Review

Cerebral Malaria: Clinicians, Extracellular Vesicles, and Therapeutically-Rational Exchange (T-REX)

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Cerebral Malaria: Clinicians, Extracellular Vesicles, and Therapeutically-Rational Exchange (T-REX)

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Abstract: As new vaccines reduce P. falciparum (Pf) cases, clinicians can focus on rescuing more children with cerebral malaria (CM). Adjunctive "therapeutically-rational exchange transfusion" (T-REX) refers to using special "Pf-resistant" donor red blood cells (RBCs) that secrete nano-sized RBC extracellular vesicles (EVs). Can some RBC EVs rapidly dislodge Pf-infected RBCs (iRBCs) sequestered in blood vessels and reduce inflammation? Does post-exchange "rebound parasitemia" reflect sequestration-reversal? We reviewed case-reports, EV and liposome studies, expert perspectives, and time-sequence data. In the AQUAMAT study, median coma-recovery time was 20 hours for artesunate. In contrast, a CM patient treated with type-O T-REX quickly began speaking (during the procedure). For other rapid-recovery CM patients, donor RBC variables had not been specified. **Despite** rapid Pf-killing, drugs need 20 hours to substantially reduce iRBC cytoadhesion while EVs can selectively bind distant inflamed tissues within 15 minutes. RBCs stressed by blood-bank storage and Pf infection secrete EVs. Post-exchange "rebound parasitemia" can occur within 60 minutes and may reflect release of sequestered iRBCs. Sequestration-reversal can be assessed by monitoring Pf parasitemia and total Pf biomass. New data suggest clinicians, working with transfusion- and laboratory-medicine physicians, can, and should, assess T-REX options and publish their findings to help clarify how RBC EVs may serve as therapeutic "decoy ligands."

Keywords: cerebral malaria; therapeutically-rational exchange transfusion (T-REX); extracellular vesicles; decoy ligands; competitive binding; coma-recovery time; CD36; PfEMP1

1. Introduction

For 50 years, front-line clinicians have been using exchange transfusions to rescue patients with cerebral malaria (CM) – a life-threatening subset of *Plasmodium falciparum* (*Pf*) malaria infections [1]. CM can involve seizures and coma – and be lethal [2]. In the 21st century, intriguing exchange-related questions have been raised and "paradoxes" remain unexplained – questions and paradoxes related to lethal *Pf*-induced cytoadhesion. How can a subset of exchange-treated comatose patients quickly recover during the procedure – even before the disease-promoting cytoadhesion of *Pf*-infected red blood cells (iRBCs) has been substantially reduced by *Pf*-killing drugs? [3] Should we risk treating CM patients with the same adhesion-promoting RBCs that may have been responsible for the onset of CM? [4] How can exchange possibly ever rapidly reverse coma given that the procedure only removes circulating RBCs and not the iRBCs sequestered in the cerebral microvasculature?

Regarding cytoadhesion, *Pf* parasites produce disease-promoting adhesive proteins (like PfEMP1) which are inserted onto the surface of iRBCs [5]. Not only are adhesive PfEMP1-expressing iRBCs pathogenic, the EVs they secrete can transport (as internal cargo) pathogenic *Pf*-related biochemicals that are critical as they interact with patient cells to promote *Pf*-disease progression [6]. Of note, both the EVs secreted by iRBCs and uninfected RBCs (uRBCs) express

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phosphatidylserine (PS) which is a key ligand in *Pf* infection [7]. Adhesive PfEMP1-expressing iRBCs can cause mechanical microvessel obstruction that can substantially reduce blood flow, damage tissues (like vascular endothelium), and trigger inflammation. Obstruction of cerebral microvessels involves binding of iRBCs, uRBCs, leukocytes, platelets, and EVs (secreted by a variety of different cells) [8]. Ligand-receptor interactions can include complex multi-agent "bridge-binding) involving combinations of cells, EVs, antibodies, and diverse biochemicals [9]. Some PS-expressing iRBCs are phagocytosed which, in turn, causes these iRBCs to release pathogenic, inflammation- and CM-promoting hemin [10]. During *Pf* infection, both iRBCs and uRBCs increase their secretion of PS-expressing EVs which can compete for binding to cell receptors [8,11,12]. To appreciate the different ways therapeutic donor RBC EVs RBCs may compete for receptor binding, see Table 1.

Table 1. PS-expressing Donor uRBC EVs Function as "Decoy Ligands" by Competing with these "Pathogenic Ligands" for Binding to PS-receptors.

"Pathogenic Ligand"	Cytoadhesion and/or Inflammation	References
the PS on the surface of iRBCs	iRBCs express PS which can interact with CD36, TIM-1, TIM-4, BAI-1, TSP, and/or integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ (to promote pathogenic cytoadherence and/or inflammation).	[13,37,38,87]
the PS on the surfaces of iRBC EVs, platelet EVs, and EC EVs	PS-expressing iRBC EVs carry pathogenic PfEMP1 (and miRNAs, etc.) as cargo which can promote inflammation. In *Pf* infection, platelet and EC EVs can also promote *Pf*-disease progression.	[6,34,82]
Hemin (released by iRBCs)	Hemin (which binds CD36) may (1) increase expression of adhesive receptors ICAM-1 and VCAM-1 on ECs and (2) promote inflammation by binding to CD36 on immune cells.	[10]
Pf-modified Band 3 on iRBCs	*Pf* parasites alter ("modify") RBC Band 3 on iRBCs in ways that promote inflammation and adhesion via modified Band 3 binding to CD36-expressing platelets, ECs, and immune cells.	[87–91]
PfEMP1 on surface of iRBCs	Some PfEMP1 proteins can bind TSP and integrin $\alpha v\beta 3$ on angiogenic ECs. Most *Pf* strains insert a CD36-binding PfEMP1 protein ("variant") onto the iRBC surface. Of note, platelet CD36 enables iRBC-platelet-EC "bridge bonding" with those cerebral ECs that do not express CD36.	[37–42]

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Of note, cytoadhesive iRBCs not only express PfEMP1 but also other pathogenic *Pf*-related "ligands" and, importantly, PS which can enable iRBCs to bind to [1] other iRBCs (auto-agglutination), [2] uninfected RBCs (uRBCs) to form "rosettes," [3] platelets ("platelet clumping"), [4] immune cells, [5] endothelial cells (ECs), and [6] thrombospondin (TSP) on connective tissue extracellular matrix [2,13]. In addition, *Pf* causes iRBCs to secrete pathogenic nano-sized extracellular vesicles (iRBC EVs) that carry as cargo *Pf*-related biochemicals. In *Pf*-infected patients, the donor uRBCs and the EVs they secrete (uRBC EVs) can "compete" with the pathogenic iRBCs and iRBC EVs. When a patient is treated via automated exchange, donor uRBCs and the EVs they secrete are delivered to the patient within 2-3 hours. The rapid, post-exchange interactions between cells, EVs, biochemicals and connective tissues are extraordinarily complex which explains why there are so many yet-to-be explained "paradoxes."

In an attempt to generate interest among front-line clinicians in optimizing exchange-for-malaria as a RBC EV "decoy ligand" strategy (analogous to recent anti-viral and anti-cancer decoy strategies [14,15], here we review data and conceptual frameworks that may help explain how some exchange transfusions are able to dramatically rescue near-death patients. Clinicians might be intrigued that there is currently no molecular explanation for why coma-recovery can be so rapid that patients begin speaking during the procedure. Having been frustrated by the gruesome deaths of children with CM, it is understandable that clinicians worldwide have repeatedly felt compelled to publish exchange-for-malaria case-reports describing stunning, unexpected recoveries – and continue to publish dramatic rescues in the 21st century [16–21]. Regarding the paradox of exchange, 25 years ago, NJ White noted, "the increasing number of case-reports and unrandomized series which suggest benefit are, collectively, difficult to ignore" and asked, "how does it work?" [22] Currently, scientists are still "desperately seeking therapies" for CM [23] at the same time the subset of exchange transfusions linked to rapid coma-recovery remains unknown.

Here we try to use "clinician-friendly" terms and concepts. "Therapeutically-rational exchange transfusion" (T-REX) refers to different options [24–30] that may optimize "conventional" exchange-for-malaria in which special *Pf*-resistant donor RBCs are used instead of nondescript "standard-issue" blood-bank RBCs that might be *Pf*-promoting RBCs (like the adhesive, PfEMP1-binding group-A RBCs) [4,31,32]. Perhaps helpful visually: The pathogenic PfEMP1 proteins expressed by iRBCs can bind the blood-group A antigen expressed by type-A RBCs, and this binding promotes cytoadhesion (iRBC-uRBC interaction called "rosetting") that promotes microvascular blood flow obstruction.

For simplicity, here "EVs" refers to the entire spectrum of different micro- and nano-sized EVs secreted by both iRBCs and uRBCs and by platelets, ECs, and other cells. Data suggest it is reasonable for clinicians to assume that *Pf* parasites have evolved so PS-expressing iRBC EVs (that carry pathogenic *Pf*-related biochemicals as cargo) generally promote *Pf* infection and are likely involved in promoting the onset of CM [2,33–35] while data using PS liposomes, which resemble PS-expressing RBC EVs [36] (Al-Jipouri-2023), suggest PS-expressing EVs (secreted by the *Pf*-resistant donor RBCs delivered via T-REX) can be therapeutic since they compete with hemin, iRBCs, and iRBC EVs for binding to PS-receptors (like CD36) [13]. Notably, PS-expressing donor uRBC EVs can also compete for binding with the PfEMP1 on iRBCs (as well as compete with the PS on iRBCs and iRBC EVs) because PfEMP1 binds to some PS-receptors (such as CD36, TSP, and integrin $\alpha v\beta 3$) [37–42].

For simplicity, we also loosely use the term "ligand-receptor binding" for all the different types of adhesive interactions involving biochemicals, cells and EVs that promote the cytoadhesion (rosetting, clumping, autoagglutination, sequestration) that can trigger inflammation and obstruct microvascular blood flow. Clinicians understand that biochemical interactions vary, may not yet be well-defined, and can involve complex multi-agent "bridge binding," etc. Regarding use of words relevant to "competitive binding" or "competitive inhibition," we note that a biochemical (like hemin) or a platelet, leukocyte, RBC, or EV ligand, etc. can "compete for" or "interfere with" the binding of some other agent to a cell receptor

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without interacting with exactly the same binding site on that receptor or without interacting in exactly the same way. We feel the term "ligand-receptor interaction" is visually and conceptually helpful for clinicians even though that term may not always be strictly correct according to biochemists. Regarding our use of the term "decoy ligand," when we refer to PS-expressing EVs secreted by donor uRBCs as therapeutic "decoy ligands" we mean data suggest their impact on target cells is different than the impact of, for example, PS-expressing EVs secreted by iRBCs (that carry pathogenic cargo) and not that there is no impact at all. That is, here, "decoy ligands" do not mean, for example, that there is complete blockade of target-cell signaling pathways – there is simply a different impact resulting in "competitive inhibition" of a pathogenic effect. Regarding pathogenic EVs secreted during *Pf* infections, in their review, Sierro and Grau noted that interfering with EV binding to target cells can reduce cell activation (not completely block activation) [34]. For context, EVs normally secreted by aging human RBCs are not pathogenic while, in contrast, EVs secreted by *Pf*-infected RBCs carry pathogenic *Pf*-related biochemicals as cargo (that can induce transcriptional changes in monocytes) [6], and the pathogenic EVs secreted by human cells stressed during *Pf* infection can promote CM [43].

Regarding questions and confusions likely to be of interest to clinicians: Are potent drugs (like artesunate) – that rapidly kill *Pf* parasites – unable to rapidly reverse iRBC cytoadhesion and rapidly reduce the high levels of EVs noted in severe *Pf* infection? Why did the parasitemia level in an exchange-treated patient (who survived) increase after the immediate (and expected) post-exchange decline? If CD36 expression on cerebral ECs is low, why does iRBC-CD36 binding seem so critical in CM?

Consideration of those questions, in turn, prompts these questions: Regarding CM patients, can recent EV research findings help explain post-exchange "rebound parasitemia" [44] and rapid coma-recovery? [20] Why does a gene deletion that reduces PS expression on iRBCs and iRBC EVs [45] and a CD36 gene polymorphism [46] protect against CM? How do *Pf*-resistant G6PD-deficient RBCs (that secrete more EVs than normal-enzyme RBCs) [47], protect against CM [48]? Why are levels of RBC EVs elevated in severe *Pf*-malaria infections? [12]. Why do higher levels of EVs correlate with the presence of neurological symptoms in CM patients? [43] What are the therapeutic implications of EV research findings that showed EVs from malaria-infected animals that were infused into healthy animals induced CM-like brain pathology [43]. These questions direct attention to the importance of EVs, PS, and CD36 in CM pathogenesis.

Regarding adjunctive exchange-for-malaria, are specific, yet-to-be-identified donor RBCs (like *Pf*-resistant type-O RBCs or "multi-gene" type-O + sickle-trait RBCs, or type-O, + C-trait + G6PD-deficient RBCs, etc.) the reason some exchanges trigger rapid coma-recovery? [20,27] Of note, In Nigeria, 5% have the HbAS + G6PD-deficient RBC combination [49]. Of note, Noulsri and Lerdwana noted that "some blood centers allow volunteer donors with thalassemic trait, glucose-6- phosphate dehydrogenase deficiency (G6PD) trait, and sickle cell trait (SCT) to donate blood if their hemoglobin values fall within acceptable ranges." [50] Is it technically feasible for front-line clinicians in SSA, for example, to immediately translate recent research findings into patient-care strategies that, when combined with careful data collection (and reporting), may advance use of RBC EVs as rapid-acting therapeutic "decoy ligands."

Here we describe (1) recent EV and ligand-receptor data, (2) a conceptual framework that may be informative for clinicians as they struggle to rescue children dying with CM, and (3) new laboratory tests and exchange-transfusion strategies. Ideally, this review will prompt some clinicians to (1) ask transfusion- and laboratory-medicine physicians for help exploiting special *Pf*-combating donor RBCs for adjunctive exchange (by opting for a T-REX option instead of risking use of *Pf*-promoting RBCs) and (2) collect and publish critical case-report data that, when integrated in the future, may help reduce CM mortality and advance EV therapeutics.

Clinicians interested in the rationale for using T-REX to rescue children with CM might find it helpful to visualize special *Pf*-resistant donor RBCs secreting *Pf*-combating, PS-expressing EVs that compete for binding to cell receptors with pathogenic hemin and PS-expressing iRBCs, iRBC EVs, EC EVs, and platelet EVs. Among the overwhelming number of ligands and receptors

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involved in *Pf* infection, data suggest key ligands are PfEMP1 (the *Pf*-disease-promoting protein generated by *Pf* parasites), hemin (released by phagocytosed iRBCs), and PS (expressed by iRBCs and most EVs), while a key patient cell receptor is CD36. In support of this over-simplification, a study found that PS liposomes (which closely mimic EVs in research studies) effectively compete with (and reduce) the binding of PS-expressing iRBCs to CD36 [13]. Among 21 CM patients, 20 of their *Pf*-parasite PfEMP1 proteins (95% of the *Pf* strains) bound to CD36 – a higher proportion than any other cell receptor studied [51]. In 2022, Bachmann and colleagues published "CD36 – A Host Receptor Necessary for Malaria Parasites to Establish and Maintain Infection" [42]. Platelet-mediated clumping is strongly linked to severe malaria, and the formation of clumps of iRBCs and platelets requires expression of platelet CD36 [52]. Relevant to platelet CD36, a review of CM noted (1) in human post-mortem brain sections from fatal CM cases, electron microscopy showed platelets had accumulated inside cerebral microvessels and (2) a dramatic increase in plasma levels of EVs was found in children with CM [53]. Regarding pathogenic CD36-binding hemin (released by iRBCs after being phagocytosed by macrophages), Banesh, Layek, and Trivedi reported that hemin plays a role in CM by triggering macrophage production of the pro-inflammatory cytokine TNF-α and noted "CD36 has strong affinity for hemin." [10]

2. Clinician Innovations: New Treatments, Studies, and Perspectives

In 1892, in sub-Saharan Africa (SSA), a military physician (Emil Steudel) successfully rescued a white European scientist with life-threatening CM and blackwater fever by deciding to transfuse the blood of a Black donor who had lived and worked in, and off the coast of, malaria-endemic East Africa [54]. A review of the context suggests Dr. Steudel felt this inter-racial transfusion was prudent because he suspected the blood from a Black African would be therapeutically superior to the blood from the patient's white colleagues. Presumably, this government physician – likely under great duress treating a renowned scientist – felt killing parasites was not enough and had noticed that malaria mortality among white expatriates from Europe was higher than among persons born in SSA.

In the 1970s, clinicians felt it was biologically plausible that exchange transfusion might be substantially more effective as a rescue adjunct than simple transfusion. In 1979, physicians reported, "the prompt reduction in the number of infected erythrocytes in our patient and the patient of Gyr et al. [1] following exchange was dramatic." [17] They noted, "patient recovered from her disease despite severe cerebral involvement and acute renal failure." [17]

In 1981, clinicians in Nigeria reported that two *Pf*-resistant RBCs – sickle-trait hemoglobin (HbAS) RBCs and G6PD-deficient RBCs – could be safely used to treat neonates who needed exchange transfusions [55]. Finding that these *Pf*-resistant RBC variants are safe is clinically significant because the prevalence of *Pf*-resistant RBCs is substantial in some *Pf*-endemic regions, and might be especially therapeutic given they are linked to reduced onset of severe *Pf* disease or death.

In 1983, physicians directed attention to adjunctive anti-adhesion strategies. They reported that "antibody can reverse in vivo sequestration as shown by the appearance of trophozoite/schizont-infected erythrocytes in the peripheral blood of an intact animal after inoculation with immune serum." [56] They noted, "the number of trophozoite/schizonts in the peripheral bloodstream increased sharply during the first half hour after serum injection" – consistent with hyperimmune serum rapidly disrupting sequestration and, thereby, releasing mature parasite-infected RBCs into the peripheral circulation. They added, "the sharp increase of circulating trophozoites/schizonts that occurs in an intact monkey within minutes after passive transfer of immune serum clearly indicates that the reversal of binding can occur in vivo as well as in vitro." [56] In 1991, with an interest in advancing development of anti-adhesion therapies, an American military physician, Ockenhouse, and his colleagues urged clarifying the roles of the receptors and ligands involved in *Pf*-induced cytoadhesion [57]. In 1999, Ho and White described how ligand-receptor interactions triggered by *Pf* infections affect inflammation and noted that cytoadherence may activate intracellular signaling pathways in ECs and iRBCs "leading to gene expression of mediators such as cytokines." [58] In

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their article titled "Desperately Seeking Therapies for Cerebral Malaria" [23], the authors cited a study [59] that showed an anti-adhesion strategy could rescue mice with experimental CM and reverse brain damage. And so, clinicians were generating interest in anti-adhesion therapies (disruption of pathogenic ligand-receptor bonds) to supplement *Pf*-killing drugs. Physicians were proposing that sequestration-reversal might reduce inflammation as well as restore microvascular blood flow.

In the 1990s, physicians continued to repeatedly advocate for exchange by publishing case-reports. In 1995, clinicians noted that, regarding a patient who "developed a systemic inflammatory response syndrome manifested as cerebral malaria, renal failure, acute respiratory distress syndrome and disseminated intravascular coagulation, an emergency blood exchange reversed the situation dramatically." [60] They added that exchange results in "improved rheology with transfused blood and reduction of microcirculatory parasitic sludge, improved oxygen-carrying capacity with transfused red blood cells and a decreased risk of disseminated intravascular coagulation. If whole blood is exchanged and not only red cells, it may also remove toxins and monokines which had been shown to play a harmful role in the pathogenesis of malaria." In 1997, clinicians in SSA published a case-report titled "Reversal of Life-Threatening Vascular Occlusion by Apheresis in Fulminating Plasmodium Falciparum Malaria" in which they noted "complications – due to vascular occlusion – can be promptly reversed with suitable apheresis. This practical approach is relatively inexpensive." [61] Interestingly, they decided to use plasma exchange following their initial RBC exchange, since they felt "the sequential process of red cell and plasma exchange is advantageous." Of note, for clinicians who agree that exchanging plasma factors is prudent, the benefits of plasma exchange can be incorporated into T-REX by using whole blood exchange of *Pf*-resistant donor RBCs instead of units of packed RBCs (RBC concentrates). [62]

In the 21st century, clinicians suggested that type-O donor RBCs would be therapeutically superior for the treatment of *Pf*-malaria patients (compared to type-A, normal hemoglobin RBCs, for example) [4,31] Rowe, et al. noted, "transfusion of non-O blood could promote rosetting; therefore, for those severe malaria patients requiring blood transfusion, it might be preferable to use group O blood whenever possible." [31] Cserti-Gazdewich et al. suggested that when treating *Pf*-infected patients, "the use of universal group O RBCs rather than type-specific RBCs may prove especially advantageous." [4] Interestingly, thoughtful, prudent donor RBC selection is consistent with the inter-racial blood transfusion used in 1892 in SSA to rescue a CM patient. [54]. That is, clinicians have been stressing the concept "for cell therapies, the cells matter." Thus, physicians have raised an important issue: If a clinician needs to rescue a child who is dying with CM and that child was born with group-A blood, for example, why treat that child with adhesion-promoting type-A RBCs that are, in part, responsible for the onset of that child's life-threatening CM? [4] Via T-REX, clinicians have proposed "converting" (temporarily) *Pf*-susceptible patients into patients protected by *Pf*-resistant type-O, sickle-trait, C-trait, or G6PD-deficient RBCs, etc. [25,27–29] If, conceptually, *Pf*-malaria is seen as primarily an "RBC disorder," T-REX might be viewed as a "host-altering" or "host-conversion" adjunct that has markedly more impact for a pathogen than a more subtle "host-directed therapy." From the perspective of the parasite, the host has changed suddenly and dramatically.

In 2011, clinicians proposed that post-exchange "rebound parasitemia" might reflect "release of sequestered infected RBCs" (sequestration-reversal). [44] They noted that when exchange transfusion was used to rescue a CM patient, "Parasitemia dropped from 30 to 15% immediately after the procedure but rapidly increased to 25% after 50 min." [44] Notably, this post-exchange "rebound parasitemia" in a CM patient is consistent with the increased level of mature parasite-infected RBCs in the peripheral circulation observed about 30 minutes after injection of hyperimmune serum in a study of malaria in monkeys. [56]

In 2014, clinicians noted that the "Pf Histidine-Rich Protein 2" (PfHRP2) level can be used to estimate the total body parasite burden while the total peripheral-blood parasite burden is calculated from the peripheral blood film parasite count. The ratio of total to circulating parasite biomass can

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estimate the degree of sequestration over time [63]. In 2023, other clinicians also described how sequential measurements of PfHRP2 and peripheral *Pf* parasitemia could be used to estimate sequestration-reversal (sequestered parasites = total parasites − circulating parasites) [64]. These advances means it is likely that sequential post-exchange measurements of PfHRP2 and peripheral parasitemia could show that post-exchange "rebound parasitemia" occurs when sequestered iRBCs are released into the peripheral circulation – ideally, in combination with sequential microscopic assessments of the maturity level (stage) of the *Pf* parasites circulating post-exchange.

3. T-REX Analogy: RBC EVs as a "Decoy Ligand Therapy" for SARS-CoV-2 Infection

Consistent with the underlying EV-based rationale for urging clinicians to consider using and evaluating T-REX options, in 2023, scientists noted that RBC EVs can inhibit SARS-CoV-2 infection in a PS-dependent manner [14]. They chose to study the TIM-1 cell receptor (a PS-receptor that also binds the PS expressed by iRBCs, iRBC EVs, and enveloped viruses) and showed RBC EVs competitively inhibit virus interaction with TIM-1 and, thereby, reduce PS-receptor-mediated viral entry. They noted, SARS-CoV-2 exploits PS-receptors "to increase their tropism and overall infectivity." [14]

Also consistent with using T-REX (as a competitive inhibition strategy), in their publication "Decoy peptides effectively inhibit the binding of SARS-CoV-2 to ACE2 on oral epithelial cells," the authors propose using spike peptides as "decoy ligands" to impede spike-ACE2 interaction [65] (Loi-2023) Interestingly, a "decoy ligand" (that competes with glutamine for binding to enzymes) is currently being evaluated as a potential adjunct for the rescue of children with CM [66]. In 2024, they noted, "Despite treatment with the highly effective antimalarial, intravenous (IV) artesunate, mortality rates are 15–25%, and children who survive often suffer severe neurological sequalae." Regarding competitive inhibition as an anti-cancer strategy, chemokine "decoy ligands" may block receptor binding and, thereby, impede intracellular signaling [15]. Consistent with T-REX as a "decoy ligand" strategy, natural receptor-antagonist ligands have been developed to block CXCR4 receptor activation in cancer.

3. "Decoy Ligands" Can Reverse (as well as Reduce) iRBC Binding

In 1985 – before ligand-receptor interactions relevant to *Pf* infection were substantially understood – physicians noted that an antibody (previously shown to react with the membranes of ECs, monocytes, and platelets) also reacted with a cell line that binds iRBCs [67]. This antibody reversed in vitro adherence of iRBCs to target cells. A recombinant peptide based on PfEMP1 was found to block and reverse adhesion of malaria-infected RBCs to CD36 under flow [68] In 2000, an anti-CD36 antibody was able to reverse the firm adhesion of infected erythrocytes, raising the possibility that antiadhesive therapy could be employed in severe falciparum malaria [69]. PS liposomes were found to inhibit the binding of iRBCs to CD36 and thrombospondin [13]. iRBCs expose PS on their surface as the parasite matures and this PS contributes to their adherence to host cells, such as ECs. PS liposomes (which resemble EVs) inhibited the binding of iRBCs to host cells, suggesting they bind to the same receptors. This suggests PS liposomes might be able to disrupt the binding between iRBCs and host cells as a "decoy-ligand" competitive-inhibition therapy [13]. Recombinant PfEMP1 peptide (a PfEMP1 "decoy") inhibits and reverses cytoadherence of clinical *Pf* isolates in vivo [70].

In the 21st century Ho noted, "an anti-CD36 antibody was able to reverse the firm adhesion of infected erythrocytes, raising the possibility that antiadhesive therapy could be employed in severe falciparum malaria." [69] "We show that anti-CD36 monoclonal antibodies (MoAbs) reverse rosetting of PRBCs from both a culture-adapted line (Malayan Camp [MC] strain) and a natural isolate, GAM425. Three MoAbs that block adherence of PRBCs to ECs or C32 melanoma cells also reversed rosetting by greater than 50% at levels of less than 1 pg/mL (OKM5, OKM8, and 8A6)." [71]

4. Method

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We reviewed *Pf*-malaria research studies and case-reports (with a special emphasis on time-sequence data), persistent confusions, EV and liposome research findings, expert perspectives and recommendations, and current transfusion- and laboratory-medicine methods.

5. Results

Regarding time-sequence data, the median "time-to-speak" (coma-recovery time parameter) in the AQUAMAT study was 20 hours for intravenous (IV) artesunate despite rapid *Pf*-killing by artemisinin drugs [72]. Based on what is known about the pathogenesis of CM, it seems reasonable to assume comatose CM patients cannot become alert and speak until a substantial proportion of the sequestration of iRBCs, leukocytes, platelets inside cerebral microvessels has been reversed. Regarding the distribution of coma-recovery times, most AQUAMAT patients were also transfused which means it is likely a small number of patients were treated with a combination of artesunate and some T-REX option. This is probable because (1) some clinicians use exchange to rescue children dying with CM and (2) the prevalence of *Pf*-resistant RBCs (such as type-O, sickle-trait, C-trait, G6PD-deficient RBCs, etc.) are substantial in some *Pf*-endemic regions. If some comatose children in the AQUAMAT study were treated with a combination of artesunate and T-REX this may have affected the range of the "time-to-speak" values (while having little impact on the median value). In contrast to the 20-hour median "time-to-speak" value in the AQUAMAT study, a type-O T-REX patient recovered so quickly she began talking during her 2.5-hour-long exchange procedure [20]. Fortunately, a study by Hughes et al. provided an explanation: *Pf*-infected RBCs remain substantially cytoadherent 20 hours after artemisinin treatment (despite rapid *Pf*-killing) due to persistent expression of PfEMP1 on the iRBC surface [3]. The impact of artemisinin drugs on RBC EV levels over time was also studied because patients with severe *Pf* disease have high levels of RBC EVs (compared to uninfected persons), and researchers found it takes artemisinin derivatives more than 20 hours to reduce the number of RBC EVs by 50% [12]. Consistent with the unexpected time delays for the therapeutic effects of artesunate, a study showed exposing iRBCs to artesunate protected iRBCs from phagocytosis by stimulating rosetting and also noted that artesunate-resistant strains formed more rosettes more rapidly [73]. And so, it is not surprising that "drug-mediated rosette-stimulation" has been proposed to explain how the ex vivo combination of artesunate with mefloquine and piperaquine resulted in increased rosetting rates [74]. In sharp contrast, regarding the kinetics of therapeutic infustions of EVs, researchers found that IV-administered EVs can selectively target, and adhere to, distant inflamed tissues within 15 minutes [75].

Our review of rapid coma-recovery, exchange-treated CM patients provided some coma-recovery and donor-RBC information. Unfortunately, only one case-report included both the coma-recovery time and the donor's ABO blood group.

In 2017, a type-O T-REX patient began talking during her 2.5-hour-long exchange procedure [20]. The clinicians did not explain if the type-O donor RBCs had been specificially requested because they expected them to more therapeutic or they were used on an emergency basis as "universal donor" group-O RBCs.

In a 1979 case-report, clinicians noted that sets of thin Giemsa-stained blood smears showed *Pf* parasites, with 20% of RBCs parasitized. "Trophozoites, mature trophozoites, schizonts (free and intracellular), and sausage-shaped gametocytes were seen. During the first day of hospitalization the patient developed progressive obtundation and coma with nuchal rigidity and bilateral extensor plantar reflexes. The mental status of the patient improved during the exchange transfusion; he awakened and was capable of answering simple questions and obeying simple orders." [16] Notable in this case-report: This CM patient quickly improved (during the procedure), but, unfortunately, donor RBC variables were not reported.

In Southeast Asia, a 21-year old woman with cerebral malaria and disseminated intravascular coagulaition (DIC) was rescued by adjunctive exchange transfusion in 1979 [76]. "Petechiae and purpuric spots were noted on the anterior abdominal wall. Blood was oozing from the venepunctures . . . coma and jaundice deepened . . . A total blood exchange was aimed by using

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8,000 ml (twice of the estimated blood volume) of citrated blood . . . No complication occurred. The total time involved was four hours . . . Immediately after the exchange, she was able to respond to simple verbal commands" [76]. The clinicians added, "this may be first reported case of full-blown DIC in malaria successfully treated by exchange transfusion." Unfortunately, donor-blood variables were not reported for this rapid (4-hour) "time-to-speak" rescue.

At Strong Memorial Hospital in Rochester, NY, clinicians re-admitted a 65-year-old woman for mental confusion following heart surgery [77]. A diagnosis of transfusion-acquired *Pf* malaria was made from a peripheral blood smear. Because of worsening encephalopathy despite antimalarial drugs, an exchange transfusion was performed. Clinical improvement occurred promptly during the exchange. Using a continuous-flow cell separator, a total of 5,000 mL of the patient's blood was removed over three hours ten minutes and replaced with whole blood. During the exchange she became alert and fully oriented. The clinicians noted they felt exchange was prudent for (1) life-threatening *Pf* infection malaria, such as encephalopathy and blackwater fever; (2) cases with levels of parasitemia greater than 100,000/cu mm; (3) elderly patients; and (4) possibly for any patient with severe *Pf* malaria whose disease is progressing despite therapy [77]. This CM patient also quickly recovered during the exchange, but, again, no donor-blood information was recorded.

A 48-year-old physician contracted *Pf* malaria while working in eastern Thailand. In the hospital, "he had a peak parasitemia of 72% RBCs infected, associated with CNS dysfunction [78]. As an adjunct to chemotherapy, a double-volume whole-blood exchange transfusion was performed on the first hospital day, dropping the parasitemia to less than 1% within 32 hours. The patient's clinical condition improved, with a prompt reversal of CNS, hepatic, and renal complications. These results, combined with those in previously reported cases, suggest that exchange transfusion should be considered more generally as a life-saving procedure . . . His confusion and somnolence continued unabated until the exchange transfusion was nearly complete, after which the patient became more alert." [78] Here, again, coma-recovery occurred during the exchange, but no donor RBC variables were reported.

In 1987, "a 38 year old white woman was admitted with a reported 80% falciparum parasitaemia acquired in Kenya." [79] The exchange transfusion in our patient was begun three hours after admission and completed two hours later. An improvement in the level of consciousness at the end of the exchange transfusion was noted in our patient and four others described. An exchange transfusion was begun in an attempt to save the patient's life. The patient became noticeably more alert during the procedure." [79]

In 1990, in Scotland, a young woman with CM was rescued using whole blood exchange [18]. Regarding her coma-recovery time, "Four hours after commencing the exchange, the patient's conscious level rose and by its completion she was fully alert and orientated with no residual focal neurological deficit . . . Our patient improved dramatically following exchange transfusion." [18] Unfortunately, donor RBC variables were not reported[18].

In 1994, in SSA clinicians used exchange to rescue a patient with severe Pf infection noting, "The patient's conscious level improved rapidly during the procedure . . . exchange transfusion is an effective and haemodynamically well-tolerated method of reducing parasitaemia in severe falciparum malaria [19]. Exchange transfusion is associated with an increase in oxygen delivery and oxygen consumption, which may partly explain the rapid improvements in conscious level which can occur in patients with cerebral malaria during exchange transfusion." [19] Unfortunately, donor and patient RBC variables were not reported.

Regarding our review of ligand-receptor data, we identified some of the ligands and PS-receptors (on cells or on vascular extracellular matrix) that are likely to be relevant for T-REX as a "decoy ligand" competitive inhibition therapy. Important "pathogenic ligands" include hemin (released by phagocytosed iRBCs), PfEMP1 (inserted onto iRBCs by the *Pf* parasites inside iRBCs), and the PS expressed by iRBCs, the EVs secreted by iRBCs (iRBC EVs), and by the platelets and ECs that are modified during *Pf* infection: See Table 1. Some key patient-cell receptors identified include CD36, BAI-1, TIM-1, TIM-4, integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, and TSP: See Tables 2–4.

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Table 2. By Competing with Hemin, iRBCs, and iRBC EVs for Binding to these PS-receptors, Donor uRBC PS EVs may Reverse Adhesion and Inflammation.

PS-receptor	Cytoadhesion	Inflammation	References
CD36 (on platelets, some immune cells and ECs)	PS on iRBCs can promote iRBC-platelet-EC "bridge binding" and immune-cell, platelet, and RBC adhesion-related microvascular obstruction.	Secreted hemin and PS on iRBCs and iRBC EVs can bind to CD36 on immune cells and promote inflammation.	[10,13,38,40,42,92–94]
BAI-1 (on some ECs and brain cells)	PS on iRBCs may promote microvascular obstruction by binding to BAI-1 on cerebral ECs.	PS-expressing iRBC EVs may increase inflammation by binding to BAI-1 on brain immune cells.	[6]
TIM-1 and TIM-4 (expressed by some immune cells)	PS on iRBCs bind TIM-1 and TIM-4 on immune cells.	PS-expressing iRBC EVs bind TIM-1 and TIM-4 on immune cells.	[6,7,95]
TSP (on ECs and extracellular matrix)	PS on iRBCs binds to TSP on ECs and extracellular matrix	PS-expressing iRBC EVs bind to TSP on ECs and extracellular matrix	[96,97]
Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ (on ECs and extracellular matrix)	PS on iRBCs can promote obstruction by binding integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ on extracellular matrix and on some ECs.	PS on iRBC EVs can promote inflammation by binding integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ on extracellular matrix and on some ECs.	[38,98–101]
Unspecified receptor (on brain astrocytes and microglia)		iRBC EVs promote inflammation by interacting with brain cells.	[102,103]

Table 3. Some Pathogenic PfEMP1 Proteins on iRBCs Bind to these PS-receptors (but must Compete for Binding with PS-expressing Donor uRBC EVs).

PS-receptor	Cytoadhesion and/or Inflammation	References
CD36	Cytoadhesion: Most PfEMP1 proteins on iRBCs bind CD36.	[39,40,42]

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(on platelets and on some ECs)	Platelet CD36 enables iRBC-platelet-EC "bridge binding" with cerebral ECs that do not express CD36.	
Integrin $\alpha v\beta 3$ (on some immune cells and on angiogenic ECs	iRBC PfEMP1-integrin binding can promote both cytoadhesion (microvascular obstruction) and inflammation.	[41,101,104]
TSP (on ECs and Extracellular Matrix)	iRBC PfEMP1-TSP binding promotes microvascular sequestration (obstruction).	[105]

Table 4. PS-expressing iRBC EVs Insert *Pf*-related Factors into Cells via these Receptors (but Compete for Binding with PS-expressing Donor uRBC EVs).

PS-receptor (and Target Cells)	**Adverse Effects of the *Pf* Factors Inserted into Patient Cells by iRBC EVs (PfEMP1, miRNAs, etc.)**	**References**
CD36 (on some ECs and immune cells; possibly on brain microglia and astrocytes)	PfEMP1 proteins incorporated into ECs and immune cells can promote inflammation	[6,13,93,94]
Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ (on some ECs and immune cells; possibly on brain microglia and astrocytes)	PfEMP1 proteins incorporated into ECs and immune cells can promote inflammation	[6,41,101,104]
TSP (on ECs and possibly on brain microglia)	PfEMP1 proteins incorporated into ECs and immune cells can promote inflammation	[6,105]
TIM-1 and TIM-4 (on immune cells and possibly on brain microglia)	Immune-cell activation	[6,7,95]
BAI-1 (on neurons and cerebral ECs)		[6]

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Relevant to the confusion surrounding the role of the CD36 cell receptor in CM, our review identified studies (and expert reviews) indicating that although cerebral ECs may express little CD36, iRBC-platelet-EC "bridge binding" within cerebral microvessels means the CD36 expressed by platelets can cause CD36-dependent microvascular obstruction in CM [9]. A study of children with malaria showed all *Pf*-parasite isolates but 1 (70 of 71) bound to CD36 [51]. Regarding PS-CD36 binding, a study found that (1) PS-expressing iRBCs bind CD36 and (2) PS liposomes compete for, and reduce this binding [13]. Of note, synthetic PS liposomes are used in studies to mimic EVs (Jayasinghe) Studies show hemin (released by phagocytosed iRBCs) binds to CD36 and promotes CM [10]. *Pf*-malaria experts concluded that the CD36 receptor is "necessary" for *Pf*-disease progression [42].

Our review found several studies (and expert reviews) that explain how the following are pathogenic: iRBCs (that express PfEMP1 and PS) and the PS-expressing iRBC EVs, platelet EVs, and EC EVs [2,35,43,80,81]. iRBCs bind both uRBCs (rosetting) and CD36-expressing platelets – pathogenic binding that obstructs microvascular blood flow in CM – while PS-expressing iRBC EVs carry pathogenic PfEMP1 internally as cargo and the binding of iRBC EVs to cells can trigger inflammation and increase the expression of adhesion receptors in the targeted cells [6,82].

The findings of a study by Wang and colleagues showed that EVs secreted by uRBCs that had been exposed to iRBCs targeted iRBCs [82]. They suggested miRNAs (that are carried as cargo inside RBC EVs) be evaluated as therapeutic agents.

Regarding data comparing the competitive inhibition potential of RBC EVs and PS liposomes, scientists noted that, in the context of virus binding (which should closely mimic the binding of iRBC EVs), "there was no significant difference in the inhibition observed between PS liposomes and RBC EVs, indicating that the antiviral effects mediated by RBC EVs are mostly dependent on PS exposure. We also further determined the relative efficacy of equal particle concentrations of PS liposomes or EVs in neutralizing viral infection. Our data revealed that equivalent doses of EVs or PS liposomes resulted in comparable levels of viral neutralization, indicating that EVs have sufficient PS to efficiently compete with viruses for PS receptor binding." [14] Given the similarity between PS liposomes and RBC EVs, it is not surprising their data showed "the presence of either PS liposomes or RBCEVs significantly inhibited viral adsorption onto cells."

6. Discussion

The absence of RBC-variable data when exchange has been used as a rescue adjunct is disappointing given that the dramatic, rapid rescues of near-death patients remains unexplained. Also unfortunate, mortality studies of exchange never considered RBC variables as confounders despite data showing that some RBCs provide 10-fold protection, and, in general for all cell therapies, the cells matter.

Given that T-REX immediately delivers donor uRBC EVs (and then the donor uRBCs continue to secrete EVs post-exchange), clinicians should be encouraged that IV-administered EVs can selectively target, and adhere to, distant inflamed tissues within 15 minutes [75]. Regarding safety, exchange-for-malaria has been used for 50 years. Regarding potential therapeutic efficacy, studies have found that persons born with *Pf*-resistant RBCs enjoy as much as 10-fold protection against onset of severe disease [84–86].

This review found that, since 1892, front-line clinicians in malaria-endemic SSA have helped advance transfusion medicine. Past innovations include thoughtful selection of donor blood for the rescue of a dying CM patient in [54], evaluation of the use of two *Pf*-resistant RBC genetic variants for exchange transfusion [55], and use of a combination of RBC exchange and plasma exchange to treat CM [61]. Now, in 2024, clinicians in SSA have the opportunity to assess T-REX options and collect unprecedented amounts of new data regarding the clinical impact of special *Pf*-resistant RBCs – and the nano-sized EVs they secrete – for the treatment of CM. Given that interest in EV therapeutics is currently intense and concerns about the emergence of drug- and vaccine-resistant *Pf* parasites are substantial, support for clinicians interested in evaluating T-REX might be surprisingly strong, including support from EV researchers as well as the global-

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health community. Furthermore, some community residents might agree to become "contactable blood donors" if they are properly informed they carry special *Pf*-combating RBC genetic variants that could be life-saving for the young children in their communities who develop CM. The blood from the subset of contactable donors can be screened for pathogens and, thereby, reduce concerns about transfusion-transmitted infection.

Regarding molecular mechanisms, EV, virus, and liposome research findings suggest competition for PS-receptor binding by PS-expressing RBC EVs may explain the rapid coma-recovery enjoyed by some exchange-treated patients (like the type-O T-REX patient rescued in 2017 [20]. Post-exchange "rebound parasitemia" surely warrants investigation to determine if it is linked to sequestration-reversal and coma-recovery [44]. Post-exchange sequential measurements of peripheral *Pf* parasitemia and plasma PfHRP2 levels should be able to (1) determine if a T-REX option can reverse iRBC sequestration and (2) help explain post-exchange "rebound parasitemia" – especially when combined with sequential microscopic assessments of the proportion of mature, late-stage *Pf* parasites in the peripheral blood [63,64].

7. Conclusions

As new anti-*Pf* vaccine strategies are likely to substantially reduce the incidence of *Pf* infections, front-line clinicians in SSA can now focus on evaluating non-drug, "patient-altering" adjuncts for the rescue of the smaller, more manageable number of children who will develop CM. After reviewing clinician-driven innovations, case-reports, and EV data, remarkable questions emerge: Do some residents of SSA carry special *Pf*-combating RBCs that could, via exchange, rescue young children dying with CM? Is it feasible and prudent to answer that question – now? If residents know they carry special, "evolution-engineered" RBCs that could save comatose children with life-threatening *Pf* malaria, would they be willing to become contactable donors? Do we now have an opportunity to increase our understanding of the role of RBC EVs in *Pf* malaria and promote EV therapeutics while trying to reduce CM mortality? Does post-exchange "rebound parasitemia" reflect sequestration-reversal and is this increase in parasitemia linked to coma-recovery ("time-to-speak")?

We feel data suggest T-REX is a biologically plausible way to optimize exchange-for-malaria. Current use of "standard issue" units of RBCs that may contain the same *Pf*-promoting RBCs that facilitated the patient's onset of CM in the first place seems misguided. That is, donor RBCs delivered via "conventional exchange" might be linked to higher onset of severe *Pf* disease and death, such as group-A RBCs (if they also contain normal hemoglobin and have normal enzyme levels). Fortunately, current laboratory-medicine techniques can clarify how donor RBC EVs delivered via T-REX impact *Pf*-disease progression. Also reassuring for clinicians, modern continuous flow apheresis machines allow clinicians to thoughtfully set the post-exchange hematocrit to the value they feel is "optimal" for the patient. Front-line clinicians are likely to be enthusiastically supported by transfusion- and laboratory-medicine physicians familiar with CM as well a EV researchers who will surely appreciate plasma samples for EV studies. To our knowledge, no other clinical strategy can so immediately and so safely provide large amounts of data regarding how readily available human-donor EVs affect a human disease. And so, this opportunity seems historically unique given that exchange-for-malaria – considered to to be a life-saving EV strategy by clinicians worldwide – has already been used for 50 years.

The findings reported here mean clinicians can confidently and justifiably ask transfusion- and laboratory-medicine specialists for help using T-REX to rescue comatose, *Pf*-infected children. The current explosion of interest in EV therapies means clinicians should also reach out to EV researchers who know how to solicit funding and explain the importance of bio-banking plasma samples.

Clinicians who decide to use T-REX options to rescue CM patients should publish case-reports – regardless of the clinical outcome. Future integration of data can improve clinical care and address our substantial knowledge gaps. Blood-bankers can explain what *Pf*-resistant RBCs are available in their hospitals or are accessible from "contactable donors" who carry *Pf*-resistant

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RBCs. Given that the prevalence of specific "evolution-engineered" *Pf*-resistant RBCs varies markedly in the different *Pf*-endemic regions of SSA means T-REX studies can provide insight into the RBC EVs secreted by several different RBC genetic variants.

With the help of transfusion- and laboratory-medicine physicians, clinicians should publish T-REX CM case-reports that include (1) exchange details (manual vs. automated continuous-flow apheresis, number of units, the post-exchange hematocrit setting, etc.); (2) donor RBC variables (ABO blood group, hemoglobin type, RBC enzyme deficiency, membrane defects, etc.); (3) the "time-to-speak" coma-recovery time; (4) measurements relevant to sequestration-reversal whenever post-exchange "rebound parasitemia" occurs (sequential post-exchange parasitemia and total *Pf* biomass values) and, ideally, sequential microscopic assessments of the distribution of parasite stages; and, of course, (5) demographic and clinical-course data. Clinicians can expect strong support from the research community given the intense interest in the EV "decoy ligand" competitive-inhibition therapies being promoted by cancer and COVID-19 researchers.

In summary, we already know (1) yet-to-be-identified subsets of exchange transfusions trigger rapid coma-recovery among near-death CM patients and (2) rapid *Pf*-killing by potent drugs does not quickly reverse cytoadhesion. Evolutionary biologists urge developing non-drug adjuncts to reduce the emergence and spread of drug- and vaccine-resistant pathogens. Our attempt here to explain ("reverse engineer") the mechanism of rapid coma-recovery provides new data and conceptual frameworks that suggest fast-acting donor RBC EVs – serving as therapeutic "decoy ligands" – may disrupt pathogenic binding by competing with iRBCs, iRBC EVs, and hemin and, thereby, quickly reverse sequestration and inflammation. It now seems prudent to determine if T-REX can optimize current use of conventional exchange-for-malaria. Data suggest evaluating T-REX as a donor RBC EV "decoy-ligand therapy" is a strategy clinicians should assume will reduce CM mortality – until proven proven otherwise.

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