

S1P Is Associated with Protection in Human and Experimental Cerebral Malaria

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Cerebral malaria (CM) is associated with excessive inflammatory responses and endothelial activation. Sphingosine 1-phosphate (S1P) is a signaling sphingolipid implicated in regulating vascular integrity, inflammation and T-cell migration. We hypothesized that altered S1P signaling during malaria contributes to endothelial activation and inflammation, and show that plasma S1P levels were decreased in Ugandan children with CM compared with children with uncomplicated malaria. Using the *Plasmodium berghei* ANKA (PbA) model of experimental CM (ECM), we demonstrate that humanized *S1P lyase (hS1PL)*^{-/-} mice with reduced S1P lyase activity (resulting in increased bio-available S1P) had improved survival compared with wild-type littermates. Prophylactic and therapeutic treatment of infected mice with compounds that modulate the S1P pathway and are in human trials for other conditions (FTY720 or LX2931) significantly improved survival in ECM. FTY720 treatment improved vascular integrity as indicated by reduced levels of soluble intercellular adhesion molecule (sICAM), increased angiotensin 1 (Ang1) (regulator of endothelial quiescence) levels, and decreased Evans blue dye leakage into brain parenchyma. Furthermore, treatment with FTY720 decreased IFN γ levels in plasma as well as CD4⁺ and CD8⁺ T-cell infiltration into the brain. Finally, when administered during infection in combination with artesunate, FTY720 treatment resulted in increased survival to ECM. These findings implicate dysregulation of the S1P pathway in the pathogenesis of human and murine CM and suggest a novel therapeutic strategy to improve clinical outcome in severe malaria.

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INTRODUCTION

Cerebral malaria (CM) is a life-threatening complication of *Plasmodium falciparum* infection (1). *Plasmodium berghei* ANKA (PbA) infection is an experimental model of CM (ECM) in mice that exhibits several of the neurological manifestations observed in human CM. Studies in both humans and animals have demonstrated that dysregulated inflammatory responses to infection and their effects on vascular endothelium

play a central role in disease progression and outcome (2).

Sphingosine 1-phosphate (S1P) is a signaling sphingolipid that regulates several cellular processes implicated in CM pathology, including inflammation and vascular endothelial homeostasis (3). S1P is produced by the phosphorylation of sphingosine by sphingosine kinases (4) and inactivated by S1P phosphatases or S1P lyase (S1PL) (5). S1P can act intracellularly (6) or function in an autocrine or

paracrine manner as an extracellular ligand for cell surface S1P receptors following release via ABC transporters (5). S1P is one of the most abundant biologically active lysophospholipids (4) and exerts its cellular effects through five G-protein-coupled receptors (S1P receptor 1 [S1P1]–5), differentially distributed and expressed on various cell types (7,8).

The main sources of S1P in blood are considered to be endothelium, platelets and red blood cells (RBCs), which store and protect S1P from degradation in plasma (9,10). A concentration gradient of S1P exists between plasma (11,12) and interstitial fluids (13) which is thought to be a major regulator of lymphocyte egress and trafficking (14). S1P also promotes T-cell egress from lymphoid organs through binding of S1P to lympho-

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cyte S1P receptors. However, at high concentrations, S1P downregulates S1P receptors and promotes closing of endothelial tight junctions, resulting in lymphocyte retention (15).

A number of agents have been used to modulate S1P receptor signaling directly (16). LX2931 specifically inhibits S1PL function, thereby reducing S1P degradation and increasing S1P bioavailability. At high concentrations, FTY720 (fingolimod) also affects S1PL (17), but its main target is S1P receptor 1 (S1P1) (18). FTY720 administration has proved beneficial in experimental disease models (19–23), and recently in human clinical trials of relapsing multiple sclerosis (24). Both S1P and FTY720 result in S1P1 internalization and recycling (25,26), FTY720 being a more efficient inducer of receptor degradation (27). This functional antagonism of S1P receptors leads to lymphocyte retention in lymphoid tissues, thus reducing the number of activated lymphocytes available to enter sites of inflammation, including the central nervous system (CNS) (28).

Based on the above observations suggesting a role for S1P in the regulation of biological processes implicated in the pathogenesis of CM, we hypothesized that S1P signaling is affected during malaria infection in a manner that impacts host immune responses deleteriously. Studying both clinical human malaria and animal models, we implicate altered S1P signaling in CM pathogenesis. Through genetic and pharmacologic strategies to modulate S1P-to-S1P receptor interactions, we show improved disease outcome associated with preserved endothelium integrity, reduced host inflammation and T-cell influx into the brain. Improved survival also was observed when FTY720 was administered following infection in combination with a subcurative dose of artesunate. These data suggest that treatment approaches to enhance S1P-mediated activity, such as administration of FTY720, may have potential clinical utility as adjunctive therapeutic strategies for individuals with malaria.

MATERIALS AND METHODS

Study Participants

Plasma samples were collected from children aged 0.6 to 10.6 years living in an area of holoendemic malaria transmission in Kampala, Uganda. Samples were derived from an observational case-control clinical study at Mulago Hospital in Kampala. Children were diagnosed with uncomplicated or cerebral malaria according to the World Health Organization (1). The study was approved by Mulago Hospital Research Ethics Committee, Makerere University Faculty of Medicine Research Ethics, Uganda National Council on Science and Technology and Toronto Academic Health Sciences Network Research. Written informed consent was obtained from all participants and/or their guardians.

Mice, Parasites and Drug Regimens

The mice with diminished S1PL activity (*hS1PL*^{-/-} mice, provided by Lexicon Pharmaceuticals, The Woodlands, TX, USA), were on a mixed genetic background (129S5/SvEvBrd and C57BL/6J) (29). Wild-type littermates were used as controls in these experiments since the response to *P. berghei* infection is mouse-strain specific. FTY720 and LX2931 experiments were performed using 6- to 8-wk-old female C57BL/6 WT animals (Charles River, Sherbrooke, QC, Canada). All animals were housed at the University of Toronto according to animal use guidelines and euthanized using an overdose of anesthetic (isoflurane). At least five animals per group were used for each experiment.

Animals were treated daily with either FTY720 (0.3 mg/kg, Selleck) or LX2931 (100 mg/kg, Lexicon Pharmaceuticals Inc.) 1 d prior to and 1, 3 or 5 d after intraperitoneal (i.p.) infection with 1×10^6 PbA parasites. The compounds were reconstituted in sterile water and administered by gavage. For artesunate (Sigma-Aldrich, St. Louis, MO, USA) treatment, mice received a single i.p. injection of 10 mg/kg diluted in RPMI medium

(Gibco, Burlington, ON, Canada) 5 d after malaria infection.

Plasma Protein and Lipid Measurements

Human S1P levels were measured in plasma samples by EIA following the manufacturers' guidelines (Echelon, distributed by Cedarelane, Burlington, ON, Canada).

For measuring murine circulating plasma cytokine levels, blood was collected from mice on days 5 and 6 after infection (p.i.) from the saphenous vein in heparinized tubes (Starstedt, Montreal, QC, Canada) or by cardiac puncture into heparin-coated syringes. Blood was centrifuged (Sorvall, distributed by ThermoScientific, Asheville, NC, USA; 16,060g for 10 min), and plasma was collected and stored at -80°C. Plasma cytokine levels for IFN γ and TNF were measured using an inflammatory cytokine CBA kit (BD Bioscience, Mississauga, ON, Canada). Levels of Ang1 and sICAM as well as TNF and IFN γ in uninfected, but treated, control mice, were measured by EIA following the manufacturer's instructions (R&D, distributed by Cedarelane). EIAs are much more sensitive than CBAs, hence the apparent high levels of TNF in uninfected mice in Supplementary Figure 2.

Brain Lymphocyte Isolation

Brains were perfused with PBS, isolated and incubated with DNase (Worthington, distributed by Cedarelane) and collagenase (Worthington) for 40 min at room temperature. Cells obtained were washed (2% FBS/PBS) and lymphocytes isolated using a 33% Percoll gradient. Lymphocytes were washed (2% FBS/PBS), treated with RBC lysis buffer (Sigma) and counted. All cells were stained for CD4 (RM4.5, Ebioscience, San Diego CA, USA), CD8 (53-6.7, Biolegend, distributed by Cedarelane), and CD3 (17A2, Ebioscience) for 20 min, washed, fixed in 1% formalin and run on an LSR II flow cytometer. Data were analyzed using FlowJo software.

Evans Blue Staining

A solution of 1% Evans blue dye was made using Evans Blue powder (Sigma-Aldrich) and PBS. The solution was filter sterilized and, on day 7 p.i., 100 μ L was injected intravenously (i.v.) (or i.p., as in Supplementary Figure 3, which explains the lighter staining) into mice and left to circulate for 1 h. Mice were euthanized and brains perfused with PBS. Brains were removed and photographed. Representative photographs were chosen based on animals with the most similar parasitemia.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism. All data which involved more than one experiment were normalized to the geometric mean of the infected but untreated group of each experiment. For analyses involving two groups, Mann-Whitney tests (MW) were performed on nonparametric data. For analyses involving three or more groups, analysis of variance (ANOVA) followed by the Bonferroni post hoc test (BpT) were performed on parametric data. Kruskal-Wallis tests (KW) followed by the Dunn multiple comparison (DMC) were performed for nonparametric data. For survival statistics, the log-rank test (LRT) and the Fisher exact test (FET) were used. For the logistic regression model involving S1P levels, hemoglobin levels and platelet counts, the SPSS program was used.

All supplementary materials are available online at www.molmed.org.

RESULTS

Plasma S1P Is Decreased in Children with Cerebral Malaria

To examine the biological relevance of S1P in human severe malaria, we first investigated plasma S1P levels in African children with CM compared with those with uncomplicated malaria (UM) (Figure 1A). Median plasma S1P levels were decreased significantly in Ugandan children with CM compared with chil-

dren with UM ($P < 0.0001$). As expected, we observed differences in hemoglobin and platelet levels between CM and UM groups (Figure 1B, C). However, after correcting for anemia and thrombocytopenia and in accordance with recent data (30), the difference in S1P levels observed remained a significant and independent predictor (logistic regression model, $P = 0.02$) of cerebral malaria relative to uncomplicated controls. The logistic regression model used fit the data well (Hosmer-Lemeshow, $P = 0.38$) and discriminated between cerebral and uncomplicated malaria (c-index = 0.963, 95% CI = 0.92–1.0).

Survival Is Improved in Mice with Decreased S1PL Activity in PbA-Induced ECM

Having established that S1P levels were decreased in children with CM, we investigated a potential causal role for S1P in CM pathogenesis by examining the outcome of infection in mice with decreased S1PL activity, resulting in increased tissue and plasma S1P levels (29). We infected

humanized S1PL deficient mice ($hS1PL^{-/-}$) with PbA (Figure 2A, left panel). Survival was improved significantly in $hS1PL^{-/-}$ mice compared with wild-type littermates (95% versus 35% survival; $P < 0.0001$) with no effect on parasite burden (Figure 2A, right panel). Thus, in ECM, as with our clinical data, increased S1P levels were associated with improved outcome.

The 35% survival rate observed in wild-type littermates differs from the 0% survival rate observed with C57BL/6 mice used in all other experiments. Animals used in this experiment were of mixed background (129S5/SvEvBrd and C57BL/6J), and since *P. berghei* infection is mouse-strain specific, we used the appropriate control of wild-type littermates in these experiments.

Treatment with Either FTY720 or LX2931 Improves Survival in ECM

We assessed the effect of modulating S1P signaling in ECM by treating mice with the S1P receptor modulator FTY720 (0.3 mg/kg/day by gavage), starting at

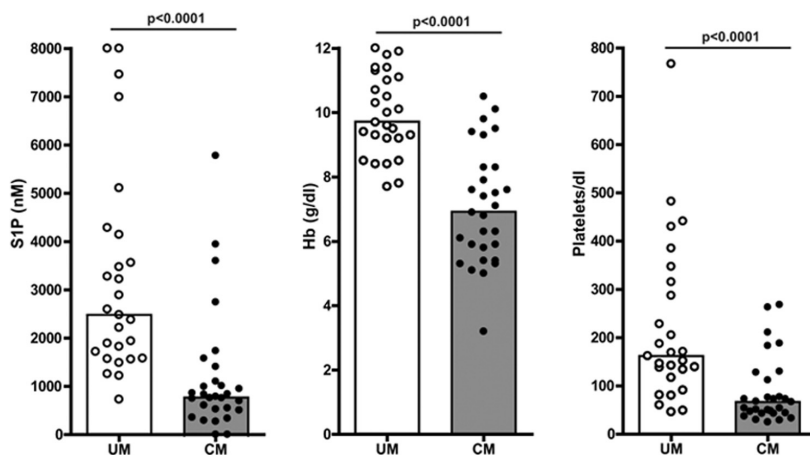


Figure 1. Plasma S1P is decreased in children with cerebral malaria. (A) S1P is decreased in children with cerebral malaria. Plasma S1P levels were assayed in Ugandan children with malaria. S1P levels were significantly lower in children with cerebral malaria (CM, $n = 29$) versus uncomplicated malaria (UM, $n = 27$), $P < 0.0001$, MW. (B) Hemoglobin levels are decreased in children with cerebral malaria. Hemoglobin levels were significantly lower in children with cerebral malaria (CM, $n = 29$) versus uncomplicated malaria (UM, $n = 27$), $P < 0.0001$, MW. (C) Platelet counts are decreased in children with cerebral malaria. Counts were measured in Ugandan children with malaria, and were shown to be significantly lower in children with cerebral malaria (CM, $n = 29$) versus uncomplicated malaria (UM, $n = 27$), $P < 0.0001$, MW.

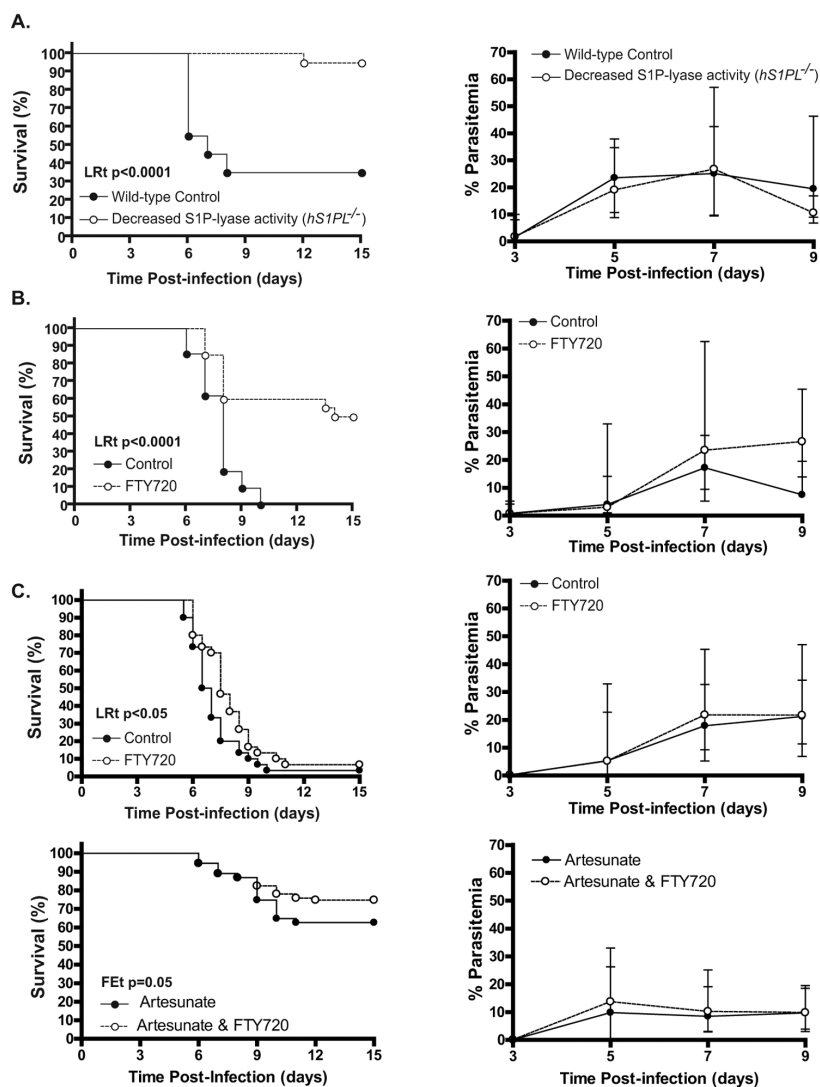


Figure 2. Modulating S1P levels improves survival in mice with *P. berghei* ANKA-induced ECM. (A) Survival (left panel) is improved, while parasite burden remains unchanged (right panel), in mice with decreased S1PL activity in *P. berghei* ANKA-induced ECM. Survival of *hS1PL*^{-/-} mice infected with 1×10^6 parasites i.p. was compared to their wild-type littermates. Decreased S1PL activity (resulting in increased bioavailable S1P) provided a significant survival advantage compared with control animals (two pooled independent experiments (IE), $n = 10$ /group/IE, LRt, $P < 0.0001$). (B,C,D) Mice were infected with 1×10^6 parasites i.p., and treated daily by gavage with FTY720 (0.3 mg/kg). Treatment with FTY720 provided a significant survival advantage if administered (B) 1 d prior to (two pooled IE, $n \geq 10$ /group/IE, LRt, $P < 0.0001$), (C) 1 d p.i. (three pooled IE, $n \geq 10$ /group/IE, LRt, $P < 0.05$) or (D) 5 d p.i. in combination with a single dose of artesunate administered on d 5 of infection (five pooled IE, $n \geq 10$ /group/IE, FEt, $P = 0.05$). Parasite burden remain unchanged between groups for all these experiments (right panels).

various points during the course of infection (Figure 2B, C, left panels). Both prophylactic (initiation of FTY720 1 d prior to infection) and therapeutic (initiation of FTY720 1 d p.i.) treatment with FTY720

significantly increased survival ($P < 0.0001$ and $P < 0.05$ respectively, Figure 2B, C). Therapeutic interventions with FTY720 initiated later during the course of infection (3–5 d) did not reach statisti-

cal significance (data not shown). Treatment with LX2931 (100 mg/kg/day by gavage) had a beneficial but less marked effect on outcome than FTY720 (Supplementary Figure 1). Prophylactic treatment with LX2931 1 d prior to infection did not lead to increased survival but did provide a significant delay in the onset of symptoms ($P < 0.0001$; Supplementary Figure 1A). However, therapeutic treatment during infection showed no significant benefit (Supplementary Figure 1C).

Parasite burdens were not significantly different between untreated and drug-treated animals over the course of infection (Figure 2, right panels; Supplementary Figure 1B, D). The effects of both compounds on survival therefore were attributed to host response modulation rather than anti-parasitic effects (although an impact of drug treatment on parasite viability and/or infectiousness was not assessed directly and could have contributed to the differences observed). We next examined the effect of FTY720 on the blood-brain barrier (BBB), which is disrupted severely in cerebral malaria (31).

Treatment of ECM with FTY720 Decreases Endothelium Activation and Enhances Blood-Brain Barrier Integrity

S1P has been shown previously to limit the effects of inflammatory mediators on endothelium (32). Secretion of S1P by endothelial cells may be critical for the maintenance of the plasma S1P gradient and vascular barrier homeostasis.

Plasma sICAM-1, a marker of endothelial activation (33), was decreased in FTY720-treated mice compared with untreated mice (Figure 3A). Elevated levels in infected but untreated mice were decreased significantly in all animals treated with FTY720 ($P < 0.0001$). Conversely, plasma Ang1, a marker of endothelium quiescence and stability, was increased in mice treated with FTY720 prophylactically compared with untreated animals ($P = 0.02$, Figure 3B). Mice treated with FTY720 but not infected with malaria showed no change in

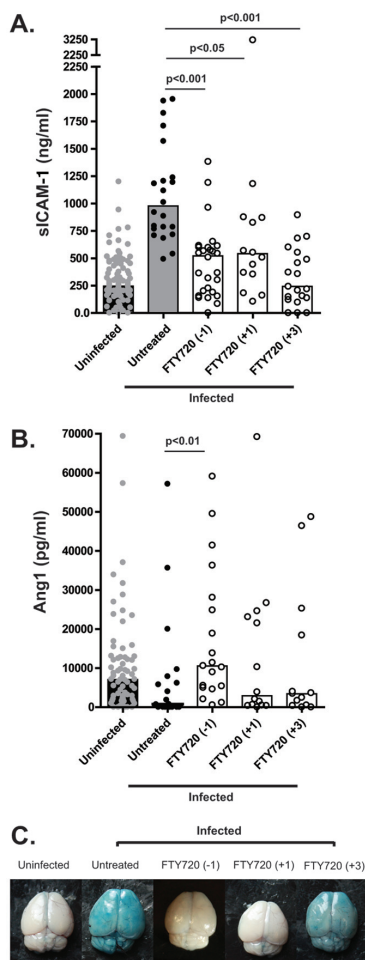


Figure 3. Treatment of ECM with FTY720 decreases endothelium activation and improves blood-brain barrier integrity. (A) Plasma levels of sICAM-1, a marker of endothelial activation, are decreased in FTY720-treated animals ($P < 0.001$, KW); for (FTY720 (-1), $P < 0.001$, FTY720 (+1) $P < 0.05$ and FTY720 (+3), $P < 0.001$; DMC, three pooled IE, $n \geq 3$ /group/IE). (B) Plasma levels of Ang1, a marker of endothelial integrity, are increased in animals treated with FTY720 prophylactically ($P < 0.01$, KW and DMC, two pooled IE, $n \geq 3$ /group/IE) but not in animals treated during infection. (C) Brains stained with Evans blue were harvested on day 7 p.i. Brains displayed are from animals with the following parasitemia: Wild type: 12.7%; FTY720 (-1): 20.6%; FTY720 (+1): 17.8%; FTY720 (+3): 11.9%. Brains from FTY720-treated animals show improved endothelium integrity (less blue dye leakage from the blood into brain parenchyma) as compared with the wild-type animal (two pooled IE, $n \geq 3$ /group/IE).

either their sICAM-1 or Ang1 levels compared with uninfected, untreated controls (Supplementary Figure 2A, B).

Studies observing Evans blue staining of the brain demonstrated that FTY720 treatment improved BBB integrity in ECM. These experiments (Figure 3C; Supplementary Figure 3) demonstrate the decrease in endothelium vascular leak apparent in 1-day-preinfection and 1-day-postinfection drug-treated animals (blue-grey, similar to uninfected control animals) compared with the untreated infected mice and animals treated on day 3 p.i. (dark blue staining). Collectively, these data indicate that FTY720 treatment preserves BBB and endothelium integrity.

FTY720 Treatment of ECM Decreases Plasma $IFN\gamma$

Both $IFN\gamma$ and TNF have been implicated in the pathogenesis of CM (34,35), and S1P has also been shown to influence T-cell $IFN\gamma$ production (28). FTY720 treatment did not affect TNF plasma levels (measured on day 5 p.i., $P > 0.05$, Figure 4A). However, $IFN\gamma$ plasma levels, measured on day 6 p.i. (Figure 4B), were decreased significantly with pretreatment and treatment 3 d p.i. ($P < 0.01$ and $P < 0.05$ respectively). This decrease was not observed in mice treated with FTY720 but not infected with malaria compared to uninfected, untreated controls (Supplementary Figure 2D). Pretreatment with LX2931 significantly decreased $IFN\gamma$ but not TNF levels measured at 5 d p.i. (Supplementary Figure 1).

FTY720 Treatment of ECM Decreases Central Nervous System Lymphocyte Numbers and Granzyme A mRNA during PbA Malaria Infection

FTY720 administration causes transient sequestration of circulating lymphocytes in lymph nodes and a sustained decrease of lymphocytes in the blood and spleen (13,14,36–38). Consistent with other studies (38), we observed a marked reduction of lymphocytes in the blood (data not shown). Importantly in the context of ECM, FTY720 can cross the BBB and be phosphorylated within

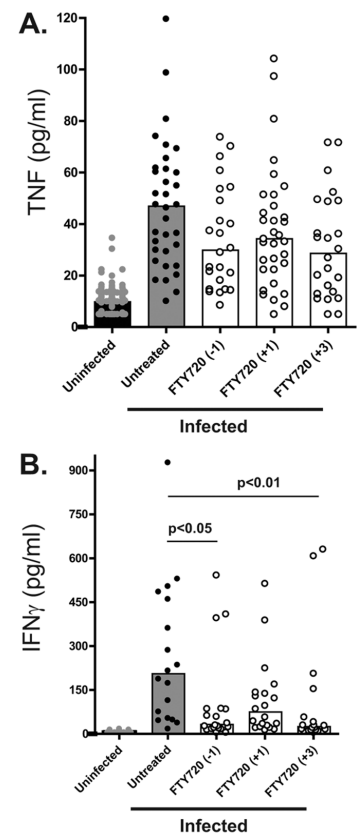


Figure 4. FTY720 treatment of ECM decreases plasma $IFN\gamma$ and TNF. (A) TNF plasma levels trended toward a decrease in the plasma of all animals treated with FTY720 ($P > 0.05$, ANOVA, three pooled IE, $n \geq 5$ /group/IE). (B) $IFN\gamma$ plasma levels were decreased in the plasma of animals treated with FTY720 1 d before infection and 3 d p.i. ($P < 0.01$ and $P < 0.05$ respectively, KW and DMC, three pooled IE, $n \geq 5$ /group/IE).

the CNS (24). Since S1P receptors are expressed on all cell types found within the CNS (25), FTY720 can restrict immune cell entry into the CNS (39) and could contribute to reduced CM pathology by limiting lymphocyte infiltration into the brain (40), a process implicated in ECM pathogenesis (41). We show that lymphocyte infiltration (both $CD4^+$ and $CD8^+$ cells) in the brain is decreased in mice treated with FTY720 1 d before infection and 1 d p.i. (Figure 5A, B, $CD4^+$: $P < 0.05$ and $P < 0.001$, $CD8^+$: $P < 0.05$ and $P < 0.01$ respectively), as is expression of

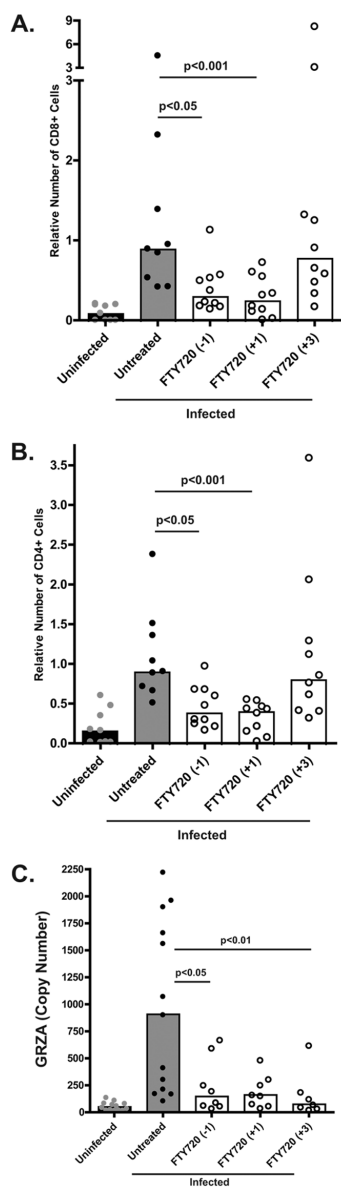


Figure 5. FTY720 treatment of ECM decreases lymphocyte numbers and granzyme A mRNA in the brain. (A) Relative CD8⁺ and (B) CD4⁺ T-cell numbers isolated from the brains of mice infected for 6 d. Numbers were decreased in animals treated with FTY720 1 d before infection and 1 d p.i. (CD4⁺: $P < 0.05$ and $P < 0.001$, CD8⁺: $P < 0.5$ and $P < 0.01$ respectively, KW and DMC, two pooled IE, $n \geq 4$ /group/IE) (C) GZMA mRNA levels were decreased in the brains of FTY720-treated animals compared with control animals: for (FTY720 (-1), $P < 0.05$, FTY720 (+1) $P > 0.05$ and FTY720 (+3), $P < 0.01$; KW and DMC, two pooled IE, $n \geq 3$ /group/IE).

granzyme A (GZMA), an inflammatory mediator released by activated T cells (Figure 5C). Decreased GZMA levels were observed in all FTY720-treated mice compared with untreated mice, although the differences achieved statistical significance for only the FTY720 (-1) and FTY720 (+3) treatment groups ($P < 0.05$ and $P < 0.01$ respectively). Brains were perfused prior to measuring lymphocyte counts in order to ensure that only sequestered and not circulating lymphocytes were included in our results.

Treatment with FTY720 in Combination with an Anti-Malarial Improves Survival in ECM

We assessed the impact of therapeutic FTY720 treatment (0.3 mg/kg/day by gavage), starting at day 5 during the course of infection (Figure 2D, left panel) when administered in combination with a subcurative dose of artesunate (10 mg/kg) on day 5. The combination treatment resulted in a significant increase in survival over artesunate therapy alone ($P = 0.05$, FET, experiment repeated five times with similar trend).

DISCUSSION

Our data implicate the S1P pathway in the pathobiology of human and experimental CM, and highlight the effects of S1P on endothelial quiescence and infection-induced inflammation in this context. S1P levels were decreased significantly in Ugandan children with cerebral malaria (Figure 1), in contrast to increased proinflammatory markers associated with severe and complicated disease (1). This is of particular interest since it has been shown recently (42) that neurocognitive functions are impaired in sphingosine kinase 1 (*Sphk1*)^{-/-} animals (animals deficient in sphingosine kinase 1); *Sphk1*^{-/-} mice have reduced plasma S1P (43). Children with cerebral malaria have decreased plasma S1P levels (see Figure 1), and also can display impaired neurocognitive functions once they have recovered from infection (44). The dysregulation of the S1P pathway therefore may contribute to this process.

Genetic approaches using *hS1PL*^{-/-} mice, demonstrated that S1P lyase deficiency conferred almost complete protection to PbA-induced ECM (Figure 2A). Treatment with either of two compounds (for example, LX2931 and FTY720) known to alter S1P signaling result in increased survival from ECM (Figure 3B, C; Supplementary Figure 1A). Interestingly, while no animals survived infection after prophylactic LX2931 treatment, despite a significant delay in mortality, 50% of animals treated prophylactically with FTY720 survived ECM (FET, $P = 0.0002$ for FTY720 treatment versus $P = 1$ for LX2931 treatment). These differences may be attributable to the observation that, unlike FTY720, LX2931 does not cross the BBB.

In order to enhance S1P bioavailability, FTY720 or LX2931 administration was preferred to that of S1P itself since FTY720 has recently been FDA approved for use in multiple sclerosis and a phase 2A study has just been completed with promising results using LX2931 to treat rheumatoid arthritis. As such, these compounds, unlike synthetic S1P, have safety profiles in humans (24,45), which would make their application as malaria adjunctive therapy imminently testable, with possible accelerated translation to clinical populations.

During FTY720 and LX2931 treatment, oral rather than intravenous administration was preferred since it has been shown that lower blood lymphocyte counts can be achieved using this route (38). Dosing was based on the preestablished range shown to reduce lymphocyte numbers in the blood (15). We did perform experiments at a higher dose (3 mg/kg/day) in an attempt to increase survival in ECM, however, this higher dose did not confer additional survival benefit (data not shown).

The administration of FTY720 had a significant impact on endothelial integrity (Figure 3). Prophylactic treatment resulted in increased Ang1 levels, a biomarker of endothelial quiescence, which may explain the enhanced survival provided by prophylactic FTY720 treatment since in humans, decreased Ang1 levels

are associated with CM (46). Increased Ang1 and S1P levels have also been associated with the maintenance of vascular integrity (47,48).

S1P has been shown recently to induce nitric oxide (NO) (49) as well as vascular endothelial growth factor (VEGF) expression (50) in endothelial cells. Notably both reduced NO and reduced VEGF levels have been associated with severe and cerebral malaria (51) and may be related to the decreased S1P levels we observed in the cerebral malaria patients (see Figure 1). Since increased bioavailable NO has been associated with resistance to malaria (52), it also would be of interest to determine whether S1P levels correlate with clinical response and outcome in human infection.

Decreases in inflammatory markers have been associated with protection from ECM (41), which suggests that reduced levels of IFN γ observed with FTY720 and LX2931 treatment may have contributed to increased survival (Figure 4; Supplemental Figure 1).

In addition to improving endothelial integrity and inflammation, the administration of FTY720 significantly reduced the number of CD4⁺ and CD8⁺ lymphocytes migrating into the brain during PbA infection (Figure 5C, D). However, other types of lymphocytes which we did not assess also may have been affected by FTY720 treatment. Although Th17 cells have not been implicated yet in malaria, FTY720 has been shown to attenuate Th17 cell accumulation in a model of neuritis (53) and in multiple sclerosis patients (54). FTY720 also has been shown to affect regulatory T cells (Tregs) by increasing the expression of IL-10 and CTLA-4 *in vitro* (55), promoting Treg activity *in vivo* (56) and more recently by modulating the reciprocal differentiation of inducible Tregs and Th1 cells (57). The role of Tregs in malaria infection remains controversial (58), and therefore the impact of S1P on this cell type during infection requires further investigation.

Despite FTY720 mainly affecting T cells (15), an effect on B and NK cells

cannot be excluded (59). NK cells have been implicated as potent IFN γ producers during malaria infection (60) and the reduced inflammation observed in FTY720-treated animals (Figure 4B) may result partially from an effect of FTY720 on NK cells. Although it was thought that myeloid cells were not affected significantly by FTY720 treatment (61), FTY720 has been shown to skew dendritic cell and macrophage responses towards anti-inflammatory profiles (28). Finally, the effect of FTY720 on other cell types in the CNS such as microglia which have been implicated in the pathogenesis of cerebral malaria (62) or astrocytes which are integral to BBB maintenance (63), and strongly express S1P receptors (64), cannot be excluded.

A recent publication implicates the S1P pathway in life-threatening infectious disease processes (65). The authors report that in an LPS model of sepsis in mice, knockdown of Sphk1 through the use of small interfering RNAs leads to recovery, characterized by decreased cellular infiltration and inflammation (65). Sphk1 phosphorylates sphingosine, resulting in S1P (4) and knocking down its function might suggest reduced S1P plasma levels (43). However, the authors did not report on extracellular or plasma S1P levels and suggest that the blockade of Sphk1 does not affect the extracellular S1P gradient required to maintain vascular barrier function (65). Our findings whereby FTY720 treatment and decreased S1PL activity improves survival to experimental cerebral malaria are based on a dysregulation of the extracellular S1P gradient. The apparent contradiction between our results therefore could be explained by the regulation of intracellular versus extracellular or plasma S1P. Furthermore, differential tissue and cell receptor subtype expression also can contribute to different or opposing roles in maintaining vascular barrier function, and may contribute to our observed results in human and murine CM.

In summary, FTY720 treatment modulated a number of pathways central to the pathophysiology of ECM. FTY720 ad-

ministration improved vascular integrity in the brain (increased Ang1 levels, reduced sICAM-1 levels and vascular leak), as well as reduced inflammation (IFN γ levels) and reduced T-cell infiltration in the brain (CD4⁺ and CD8⁺ cells). Its administration in combination with a subcurative dose of artesunate during infection led to a biologically significant increase in ECM survival.

A recent report has questioned the utility of the experimental CM model, however this report has been challenged by the research community with most investigators agreeing there are a number of features shared between human (and especially pediatric CM) and murine CM (66,67). Moreover, our approach obviates this criticism by first establishing the biological relevance of the S1P pathway to human disease by studying informative patient populations before moving to animal models to examine mechanism and causality.

Using human clinical samples combined with genetic approaches in hS1PL^{-/-} mice, as well as compounds which modulate the S1P pathway, we provide strong evidence implicating the S1P pathway in malaria pathogenesis. Considerable malaria research has focused on host protein rather than lipid pathways, which we have shown can play an important role in infection. The S1P signaling cascade may represent a novel target for adjunctive therapeutics for severe and cerebral malaria. We have observed a small increase in survival in animals treated therapeutically with FTY720 in combination with a subcurative dose of anti-malarial (FET, $P = 0.05$) as compared to animals treated with the antimalarial alone (Figure 2D, left panel). Given that FTY720 (68) and LX2931 have an established safety profile in human trials (FTY720 has recently been approved by the FDA for use in the treatment of multiple sclerosis), this hypothesis could be directly tested.

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DISCLOSURE

T Oravec, CAM Finney, and KC Kain are inventors in pending patent applications owned by Lexicon Pharmaceuticals Inc. for the use of both LX2931 and FTY720 in the treatment of human cerebral malaria. Lexicon Pharmaceuticals Inc. provided support for experiments involving LX2931 and employs T Oravec. This work was supported by a CIHR Team Grant in Malaria (KC Kain), operating grant MT-13721 (KC Kain), Genome Canada through the Ontario Genomics Institute (KC Kain), and CIHR Canada Research Chairs (KC Kain, WC Liles). CAM Finney was supported by a post-doctoral fellowship from the Canadian Department of Foreign Affairs and Trade. C Cserti-Gazdewich was funded by the International Society of Blood Transfusion Foundation (Amsterdam, the Netherlands), and the National Blood Foundation (Bethesda, MD, USA).

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