0.001 (Figure 4E). Expression of Fas mRNA was significantly increased in saline-treated (2.5-fold) and ATV-treated (1.9-fold) mice, p<0.001 (Figure 4F). There were no significant changes in the expression of Fas mRNA in the brain tissue of ARM- and ATV/ARM-treated mice compared with untreated mice (Figure 4F). Expression of Fas mRNA in ARM- and ATV/ARM-treated mice were significantly lower when compare with saline-treated mice, p<0.001 (Figure 4F).

**Treatment with Atorvastatin modifies cytokine and chemokine expression profiles associated with murine ECM**

Multiplex immunoassay of panels of cytokines and chemokines associated with murine ECM [44] were performed with milliplex MAP mouse cytokine/chemokine panel (Millipore, MA) to determine the protective effect of ATV against murine ECM and its dampening effect on CXCL10 levels. The milliplex assay compared ATV-treated and saline-treated mice on day 11 post infection. Comparing the cytokine/chemokine profiles of ATV-treated with saline-treated infected mice revealed 20 down-regulated and 9 up-regulated genes with fold changes of >1.5.

Pathway analysis revealed a down-regulation of pro-inflammatory cytokines/chemokines in the panel with the exception of IL-15 and CXCL5 (Figure 5). The down-regulated cytokines/chemokines includes IFN-γ, TNF-α, IL-1α, IL-1β, IL-6, IL-12, IL-17, MCP-1, MIP-1α, MIP-1β, RANTES, eotaxin, and CXCL9 (Figure 5). Additionally, IL-2, IL-4, IL-10, GM-CSF, M-CSF, and CXCL1 were also down-regulated.

In contrast, a network of anti-inflammatory (IL-5 and IL-13) and growth factors (VEGF, G-CSF, IL-3, IL-7, and IL-9) were up-regulated in the ATV-treated compared to the saline-treated infected mice (Figure 5). Furthermore, using the Ingenuity Pathway Analysis software for core analysis it was noted that these networks of up-regulated and down-regulated cytokines and chemokines in ATV-treated mice modulates multiple biological and physiological processes during murine ECM pathogenesis. The analyses indicated that treatment with ATV enhanced or regulated biological processes such as survival and proliferation of neuroglia (p<3.27E-15), angiogenesis (p=4.23E-20), vascular endothelial cells and blood vessel development (p<7.15E-15), and production of nitric oxide (p=3.31E-20) (Table 1) which are essential processes required for recovery from murine ECM.

**Atorvastatin regulates CXCL10 through HO-1**

To determine the pathway(s) through which ATV modulates the expression of CXCL10, pathway analysis was used to explore the possible relationship between ATV and CXCL10. Pathway analysis identified five possible pathways that ATV could utilize for regulating CXCL10 expression. Atorvastatin inhibits activation of NFκB/p65 (RELA) [56,57], NFκB complex [58,59] and STAT1 [59] which are transcription factors that activate the transcription of CXCL10 gene to mRNA [60–67] (Figure 6). Atorvastatin increases expression of heme oxygenase-1 (HO-1) [68] which inhibits activation of NFκB complex [69], STAT1 [70] and expression of CXCL10 [71] (Figure 6). Atorvastatin stimulates production of nitric oxide (NO) [72], which inhibit active NFκB/p65 and NFκB complex [73–76]. Furthermore, nitric oxide decreases expression of CXCL10 [77] and increases induction of HO-1 [78–80] (Figure 6).

We have previously shown that heme/HO-1 and CXCL10 are directly involved in the pathogenesis of CM and that HO-1 modulates CXCL10 expression in vitro [81]. Therefore, we validated the ATV/HO-1/CXCL10 pathway by determining the expression of HO-1 and CXCL10 as well as CXCR3 in the brain tissue of the mice with late stage ECM receiving different drug treatments. Atorvastatin or ATV/ARM treatment increased HO-1 expression and decreased CXCL10 expression in the brains of the mice on day 11 when compared with untreated or saline-treated mice, p<0.001 (Figure 7A and B). Expression of HO-1 mRNA in brain tissue of the ARM group was decreased (Figure 7A). However, CXCR3, a receptor of CXCL10 mRNA expression, was increased among the ATV treatment group alone when compared with untreated or saline-treated mice, p<0.001 (Figure 7C).

**Table 1. The table displays biological processes or functions enhanced by Atorvastatin treatment identified by Ingenuity Pathway Analysis as significantly (p<0.05) associated with the dataset.**

| Biological Processes Enhanced by Atorvastatin Treatment | # of gene products | P-value |
|-------------------------------------------------------|--------------------|---------|
| Survival of neuroglia                                 | 8                  | 3.27E-15|
| Proliferation of neuroglia                            | 11                 | 5.97E-18|
| Angiogenesis                                          | 19                 | 4.23E-20|
| Endothelial cell development                          | 12                 | 7.15E-15|
| Development of blood vessel                           | 21                 | 7.27E-22|
| Production of Nitric Oxide                            | 14                 | 3.31E-20|

The column ‘# of gene products’ gives the number of cytokines/chemokines from the dataset obtained from the network.

doi:10.1371/journal.pone.0060898.0001
Discussion

Blood-brain barrier disruption and dysfunction are among the major features of human CM [82,83]. Blood-brain barrier disruption is detected in children and adult patients with CM studied by in vivo imaging techniques [84–86], and are of prognostic value in these patients [87]. The exact cause of blood-brain barrier dysfunction and damage during human CM pathogenesis is unclear. The correlation between the number of brain petechial hemorrhages and poor clinical outcomes has led to the proposal that blood-brain barrier disruption may be partially due to vascular endothelial apoptosis caused by granzyme B and

Figure 6. Schematic model of signaling pathways ATV utilize to modulate the expression of CXCL10 identified by Ingenuity Pathway Analysis. In response to various stimulus, transcription factors, STAT1, NFkB, and RELA, are activated resulting in the transcription of CXCL10 gene to mRNA [57–64]. Atorvastatin inhibit activation of RELA (NFkB/p65) [56,57], NFkB complex [58,59] and STAT1 [59]. Atorvastatin increases expression of HO-1 [68] which inhibit activation of NFkB complex [69], STAT1 [70] as well as expression of CXCL10 [71]. Atorvastatin stimulates production of nitric oxide (NO) [72], which inhibit active NFkB/p65 and NFkB complex [73–76], ATV = atorvastatin; IRBC = infected red blood cells; HO-1 = heme oxygenase-1; NO = nitric oxide; RELA = v-rel reticuloendotheliosis viral oncogene homolog A (avian); NFkB = nuclear factor of kappa light polypeptide gene enhancer in B-cells; STAT1 = signal transducer and activator of transcription 1.

doi:10.1371/journal.pone.0060898.g006

Figure 7. mRNA expression of HO-1 (A), CXCL10 (B), and CXCR3 (C) in whole brain homogenates of control, ARM, ATV, ATV/ARM-treated Plasmodium berghei ANKA-infected mice (n = 5 per group). Expression, measured by real-time PCR on day 11 post infection, is relative to the mean expression value in untreated mice day 5 post infection and normalized on HPRT values. Treatment with ATV or ATV/ARM increased HO-1 expression and decreased CXCL10 expression in Plasmodium berghei ANKA-infection mice brain tissue. The mean expression values were determined to be significantly different using one-way ANOVA with Holm-Sidak post-tests method for all pairwise multiple comparison. A p value of <0.05 was considered significant. Asterisks (*) denote statistically significant change compared with ctrl D5 and section sign ($) denote statistically significant change compared with ctrl D11. Note the different scales used on each graph. Ctrl = control; D = day; ARM = artemether; ATV = atorvastatin.

doi:10.1371/journal.pone.0060898.g007
perforin-mediated cytotoxicity [88,89]. Our previous studies indicate that CXCL10 levels are remarkably elevated in plasma and cerebrospinal fluid of patients who died from CM than those who survive the disease after treatment confirming the observation linking elevated CXCL10 in CM patients with poor prognosis [36–39]. Additionally, CXCL10 was an independent chemokine predictor of fatal CM in Ghanaian children and Indians who died of CM [36–39]. These observations in human CM were confirmed in murine studies, which revealed that mice deficient in the CXCL10 gene were partially protected against murine ECM when infected with *P. berghei* ANKA [44]. Campanella et al. [44] observed that CXCL10 was the initial chemokine induced in the brain during the onset of murine ECM and was highly expressed in the brain of mice infected with *P. berghei* ANKA at the late stage of ECM. Although, the murine malaria encephalopathy does not completely mimic human CM, both observations in human and mice demonstrate that CXCL10 is a strong indicator/predictor of CM in both human and murine ECM. However, the effects of CXCL10 on brain cells were unknown although the disease was associated with the brain tissue. Here, we have demonstrated that CXCL10 induces apoptosis in HBVECs as well as neuroglia cell in a dose-dependent manner. The apoptotic assay revealed that CXCL10-induced cell death occurs at a minimum threshold concentration of 0.002 µg/ml, which is within the physiologically relevant range of concentrations found in patients who died of CM [36,37]. This evidence suggests that increased production of CXCL10 in CM patients may contribute to the neuro-pathogenesis and blood-brain barrier damage associated with CM. Additionally, the study indicated that neuroglia cells are more susceptible to CXCL10-induced apoptosis than HBVECs. This suggests that CM may be initiated or amplified by CXCL10 interaction with the brain cells resulting in changes in the blood-brain barrier and cerebrospinal fluid, which would allow other cytokines and malaria antigens to enter the brain compartment from which they would be normally excluded [90]. This would lead to the activation of microglia or damage to astrocytes, a phenomenon that has been observed in human CM and murine ECM [91–94]. Astrocytes are critical in regulating interstitial fluid milieu contributing to the maintenance of the blood-brain barrier and synthesis of neuro-protective molecules. Thus, impairment of astrocyte function may cause disruption of neuronal activity [90]. We propose that increased expression of CXCL10, a potent anti-angiogenic and apoptotic protein observed in CM patients, contributes to activation and apoptosis in endothelial cells, which perturbs the blood-brain barrier, and subsequently directly or indirectly induces neuroglia apoptosis.

Despite optimal anti-malarial treatment and advances in malaria eradication, the mortality rate associated with CM remains unacceptably high. Quinine and artemisinin derivatives (artemunate and artemether) are the drugs of choice for treatment and management of human CM [95,96]. The rational for selecting ARM for this study is based on a previous study demonstrating that ARM was significantly effective against murine ECM than quinine, artemisinin, and artesunate [48]. However, the mortality rate was unacceptably high as high as 30% despite treatment with ARM [48]. This relatively high mortality rate may be due to low efficacy of ARM against *P. berghei* ANKA or other unexplained effects of ARM on compromised blood-brain barrier at late stage ECM. Additionally, targeting parasite eradication without considering secondary effects such as tissue damage and neurological complications may be inadequate for preventing the mortalities. Thus, the need for developing innovative therapeutic strategies to effectively reduce CM-associated mortality is urgent. The current study focused on adjunctive therapy in combination with anti-malarial agents in late stage *P. berghei* ANKA murine ECM rather than early stage treatment. We used ATV adjunctively with ARM treatment of established infections and iteratively repurposed a drug selected for its ability to modulate CXCL10 and inflammatory responses in murine ECM. Using advanced bioinformatics, the study explores the effectiveness of this approach as a potential adjunctive therapy for human CM in a clinical setting.

Our previous human studies indicate that increased levels of potent anti-angiogenic and apoptotic factor CXCL10 are tightly associated with fatal human CM [36–38,97,98]. In murine ECM, removing the effect of CXCL10 by knocking out the CXCL10 gene improved survival [44,99]. Together, these findings suggest that CXCL10 plays an important role in the pathogenesis of the disease. Applying the inhibitory role of ATV against CXCL10 in murine ECM treatment, we have demonstrated that ATV administered at the late stage of murine ECM reduced the production and effect of CXCL10 and increased survival.

Although other statins have been used in murine ECM, these studies led to significant delays in mortality or failed to improve survival [100–102]. Most of these statin treatments tested in murine ECM were not evaluated with or compared to effective anti-malarial drugs in the context of use in treatment of human *falciparum* malaria [101–104]. Rather, these statins were assessed for their anti-parasitic properties against *P. berghei* ANKA in murine ECM [101–104]. For example, simvastatin was evaluated for its effect on parasitemia during murine ECM with no comparison with an effective anti-malarial drug [101,102]. These studies concluded that simvastatin had no relevant effect on parasite growth inhibition and clinical outcome of murine ECM [101,102]. Furthermore, some studies typically examined survival benefits following administration of statin treatments to mice before the development of neurological signs. In support of this approach, a recent study using ATV alone therapeutically had no effect on survival in a murine ECM. However, a prophylactic scheme employing ATV together with melfloquine, rather than melfloquine alone, yielded a significant delay in mortality and onset of murine ECM symptoms [100]. This prophylactic strategy prevents the development of neurological signs but does not reverse established CM pathologies observed in clinical practice. Using a prophylactic may not be clinically relevant since most CM patients develop neurological symptoms or are at the late stages of the disease when they present at the clinics. The criteria used for initiating treatment in our study were strict and only mice with late stage ECM presentations that developed neurological signs were included in the study. We evaluated ATV as an adjunctive treatment along with an anti-malarial (ARM) in mice after they have developed ECM. Recently, Reis et al. [104] evaluated the therapeutic effect of lovastatin in murine ECM and determined that lovastatin decreased neuro-inflammation and prevented blood-brain barrier dysfunction in murine ECM [104]. However, lovastatin treatment failed to improve survival in mice with ECM [104]. The difference between ATV and other statins evaluated in murine ECM is that, ATV is a potent inhibitor of CXCL10 in humans [45]. In addition, our previous human studies indicated that increased levels of CXCL10 were tightly associated with fatal human CM [36–38,97,98]. Nevertheless, CXCL10 has also been found to be associated with murine ECM [44,99]. The rational for using ATV in the current study was to assess its effect on CXCL10 expression in murine ECM. We repurposed ATV to reduce production of CXCL10 as an adjunctive therapy in murine ECM. In our study, ATV treatment given after presentation of neurological signs increased or improved survival of mice with ECM. This effect is likely due in part to reduction in anti-angiogenic and apoptotic factors including CXCL10 and
enhancement of the angiogenic growth factor vascular endothelial growth factor (VEGF) which is associated with fatal human CM [36–30].

High levels of CXCL10 may contribute to vascular injury resulting in breakdown of the blood-brain barrier and accumulation of leukocytes to cause local hyper-inflammation [37]. Although parasite sequestration is the most common feature of patients succumbing to CM, postmortem examination has also revealed intra- and peri-vascular pathology including the presence of leukocytes within brain blood vessels in human CM [105]. These findings suggested that sequestration of host leukocytes might also contribute to the pathogenesis of some human CM cases [106]. Recent findings revealed that increased levels of several inflammatory chemokines including MIP-1α and MIP-1β and CXCL10 are associated with increased risk of severe malaria [36–38,107], suggesting a role for leukocytes trafficking in etiology of human CM [106]. Similar to humans, parasitized red blood cells have been found to accumulate in brains of susceptible mice during murine ECM [108]. Since both human and murine vascular obstruction, either by parasitized red blood cells, leukocytes or by both, appears to be associated with features of CM, the P. berghei ANKA model is useful for analysis of the relationship between reversal of microvessel blockage and inflammation in human CM [24]. In the present study, ATV effectiveness in recovering mice with ECM was associated with a marked decrease in leukocytes accumulation in brain microvessels resulting in rapid down-regulation of brain inflammation. This suggests that ATV may play a role in reversal of vessel blockage and inflammation in human CM.

CXCL10 is pathologically involved in both human and ECM [36–38,44]. CXCL10 has also been reported to play a role in CNS neuronal injury, dementia, inhibition of angiogenesis, and interfere with VEGF function [40,41,43]. Furthermore, CXCL10 inhibits angiogenesis and regeneration of damaged blood capillaries needed for CM recovery thereby exacerbating severe outcomes [36]. Vascular endothelial growth factor stimulates endothelial cell growth, migration, and enhances vascular permeability [37]. VEGF is important in brain tissue repair and in CNS wound healing [109,110]. In addition, a protective effect against human CM mortality has been associated with VEGF levels as higher levels of VEGF decreased the risk of human CM mortality [37]. Together with VEGF, the angiogenic factors angiopoietin-1 (biomarker of endothelial quiescence and stability), and angiopoietin-2 (marker of vascular barrier breakdown) are major regulators of vascular inflammatory response, endothelial activation, and endothelial integrity [111]. These angiogenic factors are now proposed as informative biomarkers of disease severity and clinical response in humans [111]. Angiopoietin-1 stabilizes vascular endothelial barrier through regulation of reversible paracellular and transcellular fluid transport mechanism by opposing the action of permeability-increasing mediators such as platelet-activating factor, bradykinin, thrombin, and histamine [112,113]. In humans, decreased angiopoietin-1 levels and increased angiopoietin-2 levels are associated with severity of CM [50–52,114,115]. Low angiopoietin-1 and high angiopoietin-2 plasma levels are associated with retinopathy, discriminate human CM and severe non-cerebral malaria from uncomplicated malaria, and predict mortality from human CM [50,52,53,114]. Atorvastatin treatment resulted in up-regulation of angiopoietin-1 and down-regulation of angiopoietin-2 suggesting enhanced survival provided by ATV adjunctive therapy. This indicates that administration of ATV had a significant impact on endothelial and blood-brain barrier integrity. Interestingly using bioinformatics and network analysis it was determined that ATV treatment regulates biological processes such as survival and proliferation of neuroglia, angiogenesis, and endothelia cell and blood vessels development, which are essential processes needed for recovery from human CM.

Parasite sequestration is necessary for parasite survival in the mammalian host and is important to downstream events such as obstruction of blood flow and in microvessels localized hypoxia [111]. Parasite sequestration may lead to localized release of bioactive toxins, which recruit inflammatory mediators that could contribute to onset of human CM [116]. Inflammatory cytokines contribute to disease by up-regulating the expression of adhesion molecules such as ICAM-1 to bind parasitized red blood cells to the vascular endothelium in humans [106]. ICAM-1, a marker of endothelial activation, plays a crucial role in pathogenesis of both human CM and murine ECM [117–119]. In humans, increased ICAM-1 levels are associated with severity of CM [117,120–122]. In murine ECM, increased expression of ICAM-1 on brain endothelial cells coincide with increases in microvascular permeability while ICAM-1 deficient mice are protected from ECM [123,124]. ICAM-1 signaling mediates endothelial activation, rearrangement of endothelial actin cytoskeleton, regulation of vascular permeability and transmigration of T cells through endothelial tight junctions into the brain parenchyma under various inflammatory conditions [118,125–127]. Consistent with decreased leukocyte adherence to the micro-vascular endothelium of ATV-treated infected mice, there was a significant decrease in mRNA expression of ICAM-1 in the brain tissues of ATV-treated mice compared with saline-treated mice at day 11. Nevertheless, perfolin and Fas, which are key mediators of blood-brain barrier damage [54,55,128,129], were down-regulated in the brain of mice treated with ATV. Perforin and Fas are also important factors in murine ECM [54]. Perforin-mediated cytosis occurs through apoptosis of activated endothelial cells leads to breakdown of blood-brain barrier during ECM [54,128,129]. Additionally, Fas-mediated apoptosis of astrocytes is a critical factor in late stage ECM pathogenesis [54,55,128]. Thus, both cytokines are critical to the pathogenesis of ECM.

A principal feature of human CM is the sequestration of parasitized red blood cells in the brain microvasculature and obstruction [130–132]. We acknowledge that murine ECM is an inflammatory syndrome with local vascular activation with inflammatory cytokines playing an essential role. There are differences and some similarities between ECM and clinical and pathological features of human CM. For instance, vascular inflammation or damage is seen in pediatric human CM cases [105]. Other studies have suggested that pro-inflammatory cytokines are involved in pathogenesis of human CM, and that increased levels of these inflammatory cytokines in the plasma and cerebrospinal fluid are tightly associated with fatal human CM [37,38,98,133]. Thus, inflammation plays an essential role in fatal CM in at least a subset of clinical patients. Therefore, studies in murine ECM should provide insight into inflammatory processes and immune responses and their role in human CM pathology [24]. Proteomic analysis showed that ATV reduces production of pro-inflammatory cytokines IFN-γ, TNF-α, IL-1β, IL-6 and IL-12 and chemokine MIP-1α, MIP-1β, and RANTES. These cytokines/chemokines have all been implicated in human CM and murine ECM, but no individual molecule has been identified as a key regulator in all settings [134]. Neutralization of IFN-γ on the incidence of ECM and prevents TNF-α overproduction [135] and several studies have shown associations between TNF-α and CM [136]. However, in a clinical trial, treatment with a monoclonal anti-TNF-α antibody did not protect against human CM and exacerbated neurological sequelae [137]. Increased levels of IL-1β,
IL-6, and IL-12 have also been reported in human CM [20,138–141]. In the brain, MCP-1 contributes to increased permeability of blood-brain barrier and increases leukocyte infiltration into CNS during inflammation process [142–144]. High levels of MCP-1 have been found in patients with neuro-inflammatory diseases, including cerebral ischemia and HIV-1 encephalitis [145,146]. However, in the current study, ATV reduced the production of MCP-1 in the ECM model. High levels or expression of inflammatory chemokine MIP-1α, MIP-1β, and RANTES has also been associated with human CM [97,107,147,148]. Interestingly, these molecules were down-regulated in murine ECM after ATV treatment.

It is widely accepted that anti-inflammatory cytokines down-regulate pro-inflammatory cytokines. Elevated levels of anti-inflammatory IL-10 have previously been reported in severe malaria [149,150]. In addition, IL-10 has been proposed to down-regulate pro-inflammatory cytokine production, resulting in a lower incidence of cerebral symptoms in mice with ECM [151]. However, in the present study treatment with ATV in murine ECM resulted in down-regulation of IL-10 and IL-4 and up-regulation of anti-inflammatory cytokines IL-5 and IL-13.

Cerebral malaria may lead to poor neurological and cognitive outcomes in surviving patients suffering significant neurological damage leading to long-term deficits [152]. There is evidence linking CM to hippocampal damage in CM patients [153,154]. Interestingly, ATV treatment selectively increased expression of neuro-protectants, IL-3, IL-15, and G-CSF. IL-3 exerts trophic action on hippocampal neurons and rescue hippocampal neurons from lethal ischemic damage [155]. IL-3 has been shown to be a potent inhibitor of neuronal death [156]. Pro-inflammatory cytokine IL-15 regulates neuro-immune responses by regulating T-cell and macrophage infiltration and activation after nerve injury [157,158]. IL-15 has been shown to regulate astrocitary, microglial and neural functions [159]. G-CSF reduces apoptosis, drives neurogenesis and angiogenesis, and attenuates inflammation [160–163]. Moreover, G-CSF is protective in stroke, Alzheimer’s disease, and spinal cord injury [164,165]. Thus, the prevention or the reversal of neurological damage with ATV treatment may result from a combination of decreased inflammatory damage to the blood-brain barrier, increased in neuro-protectants and reduced obstructive plugging of micro-vessels. Therefore, it is likely that ATV may prevent cognitive impairment in mice recovering from ECM.

Reproduction of Plasmodium parasites in red blood cells causes release of free heme, which increases the expression of the anti-inflammatory enzyme HO-1 [104]. Increased expression of HO-1 prevents mortality in murine ECM and blood-brain barrier disruption, brain microvascular congestion and neuro-inflammation [166]. Previous reports have shown that statins induce HO-1 expression [167,168]. In the present study, we also observed that ATV increased expression of HO-1 in mice with ECM, suggesting that some of protective effects of ATV resulted from increased in HO-1 expression and potentially contributing to the decrease in the inflammatory response and oxidative damage [104]. Atorvastatin treatment resulted in up-regulation of HO-1 and down-regulation of CXCL10 in the brain of mice with ECM. In addition, HO-1 modulates CXCL10 expression [81]. Furthermore, ATV specifically modulates the expression of CXCL10 through inhibition of transcription factors (NFkB and STAT1) and increased expression of HO-1 and nitric oxide (NO) [58,59,68,72,77]. Studies indicate that NO protects against murine ECM and has been utilized in previous clinical trials as adjunctive therapy [169–172]. Bioinformatic analysis revealed that ATV stimulates production of NO in mice with ECM. Nitric oxide decreases expression of CXCL10 [77]. These data support the hypothesis that reducing the production of a potent anti-angiogenic and apoptotic factor CXCL10 may represent an attractive adjunctive therapy for CM and encourage further testing of atorvastatin-like molecules or drugs in the clinical setting.

The consistent failure of adjunctive immunomodulatory treatments in human CM against murine ECM has been discussed [13]. Among the many possible explanations, this may be due to interventions not targeting the range of functions central to pathophysiology of human CM. Alternatively, the ability of a drug candidate such as ATV to impact a range of functions or pathways central to pathophysiology of both human CM and murine ECM increases the likelihood that the effect will translate to human CM. For example, ATV would modulate CXCL10 expression, inflammatory mediators, and improve vascular and blood-brain barrier integrity in the brain. Therefore, while the ECM model may not completely mimic human CM, there are significant hallmarks of CM between the human and murine ECM. Further, we have identified an adjunctive therapeutic strategy that could effectively reduce CM-mortality. In the absence of an effective adjunctive therapy, we suggest the urgent need further study this drug.

Although the use of statins in children is still being debated [173], there is evidence of efficacy and safety of statins in children with familial hypercholesterolemia [174–176]. However, the protective effect of ATV associated with ARM treatment demonstrates that agents with ATV-like properties that target potent anti-angiogenic and apoptotic factor CXCL10 and other cytokines/chemokines involved in human CM pathogenesis are urgently needed. Thus, FDA-approved drugs for oral treatment of high cholesterol, ATV, or ATV-like molecules/drug may present a novel class of adjunctive therapeutics for CM management in intensive care units, using a combination of anti-malarial and anti-inflammatory drugs that dampen host pro-inflammatory factors. The major concern may be the cost of delivery and/or availability of atorvastatin-like agents in low-income countries where malaria is endemic. Deciphering the molecular mechanisms by which ATV prevents CM-associated mortalities, controls CXCL10 and other molecules associated with pathogenesis of the disease could provide new drug targets and efficacious ways to improve the outcome of patients suffering from CM.

Methods

Cell culture

Human neuroglia cells were obtained from the American Type Culture Collection (M059K, ATCC, Manassas, VA) and cultured at 37°C with 5% CO2 in Complete Serum-free Medium Kit (Cell Systems, Kirkland, WA) supplemented with 2% fetal bovine serum (FBS; ATCC, Manassas, VA) and 100 U/ml of streptomycin, 100 U/ml of penicillin (Gibco, Grand Island, NY) and were harvested and passaged at about 70–90% confluence. At confluence, HBVECs (2×10^5 cells/ml) were transferred into 35-mm tissue culture dish containing collagen-coated cover slip and incubated at 37°C in 5% CO2 for 24–48 hours.

Human neuroglia cells were obtained from the American Type Culture Collection (M059K, ATCC, Manassas, VA) and cultured at 37°C under 5% CO2 in a humidified atmosphere in D-MEM/F-12 medium (ATCC, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum and 100 U/ml of streptomycin, 100 U/ml of penicillin. At about 90% confluence, the cells were transferred into a 35-mm tissue culture dish containing a collagen-coated cover slip at a density of 2×10^5 cells/ml and incubated at 37°C in 5% CO2 for 24–48 hours.
TUNEL assays
Apoptotic effect of recombinant human CXCL10 protein (R&D Systems, Minneapolis, MN) on HBVECs and neuroglia cells were determined in dose-dependent manner (0.002, 0.004, 0.010, and 0.020 μg/ml). DNA damage was determined as a means of assessing apoptosis using terminal deoxynucleotidyl transferase dUTP nick ending labeling (TUNEL) assay with In Situ Cell Death Detection Fluorescein Kit (Roche Diagnostics, Indianapolis, IN) which detect and quantify apoptosis at single cell level. Briefly, the cells were fixed in 4% paraformaldehyde in phosphate buffered saline for 1 hour and permeabilized with Triton X-100 at 4°C for 2 min and then incubated with enzyme solution and labeling solution (TUNEL reaction mixture) for 60 min at 37°C. The cells were visualized by epifluorescence on a computer-controlled Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY) linked to Magnafire 2.1C (Olympus America, Melville, NY) and Image-Pro Plus 4.1 (Media Cybernetics, Silver Springs, MD) imaging software via a charged coupled device (CCD) camera (MC 100 SPOT 60910; Photonic Science, East Sussex UK) to determine the degree of apoptosis. All experiments were done in triplicate.

Caspase activation assay
Homogeneous Fluorimetric Caspase Assay (Roche Diagnostics, Indianapolis, IN) was used to investigate whether apoptosis occurred by a caspase-mediated mechanism in HBVECs and neuroglia cells treated with different concentration of recombinant human CXCL10 proteins (0.002, 0.004, 0.010, and 0.020 μg/ml) according to manufacturer’s instructions. The release of rhodamine, which is an indicator of caspase activity in the assay, was detected by fluorescence, and fluorescence intensity was used to compare caspase activation across different concentration treatments. The homogeneous caspase assay was carried out to investigate caspase activation in HBVECs and neuroglia cells upon incubation with CXCL10 proteins for 24 hours. Briefly, the cells were incubated in caspase substrate at 37°C for 4.5 hours. The cleavage of the substrate by activated caspase and the release of fluorescence was determined fluorometrically at 521 nm using Spectra Max 190 fluorescence microplate reader (Molecular Devices Corp., Sunnyvale, CA). The Relative Fluorescence Units (RFU) signal is converted to nM free rhodamine via standard curve.

Ethics statement
This study was carried out in strict accordance with the recommendations in the guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Usage Committee (IACUC) of Morehouse School of Medicine ( Permit Number 09-06).

Mice and Plasmodium berghei ANKA parasites
Six- to eight-week-old C57BL/6J mice were purchased from Charles Rivers Laboratories (Wilmington, MA). Mice were housed at Morehouse School of Medicine in groups of no more than four per cage on 12 h light/12 h dark cycle with access to food ad libitum and water. Mice were allowed to adapt to their new environment for 3 days before experimentation. All experimental protocols were reviewed and approved by the Morehouse School of Medicine Institutional Animal Care and Use Committee. All procedures were performed in accordance with national regulations on animal experimentation and welfare, and the Care of Laboratory Animal Resources (CLAR) guideline was followed to minimize animal pain. The P. berghei ANKA was used (a kind donation of MR4, Manassas, VA; deposited by T.F. McCutchan; MR4 reagent number MRA-311). The parasite was propagated in C57BL/6J mice, and in each experiment, a fresh blood sample was obtained from a passage mouse and a suspension, containing 1 × 10⁷ parasitized red blood cells in 100 μl was injected intraperitoneally in each mouse of the experimental groups. Mice were checked several times daily for mortality and the development of neurological symptoms indicative of murine ECM, such as ataxia, loss of reflex, and hemiplegia. All murine ECM experiments were terminated 19 days after infection. Parasitemia was checked beginning on day 5 after infection. Parasitemia was monitored by Giemsa-stained blood smears using light microscopy at ×100 magnifications with an oil immersion lens (Nikon Eclipse E200). Parasitemia was checked and quantified by counting the number of parasitized red blood cells in at least 1,000 red blood cells. After treatment, we considered dead parasites as those that presented as a mass of condensed matter inside the red blood cells and could no longer be clearly distinguish based on typical morphology.

Clinical assessment
Simple behavioral tests (transfer arousal, locomotor activity, tail elevation, wire maneuver, contact righting reflex, and righting in arena) adapted from the SHIRPA protocol [177,178] was used to provide a better estimate of the overall clinical status of the mice during infection. Murine experimental cerebral malaria (ECM) was defined as the presentation of one or more of the following clinical signs of neurological involvement: ataxia, limb paralysis, poor righting reflex, seizures, roll-over, and coma [48]. Clinical signs of murine ECM was evaluated and used for scoring disease severity [179]. Infected mice show presence of parasites as well as murine ECM signs on day 5 or 6 post-infection [48].

Drug treatment regimens
The present study was designed to treat P. berghei ANKA-infected mice presenting clinical signs of murine ECM. This study sought to determine the efficacy of adjunctive therapy by reducing CXCL10 levels in recovery of mice with late stage ECM. The mice were treated after they were diagnose with murine ECM on day 6 with one of the following drug regimens intraperitoneally. (i) Artemether (Sigma-Aldrich, St. Louis, MO) was prepared in coconut oil and was administered at 25 mg/kg. (ii) Atorvastatin (Sigma-Aldrich, St. Louis, MO) was dissolved in saline and delivered at 25 mg/kg.

Experimental design
A group of 44 infected mice was used for survival experiments. To obtain significant data, 11 mice were included in each group. After diagnosis of murine ECM on day 6, mice were randomized in 4 groups. At days 6, 7, 8, and 9 post infection, 11 mice received saline (control group), 11 mice received 25 mg/kg/day of artemether (ARM group), 11 mice received 25 mg/kg/day of atorvastatin (ATV group), and 11 mice received the drug combination atorvastatin 25 mg/kg/day and artemether 25 mg/kg/day (ATV/ARM group). Each injection subsequent to the initial dose was given in the morning.

Tissue extraction, sample preparation, and histopathology
Mice were anesthetized by isoflurane inhalation and euthanized on days 5 and 11 after infection. Mice were perfused with 10 ml of cold sterile phosphate buffered saline to clear vessels of blood and...
brains were collected and processed for histology. Brains were stored in formalin for fixation, embedded in paraffin, and sectioned at 10 μm. Sagittal sections of the brain were mounted on glass slides and stained with hematoxylin-eosin (H&E). The number of leukocytes in each vessel of each section was quantified by using an ocular grid calibrated with a ×400 magnification in a Zeiss Axioskop 2 plus microscope (Carl Zeiss Microscopy, NY). The whole area of each section was similarly quantified with the grid calibrated at ×40 magnification. Pictures were taken with a Zeiss AxioCam HRc camera (Carl Zeiss Microscopy, NY).

Measurement of cytokine and chemokine levels in serum
To determine the effect of drug treatments on CXCL10 as well as levels of known pro- and anti-inflammatory cytokine and growth factors associated with CM, serum cytokines were measured from pre-treatment (day 5) and post-treatment (day 11) blood draws using a commercially available multiplex bead-based cytokine assay (Millipore, MA) coupled with the Luminex system (Austin, TX). Thirty-two analytes (CCL1, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, CXCL10, CXCL11, IFN, CXCL5, M-CSF, CCL2, CXCL9, CCL3, CCL4, MIP-2, CCL5, TNF-α, VEGF) were analyzed. Biological factor levels were measured using optimal concentrations of standards and antibodies according to the manufacturer’s instructions. The data was analyzed usingIngenuity Pathway Analysis software.

RNA extraction and reverse transcription (RT)-PCR
Whole brain tissue from each group of mice were collected and immediately homogenized in Trizol reagent (Life Technologies, Gaithersburg, MD). Total RNA was extracted using RNeasy Mini Kit (QIAGEN, CA). Briefly, chloroform (0.2 ml) was added to the homogenate, and the lysate mixed thoroughly. After centrifuging at 12,000 g for 20 min at 4°C, the aqueous layer was transferred to a new tube. RNA was precipitated with 500 μl of isopropanol and pelleted by centrifuging at 12,000 g for 20 min at 4°C. Any contaminating genomic DNA was removed by DNase treatment using RNase-Free DNase Set (QIAGEN, CA) according to contaminating genomic DNA was removed by DNase treatment. The whole area of each section was similarly quantified with the grid calibrated at ×40 magnification. Pictures were taken with a Zeiss AxioCam HRc camera (Carl Zeiss Microscopy, NY).

The quantitative real-time PCR assay was performed using Bio-Rad C1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA). Approximately 20 ng of cDNA was used in each 25 μl PCR reaction using the Bio-Rad iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA) and 50 μM of each primer. After a 15 min incubation at 95°C, amplification was achieved by 39 cycles of a 1 s denaturation incubation at 95°C, followed by a 50 s annealing incubation at 55°C and 30 s extension incubation at 72°C. The identity and purity of the PCR product was confirmed by using dissociation curves and by checking the melting temperature of the PCR product, independently of the PCR reaction. To determine the relative amount of target cDNA present, the cycles to threshold (Ct) values of the target genes were compared with the basal expression of the housekeeping gene, hypoxanthine guanine phosphoribosyltransferase (HPRT). The average amount of HPRT present in each mouse group was used to normalized the quantity of target mRNA sequence against total RNA in each reaction. The differences in Ct values between HPRT and target gene of day 11 after infection of each group were compared with day 5 after infection untreated control samples to determine the relative change in mRNA expression.

Pathway analysis
To determine the regulatory pathways linked to CXCL10 and modified by ATV as well as its effects on disease processes, network and pathways analyses were performed using the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Inc., Redwood City, CA; www.ingenuity.com). In addition, we identified up- and down-regulated genes that are significantly associated with ATV treatment on day 11 after infection relative to saline treatment on day 11 after infection. Briefly, each gene identifier was mapped to it corresponding gene object in the Ingenuity Pathway Knowledge Base. Day 11 protein fold-change of ATV-treated mice relative to saline-treated mice was overlaid onto global molecular networks in the Ingenuity Pathway Knowledge Base. These networks were ranked based on the significance score value calculated by the Ingenuity Pathway Analysis software. Networks of focus genes were algorithmically

| Table 2. Primer sequences used. |
|--------------------------------|
| **Target gene or mRNA** | **Primer 5 ' - 3 '** | **Forward** | **Reverse** |
| HPRT | GCTTCTTCCGTGTTAAGCAGCTACA | CAAACTGTGTGAACTTCTCAATTC | GTCAGGGTTGTCAGCTTTG |
| ICAM-1 | GCTCCGGCTTCTTCGATCTT | GTTGGCGTGTCAGCTTTG |
| Perforin | TGGCCCCATTTGTTGTGAAG | AGTCTCCACAGATGCTTCG |
| IFN-γ | CAGCAACAGGAAGGCAAAA | GCTGGATTCCGGCAACAG |
| FasL | ACCACCACTCCATGTCACACTA | CACCGTAGCCACAGATTTG |
| CXCL10 | GAGGCTCCGGCTGCAACTG | GCTTTCTATGGCCCTCTCAT |
| CXCR3 | AATGCGACAGGAGCTGTACAT | ATCGAGAGACGATATCAAG |
| HO-1 | GCCAGCAGGAGGATACAT | CTTCAAGGGAGTATGATA |
| ANG-1 | ATGGTTGCTCAAAACCACAG | TTCAAGTGCGGATTTGT |
| ANG-2 | ATGGTGGCAGAAGCCAGACA | GCAAGCTCGAGTCTGTCG |

doi:10.1371/journal.pone.0060898.t002
generated based on their connectivity and their association with biological function and/or disease most significant for the dataset. The Fisher’s exact test was used to compare the different survival curves. A p-value of <0.05 was considered significant. SigmaPlot (version 11.0) with SigmaStat (version 3.5) integration (Chicago, IL) and SAS version 9.2 (Cary, NC) software for windows were used for statistical analysis. GraphPad Prism version 6 (La Jolla, CA) for windows was used to generate all the graphs.

Acknowledgments

We thank Morehouse School of Medicine (MSM) Center for Laboratory Animal Resources staff for technical assistance in animal experiments. Plasmodium berghei ANKA parasites were obtained through the MR4 as part of the BEI Resources Repository, NIAMD, NIH: Plasmodium berghei ANKA, MRA-511, deposited by TF McCutchan.

Author Contributions

Conceived and designed the experiments: NOW WS JKS. Performed the experiments: NOW WS SP. Analyzed the data: NOW WS LA JP ML. Contributed reagents/materials/analysis tools: NOW WS LA JP SP ML VB JKS. Wrote the paper: NOW WS JKS.

References

1. World Health Organization (2012) World Malaria Report 2011.
2. Dondorp AM, Fanello CI, Heiniksen KC, Gomes E, Seni A et al. (2010) Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial. Lancet 376: 1647–1657.
3. Idro R, Marsh K, John CG, Newton CR (2010) Cerebral malaria: mechanisms of brain injury and strategies for improved neurocognitive outcome. Pediatr Res 68: 267–274.
4. Idro R, Kakoosa-Mwinegi A, Balevjesa S, Mirenge B, Mugasha C et al. (2010) Severe neurological sequelae and behaviour problems after cerebral malaria in Ugandan children. BMC Res Notes 3: 104.
5. John CG, Bangirana P, Byarugaba J, Opoka RO, Idro R et al. (2008) Cerebral malaria in children is associated with long-term cognitive impairment. Pediatrics 122: e92-e99.
6. Dondorp A, Nesten F, Stepniweska K, Day N, White N (2005) Artesunate versus quinine for treatment of severe falciparum malaria: a randomised trial. Lancet 366: 717–725.
7. Higgins SJ, Kain KC, Liles WC (2011) Immunopathogenesis of falciparum malaria: implications for adjunctive therapy in the management of severe and cerebral malaria. Expert Rev Anti Infect Ther 9: 803-819.
8. Langhorne J, Buffet P, Galinski M, Good M, Harty J et al. (2011) The relevance of non-human primate and rodent malaria models for humans. Malar J 10: 23.
9. Hunt NH, Grau GE, Engebret C, Barnum SR, van der HH et al. (2010) Murine cerebral malaria: the whole story. Trends Parasitol 26: 272-274.
10. Riley EM, Couper KN, Helmbly H, Hafalla JC, de Souza JB et al. (2010) Neuropathogenesis of human and murine malaria. Trends Parasitol 26: 277-287.
11. Carvalho LJ (2010) Murine cerebral malaria: how far from human cerebral malaria? Trends Parasitol 26: 271-272.
12. de Souza JB, Hafalla JC, Riley EM, Couper KN (2010) Cerebral malaria: why experimental murine models are required to understand the pathogenesis of disease. Parasitology 137: 753–772.
13. White NJ, Turner GD, Medana IM, Dondorp AM, Day NP (2010) The murine cerebral malaria phenomenon. Trends Parasitol 26: 11–15.
14. Engveldt C, Behouze E, Gruner AG, Renia L (2005) Experimental models of cerebral malaria:Curr Top Microbiol Immunol 297: 103-143.
15. Franke-Fayard B, Jane CJ, Canha-Rodrigues M, Ramesar J, Buscher P et al. (2005) Murine malaria parasite sequestration: CD56 is the major receptor, but cerebral pathology is unlikely to sequestration. Proc Natl Acad Sci U S A 102: 11411–11417.
16. Lamb TJ, Brown DE, Potocnjak AJ, Langhorne J (2006) Insights into the immunopathogenesis of malaria using mouse models. Expert Rev Mol Med 8: 1–22.
17. Lou J, Lucas K, Grau GE (2001) Pathogenesis of cerebral malaria: recent experimental data and possible applications for humans. Clin Microbiol Rev 14: 810–20, table.
18. Pampolna A, Ferreira A, Balla J, Jeney V, Balla G et al. (2007) Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. Nat Med 13: 703–710.
19. Renia L, Potter SM, Mauduit M, Rosas DS, Kayibanda M et al. (2006) Pathogenic T cells in cerebral malaria. Int J Parasitol 36: 547–554.
20. Brown H, Turner G, Rogerson S, Tembo M, Mwenechanya J et al. (1999) Cytokine expression in the brain in human cerebral malaria. J Infect Dis 180: 1742–1746.
21. Hancer A, Movila A, Baer K, Mikhajlyazak S, Kuppe SH et al. (2012) Neuroimmunological blood brain barrier opening in experimental cerebral malaria. PLoS Pathog 8: e1002962.
22. Mackey IJ, Hochmann A, June CH, Contreras CE, Lambert PH (1980) Immunopathological aspects of Plasmodium berghei infection in five strains of mice. II. Immunopathology of cerebral and other tissue lesions during the infection. Clin Exp Immunol 42: 412-420.
23. Cox D, McConkey S (2010) The role of platelets in the pathogenesis of cerebral malaria. Cell Mol Life Sci 67: 557–568.
24. Craig AG, Grau GE, Jane C, Kazura JW, Milner D et al. (2012) The role of animal models for research on severe malaria. PLoS Pathog 8: e1002401.
25. Zimmerman GA, Castro-Faria-Neto H (2010) Persistent cognitive impairment after cerebral malaria: models, mechanisms and adjunctive therapies. Expert Rev Anti Infect Ther 8: 1209-1212.
26. Nie CQ, Bernard NJ, Norman MU, Amante FH, Lundie RJ et al. (2009) IP-10-mediated T cell homing promotes cerebral inflammation over splenic immunity to infection. PLoS Pathog 5: e1000369.
27. Faillie D, El-Aassaf F, Assiti MG, Fusi T, Combes V et al. (2009) Platelet-endothelial cell interactions in cerebral malaria: the end of a cordial understanding. Thromb Haemost 102: 1093-1102.
28. Faillie D, Combes V, Mitchell AJ, Fontaine A, Juhan-Vague I et al. (2009) Platelet microparticles: a new player in malaria parasite cytoadherence to human brain endothelium. FASEB J 23: 3494–3508.
29. Haldar K, Murphy SC, Milner DA, Taylor TE (2007) Malaria: mechanisms of erythrocytic infection and pathological correlates of severe disease. Annu Rev Pathol 2: 217-249.
30. Grau GE, Mackenzie CJ, Carr RA, Redard M, Pizzolato G et al. (2003) Platelet accumulation in brain microvessels in fatal cerebral malaria. J Infect Dis 187: 461–466.
31. McIntosh PD, Bier JC, Oster CN, Onyango FK, Olo OJ et al. (1997) Dose- and time-dependent relations between infective Anopheles inoculation and outcomes of Plasmodium falciparum parasitemia among children in western Kenya. Am J Epidemiol 145: 945–956.
32. Day NP, Heim TT, Scholzarth T, Lee PF, Chong LK et al. (1999) The prognostic and pathophysiologic role of pro- and anti-inflammatory cytokines in severe malaria. J Infect Dis 180: 1208–1297.
33. Mainland K, Kagioul S, Opoka RO, Engoru C, Olupot-Olupot P et al. (2011) Mortality after fluid bolus in African children with severe infection. N Engl J Med 364: 2483-2495.
34. John CG, Kastamba E, Mugarura K, Opoka RO (2010) Adjunctive therapy for cerebral malaria and other severe forms of Plasmodium falciparum malaria. Expert Rev Anti Infect Ther 8: 997–1008.
35. Mishra SK, Newton CR (2009) Diagnosis and management of the neurological complications of falciparum malaria. Nat Rev Neurol 5: 189–198.
36. Wilson NO, Jain V, Roberts CE, Lucchi N, Joel PK et al. (2011) CXCL4 and CXCL10 predict risk of fatal cerebral malaria. Dis Markers 30: 39–49.
47. Hua Y, Xi G, Keep RF, Hoff JT (2000) Complement activation in the brain.

46. Goldstein JL, Brown MS (2009) The LDL receptor. Arterioscler Thromb Vasc.

45. Grip O, Janciauskiene S (2009) Atorvastatin reduces plasma levels of...a complex signal-transduction problem. Biochem Soc Trans 33: 669–672.

44. Campanella GS, Tager AM, El Khoury JK, Thomas SY, Abrazinski TA et al. (2009) Atorvastatin attenuates angiotensin II-induced inflammatory actions in...a complex signal-transduction problem. Biochem Soc Trans 33: 669–672.

43. Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE et al. (2002) IFN-...nuclear translocation of IkappaBalpha. J Biol Chem 272: 30969–30974.

42. Asensio VC, Maier J, Milner R, Boztug K, Kincaid C et al. (2001) Interferon-...nuclear translocation of IkappaBalpha. J Biol Chem 272: 30969–30974.

41. Galimberti D, Schoonenboom N, Scheltens P, Fenoglio C, Bouwman F et al. (2000) Intrathecal chemokine synthesis in mild cognitive impairment and Alzheimer disease. Arch Neurol 63: 538–543.

40. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

39. Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE et al. (2002) IFN-...inhibiting factor p105 precursor. J Biol Chem 277: 34223–34228.

38. Hua Y, Xi G, Keep RF, Hoff JT (2000) Complement activation in the brain. Antimicrob Agents Chemother 55: 1383–1390.

37. Jain V, Arnam HB, Tongeun JE, Red RM, Wilson NO et al. (2000) Plasma IP-...-induced viral protein 10 (IP-10; CXCL10)-deficient mice reveal a role for...me required for the development of murine cerebral malaria. Proc Natl Acad Sci U S A 105: 401–406.

36. Grippo O, Januskauskiene S (2009) Ato...hcytokine gene expression by astrocytes in vivo and in vitro. J Virol 75: 7067–7073.

35. Galbreth D, Schoonenboom N, Scheltens P, Fenoglio C, Bouwman F et al. (2000) Intrathecal chemokine synthesis in mild cognitive impairment and Alzheimer disease. Arch Neurol 63: 538–543.

34. Diaz-Alberto Y, Pimoule S, Pichot V, Fargue X, Rouzafel A et al. (2009) Heme...activity on mononuclear cells in the cerebrospinal fluid of patients with viral meningitis is mediated by interferon-gamma induced protein-10 and...ative inflammatory effects of acute alcohol on IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

33. Liu P, Li K, Garrard RP, Brasier AR (2008) Respiratory syncytial virus...inhibiting factor p105 precursor. J Biol Chem 277: 34223–34228.

32. Vogel SN, Feunon M (2005) Toll-like receptor 4 signaling: new perspectives on the cellular-specific functions of CXCL chemokine ligand 9 mediated by IFN-gamma microglobin in the central nervous system is determined by the myeloid transcription factor PU.1. J Immunol 175: 1864–1877.

31. Vogel SN, Feunon M (2005) Toll-like receptor 4 signaling: new perspectives on the cellular-specific functions of CXCL chemokine ligand 9 mediated by IFN-gamma microglobin in the central nervous system is determined by the myeloid transcription factor PU.1. J Immunol 175: 1864–1877.

30. Frere JD, Khoury JK, Thomas SY, Abrazinski TA et al. (2009) Atorvastatin attenuates angiotensin II-induced inflammatory actions in...a complex signal-transduction problem. Biochem Soc Trans 33: 669–672.

29. Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE et al. (2002) IFN-...nuclear translocation of IkappaBalpha. J Biol Chem 272: 30969–30974.

28. Asensio VC, Maier J, Milner R, Boztug K, Kincaid C et al. (2001) Interferon-...nuclear translocation of IkappaBalpha. J Biol Chem 272: 30969–30974.

27. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

26. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

25. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

24. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

23. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

22. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

21. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

20. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

19. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

18. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

17. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

16. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

15. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

14. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

13. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

12. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.

11. Conroy AL, Lafferty EI, Lovegrove FE, Krudsood S, Tangpukdee N et al. (2009) Atorvastatin attenuates cytokine-induced IL-10 induction involving p38 MAPK activation in monocytes. J Immunol 177: 2592–2600.
104. Reis PA, Estato V, da Silva TI, d’Avila JC, Siqueira LD et al. (2012) Statins but potentiate artesunate. Antimicrob Agents Chemother 52: 4203–4204.

105. Taylor TE, Fu WJ, Carr RA, Whitten RO, Mueller JS et al. (2004) MCP-1 in endothelial cell tight junction ‘opening’: signaling via Rho and Rho kinases. J Immunol 165: 3375–3383.

106. Mehta D, Malik AB (2006) Signaling mechanisms regulating endothelial cell permeability. Physiol Rev 86: 279-367.

107. Ochiel DO, Awandare GA, Keller CC, Hittner JB, Kremsner PG et al. (2005) Cerebrospinal fluid cytokine levels and cognitive impairment in cerebral malaria. Ann Trop Med Hyg 78: 190-205.

108. Surfo BY, Wilson NO, Bond VC, Stiles JK (2011) Plasmodium berghei ANKA infection increases Fop3 α, IL-10 and IL-6 in CXCL-10 deficient C57BL/6 mice. Malar J 10: 69.

109. Jones KL, Donegan S, Lalloo DG (2007) Artemesine versus quinine for treating severe malaria. Cochrane Database Syst Rev CD005967.

110. Krum JM, Khaibullina A (2003) Inhibition of endogenous VEGF impedes malaria-induced vascular clogging, mononuclear cell margination, and enhanced vascular permeability in the pathogenesis of human cerebral malaria. Ann Trop Med Hyg 51: 642–647.

111. Gay F, Zougbede S, N’dilimabaka N, Rebollo A, Mazier D, Moreno A (2012) Vascular remodeling, mononuclear cell margination, and enhanced vascular permeability in the pathogenesis of human cerebral malaria. Ann Trop Med Hyg 51: 642–647.

112. Mehta D, Malik AB (2006) Signaling mechanisms regulating endothelial cell permeability. Physiol Rev 86: 279-367.

113. van Nieuw Amerongen GP, van H, V (2002) Targets for pharmacological intervention of endothelial hyperpermeability and barrier function. Vasc Pharmacol 39: 257–272.

114. Conroy AL, Glover SJ, Hawkes M, Erdman LK, Seydel KB et al. (2012) Angiopoietin-2 levels are associated with retinopathy and predict mortality in Malawian children with cerebral malaria: a retrospective case-control study. Crit Care Med 40: 1952–1959.

115. Erdman LK, Dhabangi A, Muaseke C, Conroy AL, Hawkes M et al. (2011) Combinations of host biomarkers predict mortality among Ugandan children with severe malaria: a retrospective case-control study. PLoS One 6: e17440.

116. Helmers AJ, Gowda DC, Kain KC, Liles WC (2009) Statins fail to improve outcome in experimental cerebral malaria: lessons from experimental murine models. PLoS Pathog 8: e1003045.

117. Chang WL, Sun G, Chen HL, Specian RD et al. (2006) Perforin mediated apoptosis of cerebral microvascular endothelial cells during experimental cerebral malaria. Int J Parasitol 36: 485–496.

118. Turner GD, Morrison H, Jones M, Davis TM, Louaresuwan S et al. (1994) Anti-immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. Ann Pathol 14: 1057-1069.

119. Akawa M, Ikie M, Barnwell JW, Taylor D, Oo MM et al. (1990) The pathology of human cerebral malaria. Ann Trop Med Hyg 43: 30–37.

120. de Souza JK, Stiles JK, Adjie AA et al. (2005) High-level cerebellar expression of cytokines and adhesion molecules in fatal, paediatric, cerebral malaria. Ann Trop Med Parasitol 99: 629–647.

121. Cserti-Gazdewich CM, Dzik WH, Erdman L, Ssewanyana I, Dhabangi A et al. (2010) Combined measurement of soluble and cellular ICAM-1 among children with Plasmodium falciparum malaria in Uganda. Malar J 9: 233.

122. Patnaik JK, Das BS, Mishra SK, Mohanty S, Satpathy SK et al. (1994) Vascular egression, mononuclear cell margination, and enhanced vascular permeability in the pathogenesis of human cerebral malaria. Ann Trop Med Hyg 51: 642–647.

123. van Hensbroek MB, Palmer A, Onyiorah E, Schneider G, Jaffar S, Dolan G et al. (1996) The effect of a monoclonal antibody to tumor necrosis factor alpha and IFN-gamma in plasma of healthy individuals and malaria patients in a holoendemic area. J Clin Lab Immunol 55: 128–140.

124. Grau GE, Herremans H, Piguet PF, Pointaire P, Lambert PH et al. (1989) Monoclonal antibody against interferon gamma can prevent experimental cerebral malaria and its associated overproduction of tumor necrosis factor. Proc Natl Acad Sci U S A 86: 5572–5574.

125. Grau GE, Taylor TE, Molyneux ME, Wirima JJ, Vassalli P et al. (1989) Tumor necrosis factor and disease severity in children with falciparum malaria. J Pathol 163: 519-526.

126. van Hooxbroek MB, Palmer A, Onyisoroh E, Schneider G, Jaffar S, Dolan G et al. (1996) The effect of a monoclonal antibody to tumor necrosis factor on survival from childhood cerebral malaria. J Infect Dis 174: 1091–1097.

127. Carney DH, Mengin J, Theiler K, Albrecht M, Brown D et al. (2005) Combined measurement of soluble and cellular ICAM-1 among children with cerebral malaria. Ann Trop Med Parasitol 99: 629–647.

128. Jakobsen PH, McKay V, Morris-Jones SD, McGuire W, van Hooxbroek MB et al. (1999) Combined measurement of soluble and cellular ICAM-1 among children with cerebral malaria: a retrospective case-control study. PLoS Med 6: e17440.

129. Schlueter CR, Aveen P, Arnesen K, Huitfeldt LB, Skulski P et al. (2003) Combined measurement of soluble and cellular ICAM-1 among children with cerebral malaria: a retrospective case-control study. PLoS Med 6: e17440.

130. Turner GD, Morrison H, Jones M, Davis TM, Louaresuwan S et al. (1994) Anti-immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. Ann Pathol 14: 1057-1069.

131. Akawa M, Ikie M, Barnwell JW, Taylor D, Oo MM et al. (1990) The pathology of human cerebral malaria. Ann Trop Med Hyg 43: 30–37.

132. Mshana RN, Boulandi J, Mshana NM, Mayombo J, Mendome G (1991) The effect of a monoclonal antibody to tumor necrosis factor alpha and IFN-gamma in plasma of healthy individuals and malaria patients in a holoendemic area. J Clin Lab Immunol 55: 128–140.

133. van Hensbroek MB, Palmer A, Onyiorah E, Schneider G, Jaffar S, Dolan G et al. (1996) The effect of a monoclonal antibody to tumor necrosis factor on survival from childhood cerebral malaria. J Infect Dis 174: 1091–1097.

134. van Hooxbroek MB, Palmer A, Onyisoroh E, Schneider G, Jaffar S, Dolan G et al. (1996) The effect of a monoclonal antibody to tumor necrosis factor on survival from childhood cerebral malaria. J Infect Dis 174: 1091–1097.
trantact in the ischemic brain after transient focal ischemia in rats. J Leukoc Biol 65: 744–749.

146. Cinque P, Vago L, Mengozzi M, Torri V, Ceresa D et al. (1998) Elevated cerebrospinal fluid levels of monocyte chemotactic protein-1 correlate with HIV-1 encephalitis and local viral replication. AIDS 12: 1327–1332.

147. Sarthou JL, Angel G, Aribot G, Rogier C, Dieye A et al. (1997) Prognostic value of the Plasmodium falciparum malaria with respiratory distress. J Infect Dis 175: 848–856.

148. Rentzos M, Rombos A (2012) The role of IL-15 in central nervous system disorders. Acta Neurol Scand 125: 77–82.

149. Kawada H, Takizawa S, Takanashi T, Morita Y, Fujita J et al. (2006) Induction of cell-mediated tissue repair. J Exp Med 203: 1781–1789.

150. Peyron F, Burdin N, Ringwald P, Vuillez JP, Rousset F et al. (1994) High levels of circulating IL-10 in human malaria. Clin Exp Immunol 95: 300–303.

151. Sarfo BY, Singh S, Lillard JW, Jr., Quarshie A, Gyasi RK et al. (2004) The role of IL-10 in Plasmodium falciparum malaria. Arch Dis Child 89: 1086–1089.

152. Idro R, Carter JA, Fegan G, Neville BG, Newton CR (2006) Risk factors for cerebral malaria: a case study. Cortex 33: 385–388.

153. Schijns OE, Visser-Vandewalle V, Lemmens EM, Janssen A, Hoogland G (2000) Serum levels of circulating IL-10 in West African patients with severe malaria. Infect Immun 68: 5199–5205.

154. Saito M, Kiyokawa N, Taguchi T, Suzuki K, Sekino T et al. (2002) Granulocyte colony-stimulating factor directly effects human monocytes and modulates cytokine secretion. Exp Hematol 30: 1115–1123.

155. Enevoldsen L, Curbet M, Scholz B, Drevin J, Christensen K et al. (2011) Induction of cell-mediated tissue repair. J Exp Med 203: 1781–1789.

156. Diederich K, Schabitz WR, Minnerup J (2012) Seizing old friends from a different angle: novel properties of hematopoietic growth factors in the healthy and diseased brain. Hippocampus 22: 1051–1057.

157. Stein EA (2007) Statins and children: whom do we treat and when? Circulation 116: 594–595.

158. Martins YC, Zanini GM, Frangos JA, Carvalho LJ (2012) Efficacy of different lipid-lowering agents for the adjunctive treatment of severe malaria. J Exp Med 203: 1781–1789.

159. Nishio Y, Koda M, Kamada T, Soneya Y, Kadota R et al. (2007) Granulocyte colony-stimulating factor attenuates neurological death and promotes functional recovery after spinal cord injury in mice. J Neurophysiol Exp Neurol 60: 724–731.

160. Rentzos M, Rombos A (2012) The role of IL-15 in central nervous system disorders. Acta Neurol Scand 125: 77–82.

161. Martirosyan V, Vardanian MK, Blumrich IH, Vardanyan PV, Arutiunyan SN et al. (2011) Induction of cell-mediated tissue repair. J Exp Med 203: 1781–1789.

162. Schabitz WR, Minnerup J (2012) Seizing old friends from a different angle: novel properties of hematopoietic growth factors in the healthy and diseased brain. Hippocampus 22: 1051–1057.

163. Martinez-Gimber RJ, Bocca L, de la Torre J, de la Torre J et al. (2010) Algorithms to predict cerebral malaria in murine models using the SHIRPA protocol. Malar J 9: 85.

164. Cuadrado P, Jarrín-González D, Pérez-Ramírez D, Villegas JC, Heussler V et al. (2006) Induction of cell-mediated tissue repair. J Exp Med 203: 1781–1789.

165. Huang Z, Guo X, Hu HK, Perillo JM (2007) IL-15 and IL-15α: alpha gene deletion: effects on T lymphocyte trafficking and the microglial and neuronal responses to facial nerve anatomy. Neurosci Lett 417: 160–164.

166. Diederich K, Schabitz WR, Minnerup J (2012) Seizing old friends from a different angle: novel properties of hematopoietic growth factors in the healthy and diseased brain. Hippocampus 22: 1051–1057.

167. Ali F, Zakkar M, Kaur K, Leligdon EA, Hamdulay SS et al. (2009) Induction of cell-mediated tissue repair. J Exp Med 203: 1781–1789.

168. Nath N, Giri S, Prasad R, Singh AK, Singh I (2004) Potential targets of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor for multiple sclerosis therapy. J Immunol 172: 1273–1286.

169. Wierzbicki AS, Viljoen A (2010) Hyperlipidaemia in paediatric patients: the role of lipid-lowering therapy in clinical practice. Drug Saf 33: 115–125.

170. Nath N, Giri S, Prasad R, Singh AK, Singh I (2004) Potential targets of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor for multiple sclerosis therapy. J Immunol 172: 1273–1286.

171. Hawkes M, Opoka RO, Namasopo S, Miller C, Conroy AL et al. (2011) Nitric oxide for the adjunctive treatment of severe malaria: hypothesis and rationale. Med Hypotheses 77: 457–464.

172. Stein EA (2007) Statins and children: whom do we treat and when? Circulation 116: 594–595.

173. Carreau V, Girardet JP, Bruckert E (2011) Statins for children with familial hypercholesterolemia: efficacy and tolerability. Paediatr Drugs 13: 267–275.