Adipose tissue parasite sequestration drives leptin production in mice and correlates with human cerebral malaria

Pedro Mejia1*†, J. Humberto Treviño-Villarreal1*†‡, Mariana De Niiz2,3,4*, Elamaran Meibalanait, Alban Longchamp1,5,6, Justin S. Reynolds1, Lindsey B. Turnbull7, Robert O. Opoka8, Christian Roussilhon9, Tobias Spielmann10, C. Keith Ozakii, Volker T. Heussler3, Karl B. Seydel11, Terrie E. Taylor11, Chandy C. John7, Danny A. Milner4,12, Matthias Marti2,4, James R. Mitchell11||

Circulating levels of the adipokine leptin are linked to neuropathology in experimental cerebral malaria (ECM), but its source and regulation mechanism remain unknown. Here, we show that sequestration of infected red blood cells (iRBCs) in white adipose tissue (WAT) microvasculature increased local vascular permeability and leptin production. Mice infected with parasite strains that fail to sequester in WAT displayed reduced leptin production and protection from ECM. WAT sequestration and leptin induction were lost in CD36KO mice; however, ECM susceptibility revealed sexual dimorphism. Adipocyte leptin was regulated by the mechanistic target of rapamycin complex 1 (mTORC1) and blocked by rapamycin. In humans, although Plasmodium falciparum infection did not increase circulating leptin levels, iRBC sequestration, tissue leptin production, and mTORC1 activity were positively correlated with CM in pediatric postmortem WAT. These data identify WAT sequestration as a trigger for leptin production with potential implications for pathogenesis of malaria infection, prognosis, and treatment.

INTRODUCTION

Cerebral malaria (CM) is a life-threatening complication of infection with Plasmodium falciparum parasites manifesting with intracerebral hemorrhages, brain edema, coma, and death (1–3). Children under the age of 5 with little or no previous exposure to P. falciparum infection are most vulnerable to CM (4), but it is currently impossible to predict which infected patients will progress to CM (5). Once the deadly syndrome has developed, no efficient treatment exists to reduce mortality and long-term neurologic disabilities even after successful elimination of circulating blood-stage parasites with antimalarial chemotherapy. While exact molecular mechanisms remain unclear, the disease appears to be the result of a complex interplay between infected red blood cells (iRBCs) and local and systemic host immune responses (6–8). On a histological level, neurovascular perturbations include increased blood-brain barrier (BBB) permeability, leukocyte infiltration, platelet activation (9), and dysregulation of the coagulation system in brain endothelium (2, 10).

Sequestration of iRBCs via cytoadherence to the microvasculature is a hallmark of severe malaria across multiple Plasmodium species (11). iRBC sequestration, which represents a strategy by the parasite to evade host immune clearance by the spleen, can lead to pathology via endothelial cell activation and/or mechanical obstruction of blood flow. In humans, sequestration in the brain microvasculature is a defining feature of CM neuropathogenesis (12, 13); but sequestration also occurs widely in organs and tissues outside of the brain. Histopathological analyses of iRBC sequestration in postmortem samples from P. falciparum–infected children who died from CM or other causes (2, 14–16) show that iRBCs sequester across most of the organs analyzed, including the gastrointestinal tract and the subcutaneous white adipose tissue (ScWAT) (2, 15, 16). iRBCs express adhesion molecules including the variable P. falciparum erythrocyte membrane protein 1 (PfEMP1) (17, 18) that cytoadhere to host endothelial cell receptors such as intercellular adhesion molecule–1 (ICAM-1), endothelial protein C receptor (EPCR), and the scavenger receptor/fatty acid transporter CD36 (19–21). In brain endothelium, ICAM-1 is activated by local or systemic proinflammatory cytokines (22), while CD36 is expressed in both endothelial and parenchymal cells in other tissues with high levels of fatty acid metabolism including in WAT and lung, but not in brain (23).

In rodent models of experimental CM (ECM) using Plasmodium berghei ANKA (PbA) in susceptible hosts including C57BL/6 mice, sequestration of iRBCs also occurs in multiple tissues including lung and adipose, both of which are largely dependent on CD36, although the corresponding ligand on PbA-infected RBCs remains to be defined (24, 25). Parasites also accumulate in brain, but whether this is due to CD36-independent cytoadherence and sequestration in the brain microvasculature (26) or the result of BBB dysfunction...
remains a matter of debate. Despite conservation of sequestration in peripheral tissues such as adipose and lung, relevance to ECM neuropathology remains questionable in large part because CD36KO mice, which fail to support CD36-dependent sequestration in major sequestering tissues, still succumb to ECM (11, 24, 25).

In addition to energy storage, adipose tissue and adipocytes are metabolically active and capable of secreting a myriad of bioactive molecules including cytokines and hormones, collectively known as adipokines, which link metabolism, inflammation, and immunity (27, 28). One of these adipokines, leptin, has central effects promoting food intake and energy expenditure, as well as peripheral effects promoting immune and endothelial cell activation (29–31). Leptin is increased upon PbA infection and required for ECM neuropathology via induction of T cell effector functions and migration capacity (32, 33). Enforced restriction of food intake in the first 3 days of blood-stage infection can ameliorate ECM symptoms without affecting peripheral parasitemia, in part, by blunting the increase in circulating leptin (33). However, neither the trigger for leptin secretion in the ECM model nor the potential role of leptin in CM pathology remains questionable in large part because CD36KO mice fail to induce ECM (34, 35), although sequestration profiles have not been reported. We observed reduced peripheral parasitemias with PbAΔSMAC, and PbK173 (Fig. 2, A and B), as well as reduced iRBC sequestration on day 6 after infection in PgWAT in ScWAT (Fig. 2C). This correlated with significantly reduced iRBC accumulation in brain (Fig. 2D), lower levels of serum leptin (Fig. 2E), and improved survival (Fig. 2F) relative to C57BL6 mice infected with WT PbA. By day 6 after infection, lung sequestration was significantly elevated in one parasite line (PbK173) and reduced in another (ΔSMAC) and thus failed to correlate with leptin levels or survival.

We next tested a model of reduced sequestration due to genetic changes in the host. As previously reported (11, 24), female CD36KO mice on a C57BL/6 genetic background infected with PbA showed a significant reduction in peripheral parasitemia (Fig. 2, A and B) and reduced iRBC sequestration in PgWAT, ScWAT, and lung relative to WT littermates (Fig. 2C) but still exhibited signs of ECM neuropathology including parasite accumulation in brain on day 6 of infection that was not significantly different than control females (Fig. 2D). This was likely due to breakdown of BBB function as evidenced by increased Evan’s blue staining in brain (Fig. 2G). Mortality occurred on a similar time frame (days 6 to 10 after infection) and with slightly less penetrance than control females, although the difference was not statistically significantly (log-rank test, P = 0.08; Fig. 2F).

RESULTS
Sequestration of iRBCs in WAT promotes tissue vascular permeability and leptin production
We evaluated parasite sequestration in different tissues of C57BL/6 mice over a time course after infection with luciferase-expressing PbA (BL6-PbA) parasites (33). Using in vivo imaging, sequestration was detectable by day 1 and increased in intensity until day 6, coinciding with the presence of severe neurological symptoms and onset of mortality (Fig. 1A). Assessment on day 6 using a more quantitative approach in organ extracts following intracardiac perfusion revealed that parasite accumulation was highest on a per milligram tissue basis in lung, spleen, and WAT, including perigonadal (Pg) and subcutaneous depots (Fig. 1B). Together, these tissues accounted for >90% of sequestered parasite burden (Fig. 1B). Consistent with in vivo imaging, substantial Pg and ScWAT parasite sequestration were detectable by day 3 and increased at least up to day 6 after infection (Fig. 1C). Histological examination of ScWAT of C57BL/6 or ubiquitin C (UBC)–green fluorescent protein (GFP) C57BL/6 reporter mice on day 6 of infection revealed intravascular localization of iRBCs rather than direct contact with adipocytes (Fig. 1D). An absence of leukocyte infiltration was also noted in hematoxylin and eosin (H&E)–stained sections of this tissue (Fig. 1D). Intravenous injection of iRBCs harboring mature parasite stages resulted in WAT accumulation detectable 3 hours after injection into naïve mice (Fig. 1E), suggesting that parasite sequestration in this tissue is independent of any preexisting local or systemic inflammation.

Circulating leptin increases upon infection and is required for full penetrance of neuropathology and mortality in the ECM model (33). However, multiple cells secrete this adipokine, and thus, the cellular source in the context of ECM is unknown. Because WAT is a major source of leptin production, we looked at local leptin expression by immunofluorescence in WAT sections obtained from perfused mice before infection (day 0) and on days 4, 5, and 6 after infection. We observed a progressive increase in local leptin expression in Pg and ScWAT during the course of infection of ECM mice (Fig. 1, F and G), with kinetics similar to that of serum leptin (33).

To determine how iRBC sequestration in the WAT vasculature could promote leptin production/secretion by adipocytes in the absence of direct interaction between iRBCs and adipocytes, we examined vascular permeability over a time course after infection via intravenous dextran administration (Fig. 1H). Leakage of dextran (70 kDa), indicative of increased vascular permeability in WAT, was detected by day 5 of infection. This correlation between WAT parasite accumulation and tissue leptin expression suggests WAT as a major contributor of circulating leptin and local parasite sequestration as the trigger for leptin production.

Sequestration of iRBCs in WAT is required for leptin production
To assess the potential role of WAT parasite sequestration in leptin production and correlation with neuropathology, we tested three different luciferase-expressing parasites with altered sequestration and/or virulence relative to WT PbA parasites. Parasites lacking genes encoding the schizont membrane–associated cytoadherence (ΔSMAC) protein or skeleton-binding protein 1 (ΔSBP1) exhibit reduced peripheral parasitemia levels and CD36-dependent lung and WAT sequestration and fail to induce ECM neuropathology (34, 35). P. berghei K173 (PbK173) iRBCs also accumulate more slowly and fail to induce ECM (36), although sequestration profiles have not been reported. We observed reduced peripheral parasitemias with PbAΔSBP1, PbAΔSMAC, and PbK173 (Fig. 2, A and B), as well as reduced iRBC sequestration on day 6 after infection in PgWAT in ScWAT (Fig. 2C). This correlated with significantly reduced iRBC accumulation in brain (Fig. 2D), lower levels of serum leptin (Fig. 2E), and improved survival (Fig. 2F) relative to C57BL6 mice infected with WT PbA. By day 6 after infection, lung sequestration was significantly elevated in one parasite line (PbK173) and reduced in another (ΔSMAC) and thus failed to correlate with leptin levels or survival.
The response to *P. bivittatus* infection in male CD36KO mice was very similar to female CD36KO mice in terms of reduced peripheral parasitemia (Fig. 2, A and B) and reduced sequestration in Pg and ScWAT homogenates harvested after perfusion on the indicated day after infection (*n* = 3 per time point; Kruskal-Wallis test with Dunn’s multiple comparisons test on the indicated day versus day 0). (D) Sequestration of iRBCs in WAT microvasculature of *P. bivittatus*-infected mice on day 6 after infection by fluorescence microscopy with mCherry-expressing *P. bivittatus* (red to visualize intact parasites) in UBC promoter–driven GFP C57BL/6 mice (ubiquitous green expression to visualize host cells) in PgWAT (left) or H&E in ScWAT in WT C57BL/6 mice (right). (E) RBCs harboring mature parasites accumulate rapidly in WAT. Sequestration was measured ex vivo in PgWAT homogenates following perfusion 3 hours after intravenous injection of saline vehicle (saline) or 5 × 10⁶ iRBCs; *n* = 4 mice per group; Mann-Whitney test. (F and G) Representative images of leptin expression and quantitation of fluorescence intensity in ScWAT (F) and PgWAT (G) of *P. bivittatus*-infected C57BL/6 on days 0, 4, 5, and 6 after infection; *n* = 10 microscopic fields per mouse tissue with two mice per group; Kruskal-Wallis test with Dunn’s multiple comparisons test against day 0. (H) Representative images of FITC-labeled dextran (70 kDa) in WAT 2 hours after intravenous injection into mice on the indicated day after infection; quantitation of dextran Fire LUTs (lookup tables) outside of vessel relative to day 0 at right; Kruskal-Wallis test with Dunn’s multiple comparisons test against day 0. All data are expressed as mean ± SD; *P* < 0.05, **P** < 0.01, ***P*** < 0.001, and ****P*** < 0.0001. MFI, mean fluorescence intensity.
Sequestered iRBCs stimulate adipocyte leptin production via cell-autonomous mTORC1 activation

Next, we investigated molecular mechanisms controlling leptin production upon parasite sequestration in WAT. The role of the nutrient/energy sensor mTORC1 in posttranscriptional regulation of leptin production in cultured adipocytes has been suggested based on inhibition with the macrolide antibiotic rapamycin (37). We thus first measured phosphorylation status of the indirect mTORC1 target S6 in PgWAT by immunofluorescence across multiple time points following infection of C56BL/6 mice with PbA. Increased S6 phosphorylation was observed on days 4 to 6 (Fig. 3A). Western blotting revealed a similar increase in phosphorylation of the direct mTORC1 target, S6 kinase (S6K), in WAT from female and male BL6-PbA mice on day 6 after infection relative to naïve mice (Fig. 3, B and C). This increase in phospho-S6K was mitigated in female WT mice treated with rapamycin or infected with PbA-ΔSMAC (Fig. 3B), as well as in male CD36KO infected with PbA (Fig. 3C), and thus correlated with reduced iRBC WAT sequestration (Fig. 2B).

Treatment of PbA-infected female C57BL6 mice with rapamycin, which offered complete protection from ECM-associated mortality without affecting peripheral parasitemia, also reduced circulating leptin levels (Fig. 3D). To investigate cell-autonomous effects of mTORC1 on adipocyte leptin production, we knocked down the mTORC1 inhibitor TSC2 in primary adipocytes using adenoviral short hairpin RNA (shRNA). Leptin gene expression and protein secretion were both significantly increased in shTSC2-infected adipocytes compared to control cells infected with scrambled shRNA (Fig. 3E). Expression of adiponectin, an adipokine produced exclusively by adipocytes, was not affected by shTSC2 infection. Primary fibroblasts, which do not express adipokines at high

Fig. 2. Parasite sequestration in WAT is required for systemic leptin production and mortality. (A) Percent peripheral parasitemia over time grouped by experiment with corresponding controls as indicated, including SBP1-deficient versus parental WT PbA into WT female BL6 (n = 10 per group), SMAC-deficient versus parental WT PbA into WT female BL6 (n = 5 per group), PbK173 versus WT PbA into WT female BL6 (n = 5 to 6 per group), and WT PbA into male or female WT (n = 13 to 16 per group) versus male (M) or female (F) CD36KO (n = 16 to 17 per group) BL6 mice. (B) Peripheral parasitemia on day 6 (above) or day 5 (below) expressed as a percent of the corresponding sex-matched control. (C to E) Day 6 organ-specific sequestration (C), brain parasite accumulation (D), and serum leptin (E) expressed as a percentage of the control for that particular experiment as indicated in (A) and grouped by parasite (top row) or host (bottom row) mutant/strain. Statistics in (B) to (E) were performed using unpaired t tests with Welch’s correction or Mann-Whitney tests between mutants/strains and their corresponding host/strain control. (F) Kaplan-Meier survival curves of mice from the indicated experimental groups infected on day 0, including the number in each group and the log-rank statistic relative to sex-matched BL6-PbA controls. (G) Evan’s blue–stained brains of the indicated sex/genotype on day 6 after infection; white numbers indicate quantification of Evan’s blue after extraction in micrograms per gram of tissue. (H and I) Circulating leptin levels of the indicated sex/genotype presented as absolute values before (day 0, green triangles) and on day 6 after infection (red triangles), with dotted lines representing individual animals (H) or as differences between circulating leptin on day 6 and day 0 (I); one-way analysis of variance (ANOVA) with Sidak’s multiple comparisons tests as indicated. All data are expressed as mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. ns, not significant; d0, day 0; d6, day 6.
levels, were used as negative controls (Fig. 3E). Together, these data suggest that mTORC1 activation in adipocytes likely contributes to increased leptin expression upon PbA sequestration in WAT.

**Parasite sequestration, mTORC1 phosphorylation, and local leptin production in human subcutaneous adipose tissue correlate with CM mortality without increased circulating leptin**

To assess the relevance of our findings in the mouse ECM model to humans, we explored the relationship between WAT parasite sequestration, leptin production, and pathology in samples obtained from human pediatric CM cases versus controls. We first measured plasma leptin levels in CM cases from Cambodia and Senegal versus uninfected controls from France and Senegal. Contrary to our results in mouse models, no significant differences in the levels of circulating leptin were detected among the groups (Fig. 4A). These results were confirmed in independent cohorts of well-defined cases of CM from Uganda and Malawi versus uninfected, uncomplicated malaria and severe malaria anemia cases (Fig. 4B). We did not find any sexual dimorphism in the incidence of CM either in the literature or in this dataset.

We next assessed leptin production on a histological level in ScWAT from previously characterized fatal CM versus *P. falciparum*–infected non-CM cases from Malawi. As previously reported (2, 15), extensive
accumulation of *P. falciparum* iRBCs specifically in ScWAT was detected in CM cases but were virtually absent in non-CM cases (Fig. 4C). Leptin levels were then assessed by immunofluorescence staining in a subset of *P. falciparum*–infected non-CM and CM cases, using uninfected American WAT samples as controls. Notably, leptin expression in ScWAT, expressed as relative fluorescence intensity, was significantly increased in CM cases relative to non-CM and uninfected controls (Fig. 4D). Consistent with results obtained in mice, phosphorylation of the indirect mTORC1 target S6 was significantly elevated in ScWAT from CM cases compared to *P. falciparum*–infected non-CM and uninfected controls (Fig. 4E), with substantial colocalization of leptin and phospho-S6 in regions of parasite accumulation (Fig. 4F). Together, these results suggest evolutionary conservation of a link between *Plasmodium*-infected RBC sequestration in WAT, activation of mTORC1, and increased local leptin expression in both humans and mice.

**DISCUSSION**

Sequestration of *Plasmodium*-infected RBCs in WAT was first described decades ago in humans and nonhuman primates (38). Although *Plasmodium* species are thought to remain within the adipose microvasculature, at least two other parasites—*Trypanosoma cruzi*, the causative agent of Chagas disease, and *Trypanosoma brucei*, the causative agent of African sleeping sickness—localize within and between adipocytes, respectively, in their mammalian hosts (39). Several
potential selective advantages of parasite localization to adipose tissue have been proposed, including ease of transmission, relative shelter from the immune system, and/or the presence of abundant energy stores during fasting. Nonetheless, the biological significance of sequestration in adipose on the host response to parasite infection, if any, remains unknown.

The major finding here is that CD36-dependent sequestration of PbA-infected RBCs in WAT of C57BL/6 mice correlated with an increase in the circulating adipokine leptin. In each model tested, including three parasite variants that failed to sequester in adipose tissue, and in both sexes of CD36KO mice that fail to support adipose sequestration, leptin levels were reduced. The same was not true of the other major CD36-dependent sequestering tissue, lung. Circulating leptin levels failed to correlate with PbA levels in this tissue, although whether the observed luciferase activity represented iRBC cytoadherence or accumulation following vascular damage/occlusion was not addressed in this study. Also relevant is the consistent correlation found between WAT parasite sequestration and brain parasite accumulation/sequestration in both experimental and human CM, with the specific exception of CD36KO female mice (discussed in greater detail below). Combined with our previous finding that increased leptin during blood-stage PbA infection in mice is required for maximum penetration of ECM neuropathology (33), these data support a model in which sequestration of iRBCs specifically in WAT contributes to ECM neuropathology likely through effects on vascular and immune activation. While WAT is the largest endocrine organ, where adipocytes are a major source of leptin under homeostatic conditions, other cell types can also produce leptin, and thus, whether WAT is the sole source of leptin or other proinflammatory cytokines relevant to ECM pathology remains a limitation of the current study.

Mechanistically, our data point to a role for mTORC1 activation in increased leptin expression in WAT in vivo. Treatment with rapamycin, a U.S. Food and Drug Administration (FDA)–approved mTORC1 inhibitor, significantly reduced serum leptin levels and protected mice from ECM even after a single dose injected late in infection. These data are consistent with previous reports showing decreased circulating leptin levels upon rapamycin treatment in mice and humans (40). While our data in cultured adipocytes supports cell-autonomous regulation of leptin expression by mTORC1 signaling, the pleiotropic effects of rapamycin in vivo on T cell migration, vascular activation, and parasite sequestration make it difficult to unravel the specific mechanism underlying reduced leptin expression. Nonetheless, these findings build on previous reports (33, 41, 42), highlighting the therapeutic potential of rapamycin to protect against CM by targeting different cell types and inhibiting multiple mechanisms of disease.

A further limitation of the study is that we do not know the mechanism by which mTORC1 is activated in adipocytes upon cytoadherence of iRBCs to CD36-expressing endothelial cells. However, failure to find schizonts outside of the adipose tissue microvasculature combined with evidence of vascular permeability suggests that leakage of parasite material upon iRBC lysis is at least one mechanism allowing parasite-induced adipocyte mTORC1 activation and increased leptin expression. Last, our data point to conservation of this phenomenon in P. falciparum–induced pediatric CM, based on the increase in leptin expression and phosphorylation of markers of mTORC1 activity in adipocytes in postmortem adipose samples. A schematic model of the cellular and molecular mechanisms by which specific parasite sequestration in WAT contributes to neuropathology in CM is presented in Fig. 5.

The biological significance of CD36-mediated sequestration in neuropathology of both ECM and CM remains unclear. Human genetic studies of CD36 deficiency have yielded conflicting results, associating with higher susceptibility to severe and/or CM in some studies (43, 44) but protection in others (45–47). CD36 is expressed only at very low levels in brain vasculature, and most P. falciparum isolates obtained from infected individuals bind to CD36 regardless of the severity of the infection (48). CD36 is highly expressed in tissues involved in fatty acid metabolism but is not up-regulated by proinflammatory cytokines associated with fatal malaria (49). In rodents, the finding that iRBC sequestration is dissociable from ECM pathology in CD36KO mice led to the conclusion that CD36-mediated sequestration in peripheral organs is not the basis of ECM (24), a phenotype confirmed here. Also consistent with this conclusion are reports that some nonsequestering PfK173 strains can still cause ECM (11, 50, 51).

The unexpected finding here that ECM susceptibility in CD36KO mice was a sexual dimorphic trait, while failure to support sequestration was not, complicates the interpretation that CD36-dependent sequestration is not the basis for ECM (11, 24). The finding is unexpected for two reasons: first, because ECM in WT mice occurs in both sexes without significant sexual dimorphism as reported here and elsewhere, and second, because although the original studies reported only female CD36KO mice (24, 25), another study reported a similar phenotype in CD36KO males with the caveat that all animals were lethally irradiated followed by bone marrow reconstitution to study the role of CD36 in hematopoietic versus nonhematopoietic cells (52).

With respect to the potential biological importance of sequestration in ECM, while it is possible that ECM is unrelated to CD36-dependent adipose sequestration, alternately, there may exist sexually dimorphic traits in female CD36KO mice that bypass a requirement for adipose sequestration specifically in this mutant. Differences in leptin signaling may be one such trait. In one report, CD36KO mice had ~2.5-fold elevated circulating leptin relative to WT mice due to loss of fatty acid uptake that normally inhibits leptin secretion by adipocytes, with females 40% higher than males (53). Here, we failed to see a significant increase in leptin in CD36KO mice but instead observed sexual dimorphism in baseline levels (reduced in females) and in the response to PbA infection (reduced at day 6 versus day 0 in males). Changes in leptin sensitivity in the absence of CD36 may also play a role (53–56). A number of other possibilities also exist, as CD36 is a major fatty acid translocase important in endothelial cells and parenchymal cells particularly in adipose and muscle under conditions of low free fatty acids availability (57) and is associated with insulin resistance and inflammation, in part, via its role in uptake of oxidized low-density lipoprotein (LDL) by macrophages. Unfortunately, although a number of additional phenotypes of CD36KO mice have been reported, including resistance to weight gain on high-fat diet and a reduction in another adipokine, adiponectin, many studies only report the results of one sex, and thus, sexual dimorphisms are difficult to assess. At the molecular level, our study is hampered by the demonstration of reduced WAT mTORC1 activation only in male CD36KO mice. However, both the proposed trigger and functional result of mTORC1 activation, notably parasite sequestration and leptin production, respectively, are significantly reduced in CD36KO females and males relative to WT mice, strongly suggesting similar mTORC1 activation status upon
infection in both sexes. Future studies are thus required to determine the mechanism of differential susceptibility to ECM between male and female CD36KO mice, which may shed further light on the uncertain role of CD36-mediated sequestration of WAT in cerebral complications of malarial infection. Notably, although the effect of rapamycin is also known to be affected by sex in other contexts, in our multiple experiments, we have not identified any sexual dimorphism in response to rapamycin treatment in terms of parasitemia and survival in WT mice.

Our data fail to support a role for increased circulating leptin in neuropathology of pediatric CM and thus merit caution with regard to extrapolation from ECM to CM regarding the importance of WAT sequestration. Nonetheless, the conservation of iRBC sequestration, mTORC1 activation, and increased local leptin expression in WAT in both humans and mice observed here remain suggestive of potential disease relevance, either functionally for the parasite or the host response or as a marker that could be exploited for therapeutic purposes. Species-specific differences in effects of iRBC WAT sequestration on circulating leptin could be related to the observation that WAT sequestration in humans appears to be limited to specific depots (2), while in mice sequestration in visceral fat was abundant and thus likely to contribute significantly to circulating leptin levels. Because adipocytes are found in intimate contact with secondary lymphoid organs, vasculature, and neuro-adipose junctions (58), it is also possible that local leptin production could exert pathogenic effects in a paracrine manner, similar to the effects of perivascular WAT-derived adipokines on cardiovascular disease (59). Last, total leptin activity rather than its circulating levels alone might be the most relevant diagnostic factor in the pathology of human CM. The soluble leptin receptor reflects total activity of leptin, and hence, future studies may determine how the ratio between leptin and its soluble receptor predicts the pathogenesis and outcome of human CM. Another adipokine, adiponectin, has anti-inflammatory properties that could counterbalance the proinflammatory effects of leptin and influence the course of acute inflammation. Further research is warranted to elucidate a role for leptin/leptin receptor and leptin/adiponectin ratios in human CM pathology and their value as prognosis and/or diagnosis biomarkers. Moreover, if confirmed, the establishment of these ratios as pathogenic factors in the development of CM could open previously unidentified avenues for therapeutic interventions.

Notably, consistent with a potential role of ScWAT in disease progression, it has been shown in postmortem studies of CM cases that endothelial cells obtained from ScWAT are similar to endothelial cells from cerebral vasculature in features relevant to CM pathology such as tumor necrosis factor–α (TNF-α)–induced expression of adhesion molecules (8). Future experiments are thus required to uncover mechanisms of depot-specific WAT sequestration in humans and its relationship with disease severity.

WAT is the largest endocrine organ in the mammalian body, and this study suggests its importance in the pathogenic consequences of malarial infection. Furthermore, our data suggest targeting ScWAT sequestration and/or leptin production, for example, with mTORC1 inhibitors such as the FDA-approved drug rapamycin, as potential new avenues for CM treatment. Last, the apparent specificity of ScWAT sequestration in CM patients and the relative accessibility of this tissue suggest potential diagnostic opportunities for the determination of CM severity and prognosis.
Materials and Methods

Human plasma and WAT samples

Archived samples in this study were part of larger studies assessing long-term neurocognitive outcomes, pathology, and inflammatory markers in children with CM and severe malarial anemia (SMA).

French, Senegalese, and Cambodian samples

P. falciparum–infected children aged less than 4 years were enrolled with a Blantyre coma score of <2 persisting for 30 min and/or at least two seizure episodes within 24 hours, with no other obvious causes of coma. Lumbar puncture was systematically performed to exclude meningitis. Children were given the appropriate treatment, as recommended by the local Ministry of Public Health. Controls consisted of plasma samples from healthy French Caucasian blood donors and malaria-negative Senegalese volunteers.

Uganda samples

Children with CM between 18 months and 12 years of age were enrolled. CM was defined as (i) coma (Blantyre Coma Scale score ≤2 in children ≤5 years or Glasgow Coma Scale score ≤8 in children >5 years), (ii) P. falciparum on blood smear, and (iii) no other known cause of coma (e.g., meningitis or encephalitis). Children with CM were managed according to the local Ministry of Health treatment guidelines at the time of the study. Children with CM were assessed for malaria retinopathy by indirect ophthalmoscopy. SMA was defined as either a hemoglobin level of <5 g/dl or a hematocrit of <15%. Controls consisted of malaria-negative and asymptomatic malaria-positive Uganda samples.

Malawi samples

Children aged 6 months to 9 years who satisfied the clinical case definition of CM were enrolled in the study after consent was obtained from a parent or guardian. CM was defined as a Blantyre Coma Score of ≤2, peripheral parasitemia with P. falciparum of any density, and no other discernible causes of coma.

Subcutaneous WAT tissue samples were cut from blocks generated as part of an existing project involving patients with pediatric CM from Malawi. Uninfected controls were obtained from discarded tissue following vascular surgery.

Mice

All experimental mice were 8 to 10 weeks of age. WT female and male C57BL/6 (000664) mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Male and female CD36KO mice on a C57BL/6 background were either purchased directly from The Jackson Laboratory (019006) or bred from heterozygous animals in our facility. Transgenic UBC-GFP C57B/6 mice were obtained from the Institute of Cell Biology, University of Bern, Switzerland. Animals were housed four to five per cage and kept under standard laboratory conditions and allowed free access to water and food at all times. All experiments were performed with the approval of the appropriate institutional animal care and use committee associated with Harvard Medical Area, University of Glasgow, or University of Bern.

Parasites and infections

All four parasite strains used, including PbA, ΔSMAC, ΔSBP1 (34, 35), and PbK173 (36), were transgenic for constitutive expression of GFP–luciferase. In some sequestration experiments, mCherry-expressing PbA parasites (line 1804 mCherry1Hyp70 from the PbA GIMO mother line 1596c1, Leiden) were used. For infections, cryopreserved parasites were thawed and passaged once in vivo before being used to infect experimental mice with 0.5 million parasitized RBCs per mouse by tail vein injection. When PbΔSMAC or ΔSBP1 parasites were used for infections, their respective parental WT lines were used as controls. Parasites from peripheral blood were stained with SYTO16, and the percentage of infected cells was calculated by flow cytometry (60). Survival was followed for up to day 12.

Parasite sequestration

Infected mice were sacrificed on the indicated day and perfused with cold phosphate-buffered saline (PBS) intracardially. The organs were harvested, weighed, and homogenized in an equal volume per milligram of tissue. Luciferase activity was measured in a 96-well luminometer (Biotek Synergy 2). For in vivo imaging, infected mice were anesthetized and injected with luciferin. Luciferase activity was detected with IVIS Lumina In Vivo Imaging System.

Rapamycin treatment

Mice were treated with vehicle (5% Tween/5% polyethylene glycol 400) or rapamycin (1 mg/kg) (LC Laboratories, Woburn, MA) by intraperitoneal injection.

ELISA measurements

Mouse serum leptin was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit from Millipore (EZML-82 K; 0.2 to 30 ng/ml), following the manufacturer’s instructions. Undiluted serum (5 μl) was used per assay. Human leptin was measured using the Human Leptin Quantikine ELISA Kit from R&D Systems (DLP00, 15.6 to 1000 pg/ml). Plasma (5 μl) diluted 1:10 in dilution buffer provided by the manufacturer (50 μl total) was used per reaction. Adiponectin and leptin were measured from supernatants of cultured differentiated primary adipocytes using the commercial mouse ELISA kits from R&D Systems (adiponectin, MRP300; 0.2 to 10 ng/ml).

Western blotting

NP-40 extracts of snap-frozen adipose tissues were separated by SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Millipore), and stained with the following antibodies (Cell Signaling Technology, Beverly, MA) at a 1:1000 dilution: rabbit (Rb) anti–β-actin, Rb anti-total p70 S6K, and Rb anti–phospho-p70 S6K (Thr389). After washing in PBS + Tween 20, membranes were incubated with horseradish peroxidase–conjugated antibodies 1:5000 diluted in 5% skim milk. Detection was performed using the SuperSignal West Femto substrate (Thermo Fisher Scientific).

Microscopy

Sections were cut from paraffinized mouse organs from PBS-perfused animals or from human adipose tissues and placed on slides. Antigen retrieval was performed by immersing the slides in 95.5°C prewarmed universal antigen retrieval solution (R&D Systems). After cooling in PBS, slides were permeabilized in 0.25% Triton X-100 in PBS, followed by blocking in a 10% normal goat serum solution supplemented with 0.2 M glycine and 1% bovine serum albumin. Primary staining was performed with anti-λ-leptin rabbit polyclonal (Abcam, ab16227) or anti–phospho-Ser235/235 S6 Alexa Fluor 488–conjugated rabbit monoclonal (Cell Signaling Technology, 4803) diluted 1:200 in blocking solution. Mouse and human WAT leptin and pS6 images were obtained in a Nikon TiE inverted microscope adapted with a Yokogawa CSU-X1 spinning disc processor and an air Plan Apo 20× (numerical aperture = 0.75). For image analysis, each individual image...
Hexamer-primed complementary DNA (cDNA) was synthesized with using TRIzol for reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis of leptin and adiponectin transcripts. In vitro experiments Primary mouse preadipocytes were differentiated as follows: After confluence, cells were incubated with a cocktail containing dexamethasone (2.5 μM), 3-isobutyl-1-methylamine (0.5 mM), and insulin (10 μg/ml) for 2 days. The medium was then changed every 2 days until complete cell bubbling was observed. Differentiated primary adipocytes were then infected with lentivirus expressing an mCherry-expressing disc confocal microscope, with a Yokogawa CSU-X1 processor and a Plan apochromatic 20×/0.8 M27 [free working distance = 0.55 μm].

Gene expression Total RNA was extracted from cultured cells using TRIzol (Invitrogen). Hexamer-primed complementary DNA (cDNA) was synthesized with the Verso cDNA Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction was performed with MyIQ (Bio-Rad) using SYBR Green. Relative expression was calculated with the ΔΔCt method. cDNA expression of each sample was standardized to RPLA13. Each sample was tested in duplicate at least twice. Primer sequences used are as follows: leptin, 5′-GAGACCCCTGTGTGGTTC-3′ (forward) and 5′-CCTGCGGTGTAATGTACATTG-3′ (reverse); adiponectin, 5′-TGTTTCTCTTTAAAGTCTGGC-3′ (forward) and 5′-CCAACTCGACAAGTTCCCTT-3′ (reverse).

Statistics Data are expressed as mean ± SD. Statistical analyses were performed in GraphPad Prism, and a P value of less than 0.05 was considered significant.

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