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Fenozyme Protects the Integrity of the Blood−Brain Barrier against Experimental Cerebral Malaria

Shuai Zhao,[†,‡] Hongxia Duan,[†] Yili Yang,[§] Xiyun Yan,*[,†,‡,∥] and Kelong Fan*[,†,∥]

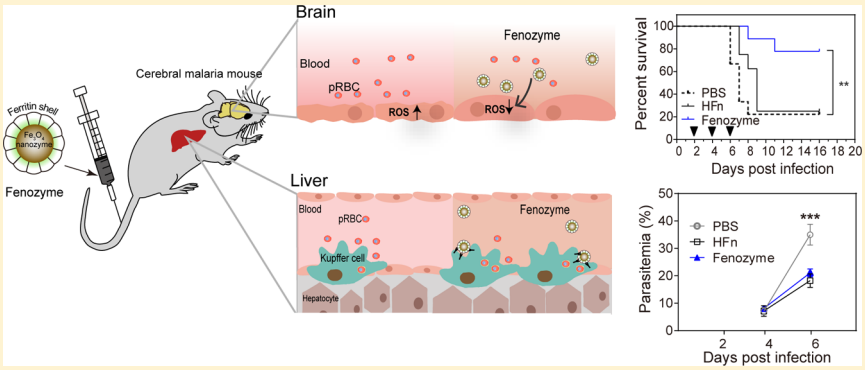
[†]CAS Engineering Laboratory for Nanozyme, Key Laboratory of Protein and Peptide Pharmaceutical, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

[‡]College of Life Sciences, University of Chinese Academy of Sciences, Beijing 100049, China

[§]Suzhou Institute of Systems Medicine, Center for Systems Medicine, Chinese Academy of Medicine Sciences, Suzhou 215133, China

[∥]Joint Laboratory of Nanozymes in Zhengzhou University, Academy of Medical Sciences, Zhengzhou University, 40 Daxue Road, Zhengzhou 450052, China

Supporting Information



ABSTRACT: Cerebral malaria is a lethal complication of malaria infection characterized by central nervous system dysfunction and is often not effectively treated by antimalarial combination therapies. It has been shown that the sequestration of the parasite-infected red blood cells that interact with cerebral vessel endothelial cells and the damage of the blood−brain barrier (BBB) play critical roles in the pathogenesis. In this study, we developed a ferritin nanozyme (Fenozyme) composed of recombinant human ferritin (HFn) protein shells that specifically target BBB endothelial cells (BBB ECs) and the inner Fe_3O_4 nanozyme core that exhibits reactive oxygen species-scavenging catalase-like activity. In the experimental cerebral malaria (ECM) mouse model, administration of the Fenozyme, but not HFn, markedly ameliorated the damage of BBB induced by the parasite and improved the survival rate of infected mice significantly. Further investigations found that Fenozyme, as well as HFn, was able to polarize the macrophages in the liver to the M1 phenotype and promote the elimination of malaria in the blood. Thus, the catalase-like activity of the Fenozyme is required for its therapeutic effect in the mouse model. Moreover, the Fenozyme significantly alleviated the brain inflammation and memory impairment in ECM mice that had been treated with artemether, indicating that combining Fenozyme with an antimalarial drug is a novel strategy for the treatment of cerebral malaria.

KEYWORDS: *Cerebral malaria, ferritin nanozyme, BBB protection, macrophage polarization, reactive oxygen species*

Malaria is a mosquito-borne infectious disease caused by protozoan *Plasmodium*, with more than 200 million cases each year and over 400 thousand deaths world wide[1]. Among the 5 species of *Plasmodium* that infect humans, *P. falciparum* often causes severe symptoms, including the life-threatening neurological complication, cerebral malaria. Despite significant progress in the development of antimalarial drugs and the artemisinin-based combination therapies, cerebral malaria still has a mortality rate of up to 30%, especially in children. Moreover, 10−24% of children patients surviving from cerebral malaria exhibited neurological sequelae and memory impairment,[2,3] underlining the importance of finding more effective and specific treatments[4].

Intense efforts have been made to understand the pathogenesis of cerebral malaria. It has been proposed that the sequestration of infected red blood cells in the brain is responsible for the development of cerebral malaria[5]. These infected cells express *P. falciparum* erythrocyte membrane

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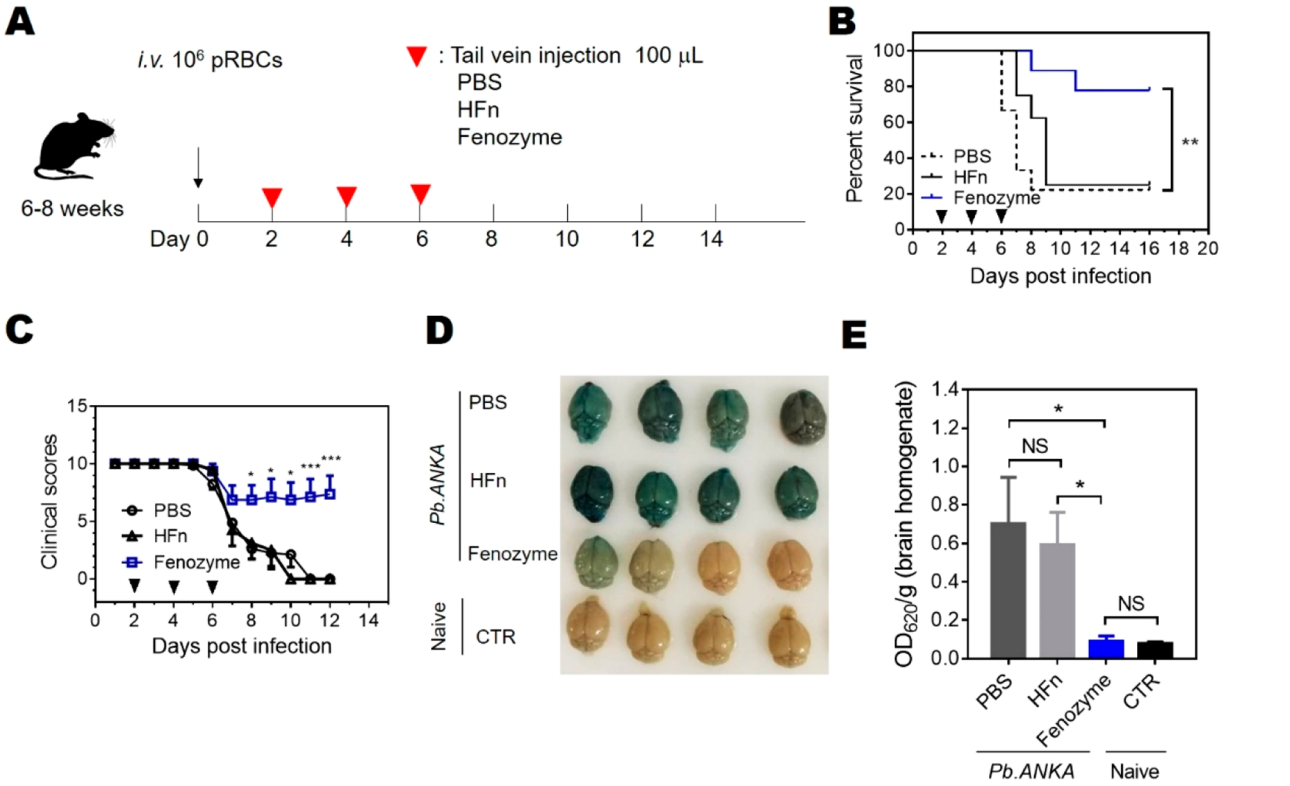

Figure 1. Fenozyme protected mice against experimental cerebral malaria. (A) Protocols for the construction and treatment of cerebral malaria models with C57BL/6J mice. Intravenous (i.v.) injection of pRBCs into mice to induce cerebral malaria and treatment of mice (i.v.) with PBS, HFn, or Fenozyme at days 2, 4, and 6. Kaplan−Meier analysis of survival (B) (log-rank Mantel−Cox test) and activity scores (gripping plus righting reflex) (C) of ECM mice treated with PBS (n = 9), HFn (n = 8), and Fenozyme (n = 9). (D) Representative Evans Blue-stained brains from PBS-, HFn-, or Fenozyme-treated ECM mice at 6 day post infection (d.p.i.) with *Pb.ANKA*. (E) Quantitative analysis of Evans Blue in PBS-, HFn-, or Fenozyme-treated ECM mice brains (n = 5). The mice in CTR groups were not infected with *Pb. ANKA*. All data were from three independent experiments and were presented as mean ± s.e.m. (*$P \leq 0.05$, ***$P \leq 0.001$, unpaired Student's t-test).

protein-1 (PfEMP1) and can engage endothelial cells membrane proteins such as the intercellular adhesion molecule 1 (ICAM-1) and endothelial protein C receptor.[5] Together with studies using the mouse model of experimental cerebral malaria, it has become evident that damage of microvessel endothelial cells and disruption of the blood−brain barrier play a critical role in the development of encephalopathy.[5−10] Interestingly, it has been showed that free heme released from ruptured parasite-infected red blood cells might produce excess amounts of reactive oxygen species (ROS) to impair endothelial cells and damage BBB.[11] Upregulation of heme oxygenase-1 and exposure to carbon monoxide, which prevent hemoglobin oxidation and the generation of free heme, suppressed the neuro-inflammation, significantly improved survival rate, and reduced cognitive dysfunction in mice with experimental cerebral malaria.[12] Thus, ROS might be a critical mediator that harms BBB during the development of cerebral malaria.

We found previously that human ferritin (HFn) can bind to HFn receptors (HFRs) expressed on BBB ECs and act as a versatile nanocarrier for disease therapy applications.[13,14] Nanomaterials with enzyme-like activities (nanozyme) have been demonstrated to be able to regulate the ROS levels in living cells.[15−17] It was also reported that the Fe_3O_4-containing nanoparticle might function as a nanozyme and exhibit catalase-like activity to suppress the ROS level *in vivo* efficiently.[18] In the present study, use was made of a Fenozyme that was composed of a HFn protein shell and Fe_3O_4 nanozyme core. When administrated to mice with experimental cerebral malaria, the Fenozyme significantly increased the survival rate by protecting the BBB ECs from the ROS damage and decreasing parasitemia via polarizing the macrophage to the M1 phenotype. Furthermore, concurrent administration of Fenozyme and antimalaria artemether alleviated encephalopathy, and memory impairment in mice survived ECM. These results demonstrated the importance of ROS in the development of cerebral malaria and indicated that combining Fenozyme with the antimalarial drug is a novel treatment strategy.

Results. *Fenozyme Protects Mice against Experimental Cerebral Malaria.* To investigate whether the reduction of ROS affects the development of cerebral malaria, we biomimetically synthesized the artificial enzyme that has the human H-ferritin nanocage containing Fe_3O_4 inside, which was named Fenozyme (Figure S1A). It existed as a nanoparticle with an intact HFn shell as assessed by a transmission electron microscope (Figure S1B,C) and exhibited catalase-like or peroxidase-like activity under physiological or acidic conditions, respectively (Figure S1D).[19] Moreover, as shown in Figure S2, Fenozymes showed sufficient stability in serum for *in vivo* applications.

We then assess the effects of the Fenozyme on mice that were given *P. berghei* ANKA (*Pb.ANKA*) parasitized red blood cells (pRBCs), an established model for experimental cerebral malaria (ECM) (Figure 1A). As shown in Figure 1B, 80% of the infected mice treated with PBS or HFn nanoparticles died

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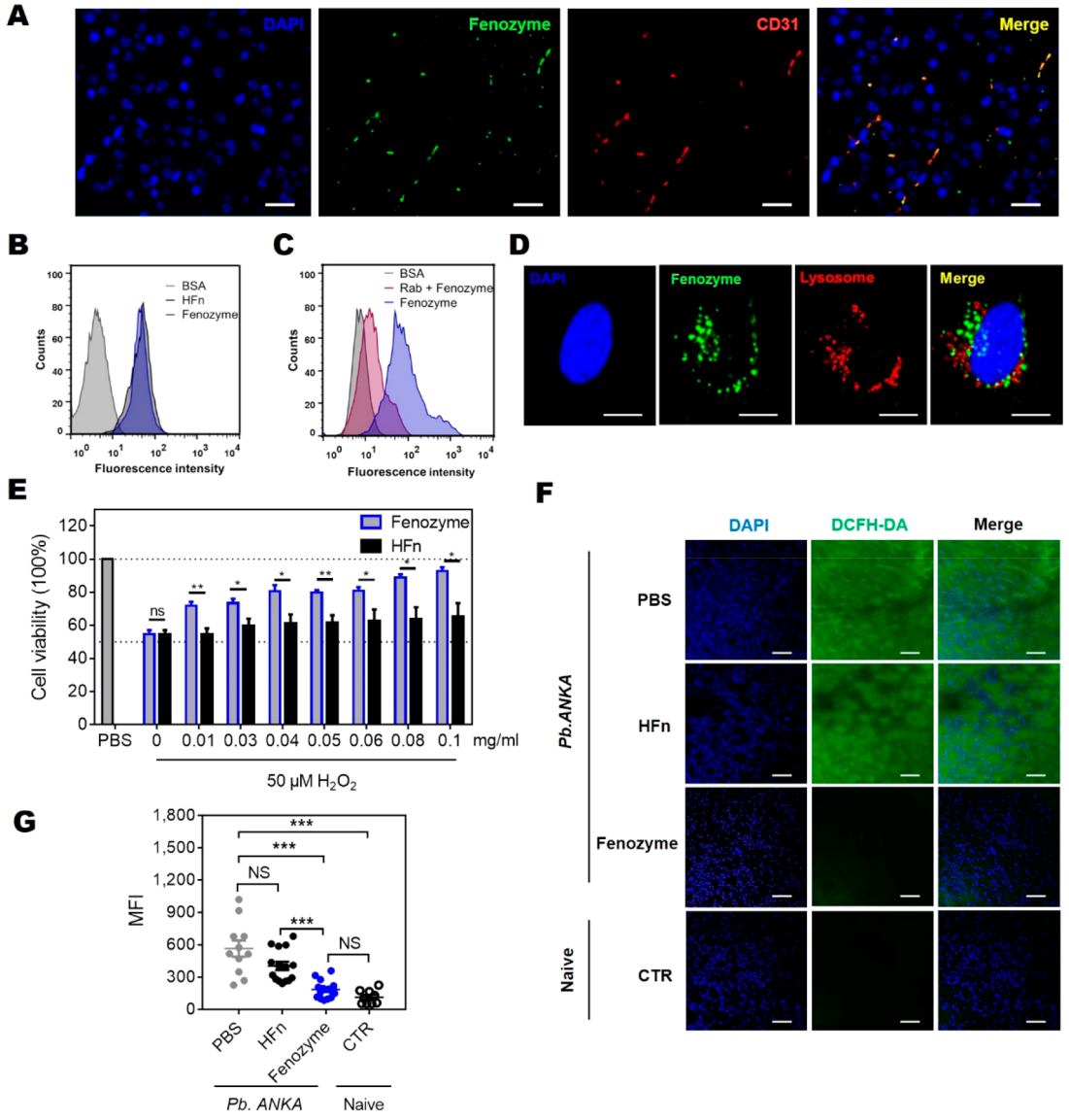

Figure 2. Fenozyme protected brain endothelial cells by scavenging ROS. (A) Brain sections from C57BL/6J were costained with FITC-Fenozyme (green) and CD31antibody (red). The scale bar represents 20 μm. (B) Flow cytometry analysis of the binding of Fenozyme and HFn to bEnd.3 cells. (C) The anti-HFR antibody blocked almost completely the binding of Fenozyme to bEnd.3 cells. (D) Confocal image of the subcellular localization of Fenozyme (green) in a bEnd.3 cell. Lysosomes were stained with the LAMP1 marker in red. The nuclei of cells were DAPI-stained (blue). The scale bar represents 10 μm. (E) The effects of indicated amounts of Fenozyme and HFn on bEnd.3 cells pretreated with 50 μM of H_2O_2. Cell viability was assessed with CCK-8. The absorption in bEnd.3 cells not exposed to H_2O_2 was regarded as 100%. (F) The ROS levels of mice brain sections, assessed by the ROS probe DCFH-DA (green). The nuclei of cells were DAPI-stained (blue). The scale bar represents 20 μm. (G) Quantification analysis of the mean fluorescence intensity (MFI) of the ROS probe in brain tissues from ECM and control mice. Data are presented as mean ± s.e.m. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, unpaired Student's *t*-test.).

after 8−10 days, whereas only 20% of these mice that received 3.75 mg/kg of Fenozyme died by day 16. It has been shown that mice with ECM have decreased activity (righting and gripping) scores, presumably due to associated encephalopathy.[20] Administration of the same amount of Fenozyme also significantly improved the activity scores in mice that survived the infection (Figure 1C). Note that the the effects of Fenozyme on both the survival and activities of ECM mice were not dose-dependent; 1.0 mg/kg and 6.5 mg/kg Fenozyme had slightly protective and improving roles (p = 0.0495 and p = 0.055, respectively), and 3.75 mg/kg of Fenozyme was the most effective (p = 0.007) (Figure S3A,B). Based on these results, all subsequent ECM treatment experiments were performed using 3.75 mg/kg Fenozyme.

It has generally been recognized that the death and abnormal activities of mice with ECM are the results of the disruption of BBB. Use was made of Evans Blue that bound to albumin in the circulation to examine the integrity of BBB. While the brains from mice with ECM were stained heavily by Evans Blue, treatment of the infected mice with Fenozyme, but not HFn proteins, decreased the brain staining significantly, to almost identical to these from uninfected mice (Figure 1D,E). Thus, Fenozymes protected BBB from damages induced by ECM, and its enzymatic activity is required for the protection.

Fenozyme Protected Brain Endothelial Cells by Scavenging ROS in the ECM Mouse Brain. It has been found recently in our laboratory that the HFn protein cage can interact with endothelial cells of BBB via binding to HFRs.[21] To understand

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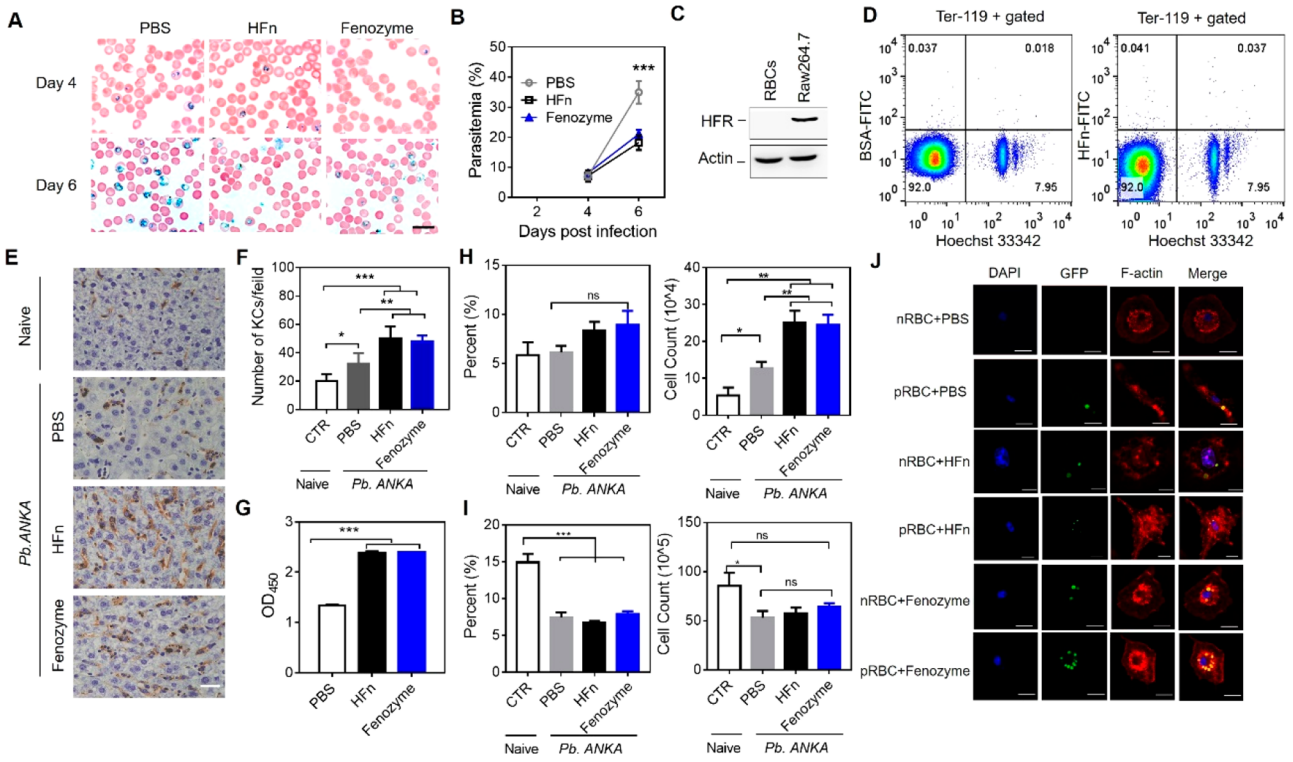

Figure 3. Fenozyme decreased parasitemia in ECM mice by enhancing the proliferation and phagocytosis of macrophages. (A) Giemsa staining of blood smears of mice infected with *Pb.ANKA* on day 4 and 6 d.p.i.. The scale bar represents 10 μm. (B) Time courses of parasitemia were assessed in Fenozyme-, HFn protein-, and PBS-treated ECM mice (each group, n = 5). (C) Western blot analysis of the HFR expression on RBCs and Raw264.7 cells. (D) Flow cytometry analysis of the binding ability of FITC-HFn with pRBCs. The Hoechst 33342 is a positive marker for pRBCs. (E) Immunohistochemistry (IHC) analysis of the number of Kupffer cells in liver sections from ECM mice at 6 d.p.i. The Kupffer cells were identified by the F4/80 antibody. Naïve healthy mice were used as a control. The scale bar represents 50 μm. (F) Quantification analysis of the number of Kupffer cells in the liver of PBS-, HFn protein-, and Fenozyme-treated ECM and control mice. (G) Proliferation of Raw264.7 cells in the presence of 0.05 mg/mL of HFn proteins or Fenozyme. (H) Percentages and counts of macrophages in the liver of infected mice treated with PBS (n = 6), HFn (n = 6), Fenozyme (n = 5), and naïve mice (n = 4) by flow cytometry analysis. (I) Percentages and counts of macrophages in the spleen of infected mice treated with PBS (n = 6), HFn (n = 6), Fenozyme (n = 5), and naïve mice (n = 4) by flow cytometry analysis. (J) Phagocytosis of HFn proteins and Fenozyme by bone marrow-derived macrophages. The nRBCs were red blood cells from uninfected GFP transgenic mice; pRBCs were red blood cells from GFP transgenic mice infected with *Pb.ANKA*. F-actin was stained by Phalloidin Red 594 (red). All data were represented of three independent experiments and were expressed as mean ± s.e.m. (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ns, no significance, unpaired Student's t-test.).

the mechanisms of protection BBB by Fenozyme in ECM mice, we examined mouse brain paraffin sections with FITC-labeled Fenozyme. As shown in Figure 2A, the Fenozyme colocalized with CD31, an adhesion molecule normally found on endothelial cells, indicating that it may act directly on BBB endothelial cells. Use was then made of the transformed mouse brain endothelial cell bEnd.3 that expressed HFR (Figure S4A). As shown in Figure 2B,C, both HFn and Fenozyme bound to the cells, and the binding of Fenozyme to bEnd.3 cells was largely blocked by an anti-HFR antibody. Moreover, the accumulation of Fenozyme in mouse brains exhibited a time-dependent manner (Figure S5A,B). Light-sheet fluorescence imaging of IRdye800-labeled Fenozyme in the whole brain section demonstrated that Fenozyme can specifically bind to the macro and micro blood vessels in the brain (Figure S5C). Taken together, the HFn shell bound HFR and directed the Fenozyme to endothelial cells of BBB in the mouse brain.

Since Fenozyme exhibits catalase-like or peroxidase-like activity depending on the pH of its environment, we examined its intracellular location after adding to cultured bEnd.3 cells. As shown in Figure 2D, the Fenozyme was quickly localized to the cytoplasmic region, not overlapped with lysosomes. Transmission electron microscope images indicated that the Fenozymes were inside the endosomes (Figure S4B), presumably through HFR-mediated endocytosis, whereas the Fe_3O_4 nanozyme without an HFn protein shell mostly localized in the lysosome. Thus, the Fenozyme apparently functions as a catalase in the endothelial cells.[22] Therefore, we added hydrogen peroxide (H_2O_2) to cultured bEnd.3 cells to mimic the oxidative stress environment. While 50 μM of H_2O_2 killed approximately 50% of bEnd.3 cells (Figure S4C), the addition of Fenozyme, but not HFn, markedly protected the cells from the damage induced by H_2O_2 (Figure 2E). The protection by Fenozyme was concentration-dependent, and 0.1 mg/mL of Fenozyme almost fully protected bEnd.3 cells from H_2O_2. Taken together, these data indicated that Fenozyme is able to use catalase-like activity to protect endothelial cells from ROS damage.

To further explore the action of Fenozyme *in vivo*, brain tissues were collected from ECM mice 6 days post infection (d.p.i.), and the ROS probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was utilized to determine the ROS level in the brain sections. Compared with that of the control, the brain section from ECM mice had a high level of ROS in the brain (MFI, 564.8 ± 75.1 vs 113.9 ± 21.5, $p < 0.001$) (Figure 2F), whereas these from ECM mice treated with Fenozyme had

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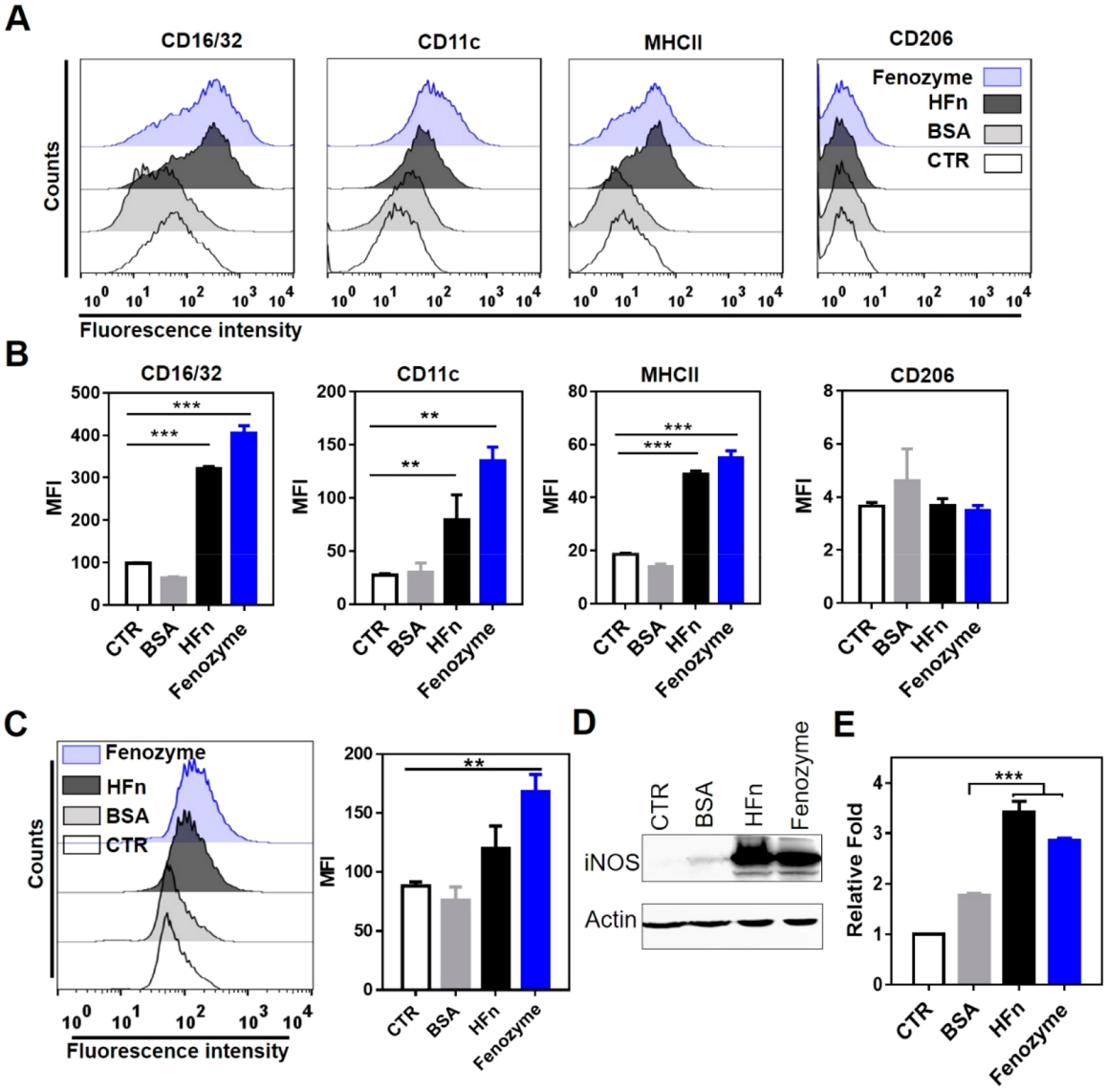

Figure 4. Fenozyme induced macrophages to M1 polarization. (A) Flow cytometry analysis of the expression of M1 and M2 markers on Raw264.7 cells treated with HFn proteins, Fenozyme, and BSA. Representative histograms are expressions of M1 markers (CD16/32, CD11c, MHCII) and M2 markers (CD206). (B) Quantification analysis of mean fluorescence density of panel A) (C) The levels of ROS in Raw264.7 cells treated with HFn proteins, Fenozyme, and BSA. The left represents histograms, and the right is the quantification analysis. (D) Western blot analysis of the expression of iNOS in Raw264.7 cells treated with HFn proteins, Fenozyme, and BSA. (E) iNOS activities in Raw264.7 cells treated with HFn proteins, Fenozyme, and BSA. All data represent one of three independent experiments and are expressed as mean ± s.e.m. (**$P \leq 0.01$, ***$P \leq 0.001$, unpaired Student's *t*-test).

little ROS. Quantification analysis of the fluorescence intensity revealed that the ROS level in the brains of the Fenozyme-treated group was similar to that of the healthy control (Figure 2G). However, brain tissues from ECM mice treated with HFn proteins exhibited similarly high levels of ROS to that of the PBS-treated group (Figure 2G). These results indicated that Fenozyme protects the integrity of BBB by scavenging ROS.

Fenozyme Decreased Parasitemia by Enhancing Proliferation and Phagocytosis of Macrophages. To fully understand the effects of Fenozyme on ECM mice, we examined the levels of *Pb. ANKA* in their blood. Parasitemia was detected 4 days after mice were infected with pRBCs. Intriguingly, administration of Fenozyme or HFn protein reduced the percentages of parasite-infected red blood cells from 34.7 ± 1.90% at day 6 to 21.1 ± 0.72% ($p = 0.0005$) and 18.2 ± 1.10% ($p = 0.0001$), respectively (Figure 3A,B). The decreased parasitemia may result from the action of HFn on red blood cells to suppress and/or kill parasites. Alternatively, HFn may act on cells of the phagocytic system, such as macrophages in the liver or spleen, which are able to eliminate pRBCs.[23,24] As HFn receptors were expressed on macrophages, but neither on RBCs by Western blot (Figure 3C) nor on pRBCs by flow cytometry (Figure 3D), we further examined the effects of HFn and Fenozyme on macrophages.

It has been reported that 80−90% of tissue macrophages in mice are settled in the liver, known as Kupffer cells,[25] and macrophages in the liver but not spleen are mostly responsible for clearing stressed erythrocytes and recycling iron.[26] We first examined Kupffer cells in liver tissues from ECM mice by immunohistochemical (IHC) staining. As shown in Figure 3E,F, livers from Fenozyme- and HFn-treated ECM mice had significantly increased numbers of macrophages compared with

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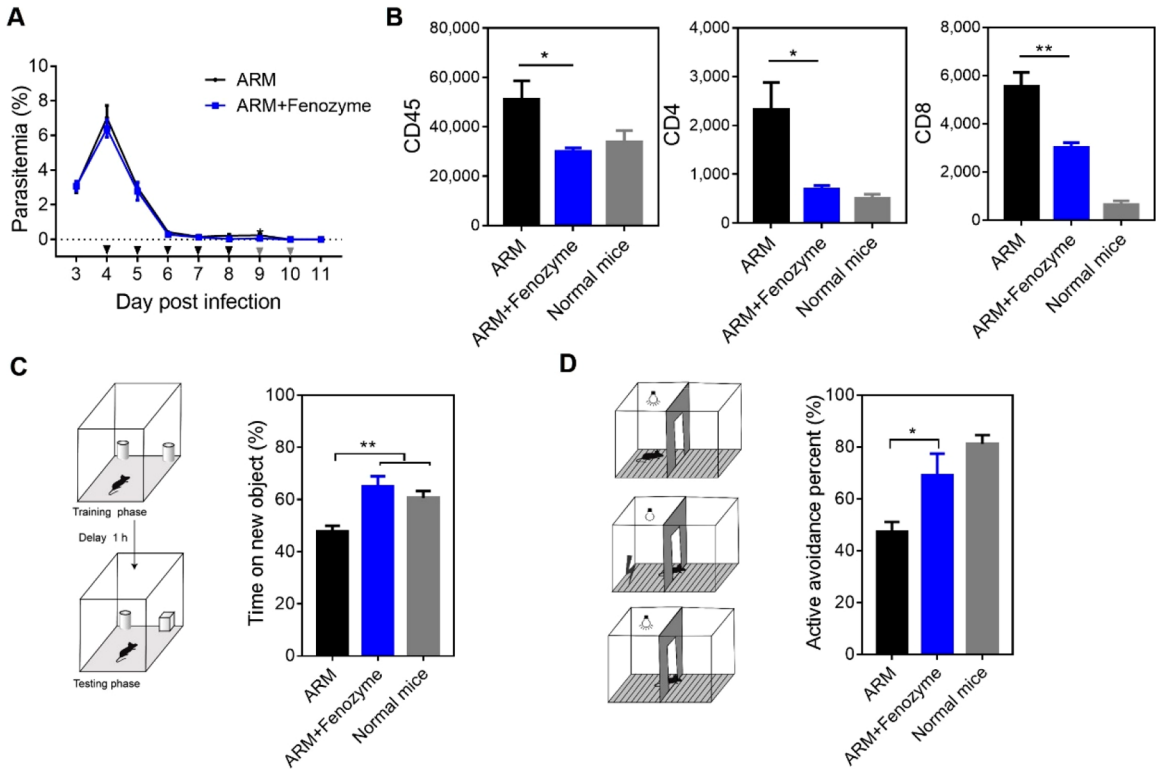

Figure 5. Fenozyme mitigated cognitive impair of artemether-treated ECM mice. (A) Time courses of parasitemia were assessed in ARM and ARM + Fenozyme-treated ECM mice (n = 8). (B) The numbers of infiltrated immune cells in the brains of ECM and uninfected mice (n = 4). (C) Time spent on the new object by ARM- and ARM + Fenozyme-treated ECM mice (n = 5). (D) Active avoidance percent by ARM- and ARM + Fenozyme-treated ECM mice in the last 30 times after 500 times of training (n = 5). Normal mice without infection were also used as a control. All data represent three independent experiments and are expressed as mean ± s.e.m. (* P < 0.05, ** P < 0.01, unpaired Student's t-test).

that of PBS-treated or uninfected groups. We also assessed the effects of HFn and Fenozyme on the murine macrophage cell line Raw264.7. As shown in Figure 3G, both of them promoted the growth of Raw264.7 cells markedly. Moreover, as shown in Figure 3H, the counts of macrophages isolated from the liver of Fenozyme- and HFn-treated ECM mice also increased significantly compared with that of PBS-treated or uninfected groups. The percentage of macrophages was also increased. In addition, we found that the macrophages in the liver but not in the spleen were increased (Figure 3H,I), suggesting that macrophages in the liver played a major role in decreasing parasitemia. Therefore, the ability of HFn and Fenozyme to stimulate macrophages may contribute to their effects on parasitemia.

Macrophages are known for engulfing cell debris and pathogens,[27] including pRBCs, which usually express unique membrane proteins that facilitate phagocytosis.[28] To evaluate the phagocytic capacity of macrophages for pRBCs, we prepared red blood cells (RBCs) from GFP-transgenic mice with or without *Pb.ANKA* infection ($pRBCs^{GFP}$ and $nRBCs^{GFP}$) and bone marrow-derived macrophages (BMDM) activated by the condition medium from cultured L929 cells. As shown in Figure 3J and Figure S6, the macrophages phagocytized $pRBCs^{GFP}$ significantly more efficiently than that of $nRBCs^{GFP}$. In the presence of Fenozyme or HFn proteins, the BMDM cells exhibited enhanced engulfment capacity toward $pRBCs^{GFP}$ as well as $nRBCs^{GFP}$. Taken together, these results indicated that Fenozyme might decrease parasitemia in ECM mice by enhancing the proliferation and phagocytosis by macrophages.

Fenozyme Induced Macrophages to M1 Polarization. Macrophages may differentiate into the M1 subtype that has pro-inflammatory and microbicidal activities or the M2 subtype that is anti-inflammatory and pro-tumorigenic. To understand whether Fenozyme affects macrophage differentiation, Raw264.7 cells were treated with Fenozyme, HFn protein, or bovine serum albumin (BSA) and analyzed for expression of M1 and M2 markers. As shown in Figure 4A,B, HFn protein and Fenozyme treatment increased the expression of M1 markers CD16/32, CD11c, and MHC class II, but not that of M2 marker CD206. By using a fluorescence probe and immunoblotting, we found an increased ROS level and elevated expression of induced nitric oxide synthase (iNOS) (Figure 4C,D) in HFn- and Fenozyme-treated Raw264.7 cells, indicative of macrophage polarization to M1.[29] Compared with that of BSA-treated cells, the total NO synthase activity in Fenozyme- and HFn protein-treated Raw264.7 cells was also markedly elevated (Figure 4E). Interestingly, Fenozyme and HFn appeared to act through activating the c-Jun N-terminal kinase (JNK), ERK, and p38-Mitogen-activated protein kinase (MAPK) signaling pathway (Figure S7).

We then ask whether Fenozyme can polarize an untransformed macrophage, the primary Kupffer cells (PKCs) from mouse livers (Figure S8A). These cells expressed HFR and interacted with FITC-labeled Fenozyme and HFn (Figure S8B,C). Similar to the M1 polarization of Raw264.7 cells, the PKCs had increased CD16/32 expression and activation of JNK, ERK, and p38 MAPK signaling after treatment with Fenozyme and HFn (Figure S8D,E). Therefore, Fenozyme can act through HFR to induce the

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polarization of both primary and transformed macrophages to M1.

Fenozyme Mitigated Brain Inflammation and Cognitive Impairment of Artemether-Treated ECM Mice. Cerebral malaria patients who survived after artemisinin-based treatment often exhibited permanent cognitive impairment.[2,3] It is conceivable that these impairments resulted from BBB disruption, brain inflammation, and neuron damages. Therefore, we firstly examined whether Fenozyme exerted protective effects on damages of BBB functions in artemether (ARM)-treated ECM mice by detecting the extent of leukocyte infiltration in brains from these ECM mice. As shown in Figure 5A, both artemether alone and artemether combined with Fenozyme effectively eliminated the parasite in the blood by day 7 in ECM mice. The brains of ECM mice treated with both artemether and Fenozyme had significantly less infiltrated $CD45^+$ cells, especially the $CD4^+$ T cells, and $CD8^+$ T cells than those of ECM mice treated with artemether alone (Figure 5B) To examine whether Fenozyme exhibited protective effects on the damages of cognitive functions in artemether-treated ECM mice, we performed the new objective recognition evaluation and the active and passive avoidance performance assessments on these mice. Compared with the uninfected mice, the ECM mice treated with artemether alone stayed significant less time with the new object, whereas the mice in the combination treatment group spent markedly more time. Thus, ECM mice treated with artemether alone had impaired memory of the old object, and including Fenozyme in the treatment protected mice from ECM-induced memory impairment (Figure 5C). In the active and passive avoidance tests, while the artemether-treated ECM mice had significantly less active avoidance than that of normal control, these treated with artemether and Fenozyme had active avoidance similar to that of control mice, demonstrating that the use of Fenozyme reduced the damage to learning and memory ability induced by ECM (Figure 5D). Taken together, these results indicated that the protection of BBB by Fenozyme not only reduced the death of mice duo to cerebral malaria, but also ameliorated impairments to the cognitive function of infected mice.

Discussion and Conclusion. Through extensive studies in animal models, cultured cells, and patients, it has become evident that the dysfunction of BBB plays a critical role in the development of cerebral malaria. A number of factors, including inflammatory cytokines and ROS, have been implied in its disruption. Using MRI and imaging techniques, it was shown recently that strong inflammatory involvement of the microvasculature in the ECM mice was accompanied by parenchymal and intraventricular inflammation of the brain, linking BBB disruption with brain damages directly.[30] As for the ROS, a type of them that appears to be involved in the disease of cerebral malaria is nitric oxide (NO). However, the role of NO is still controversial. Some reports claim that cerebral malaria is probably induced by high amounts of NO production,[31] while others support the idea that cerebral malaria results from a low bioavailability of this compound.[20] For other types of ROS, H_2O_2-related hydroxyl radicals and superoxide radicals are confirmed involved in the pathological process of cerebral malaria. It is reported that malaria infection induces the generation of hydroxyl radicals and H_2O_2, which is probably the main reason for the induction of oxidative and apoptosis.[32,33] Employing an immunofluorescence assay, we confirmed that the ROS level increased in the brain endothelial cells of ECM mice (Figures S9 and S10). As for the superoxide radicals, the superoxide dismutase (SOD) catalyzes them to produce H_2O_2 and O_2. Importantly, malaria patients have been reported to have a lower catalase activity than healthy controls but a higher SOD activity,[34] thus resulting in the accumulation of H_2O_2. Moreover, it is reported that free heme released from the hemoglobin of ruptured parasites infected red cells may continuously undergo autoxidation, producing super oxide, which dismutates into H_2O_2 and is a potential source for subsequent oxidative reactions in endothelial cells.[35,36] Thus, the H_2O_2 may be the major ROS resource for the damage of endothelial cells (Figures S9 and S10). However, the exact pathogenic mechanisms of CM in patients remain not well understood, and no specific targeting therapies have been developed to prevent the encephalopathy.

In the present study, we showed that Fenozyme significantly reduced the mortality of mice with ECM through its ability to interact with BBB endothelial cells and scavenge reactive oxygen species. These data provided strong evidence that the generation of ROS plays an essential role in BBB disruption of ECM mice and could be a target for therapeutic intervention of cerebral malaria. The catalase activity of Fenozyme contributes to the BBB protection of a cerebral malaria model, which further confirmed the important role of H_2O_2 in the pathological process of cerebral malaria. Noteworthily, Fenozyme is effective in alleviating neurological sequelae in ECM mice that survived artemether treatment, indicating that its combination with artemether could be a novel strategy for treating cerebral malaria.

Ferritin, an iron storage protein, is a self-assembly nanocage consisting of 24 subunits, with an outer diameter of 12 nm and an inner cavity of 8 nm, which is capable of storing as many as 4500 ions.[37] Because of the unique protein architecture and physicochemical properties, ferritin has been extensively used as versatile nanocarriers for disease theranostic applications.[13,14] Recently, we found that HFn possesses an intrinsic tumor-targeting ability via interacting with HFRs.[21,38,39] On the basis of this finding, we developed several HFn-based tumor imaging and therapeutic strategies.[13,14,39] Interestingly, we also found that HFn can specifically bind to BBB endothelial cells through expressed HFRs.[21] As the biocompatible iron oxide (Fe_3O_4) nanoparticle exhibits catalase-like activity that can efficiently suppress the ROS level *in vivo*, we biomimetically synthesize the Fenozyme that consists of HFn protein shells and an Fe_3O_4 nanozyme core, which is stable and can be prepared economically on a large scale. Given its effectiveness in ECM and the wide expression of HFRs, it is conceivable that the Fenozyme might be employed as a therapeutic agent in many ROS-involved inflammations.

Unexpectedly, we found that both HFn and Fenozyme stimulated the polarization of macrophages and enhanced their phagocytosis for pRBCs. Our data indicated that they activate ERK, p38, as well as JNK signaling pathways. Consistent with the results, it has been reported that the JNK signaling activation promoted the polarization of macrophages to the M1-like phenotype.[40−42] It is evident that the tyrosine phosphorylation motif of the HFR intracellular domain may participate in the signaling process.[43] However, Fenozyme, but not HFn, protected mice from the lethality of ECM, indicating macrophage polarization alone is not sufficient in treating cerebral malaria. Noteworthily, the ECM model did not involve the liver stage of malaria. Recently a paper report that Hyperplastic Kupffer cells and portal tract inflammation are two main features found in the liver tissues of severe *P.*

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falciparum malaria cases.[44] It is interesting to examine whether activation of Kupffer's cells by HFn and Fenozyme affects the replication of malaria in hepatocytes, which may provide further rationale to use the Fenozyme in the treatment of malaria in patients.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.9b03774.

Additional data and experimental details of preparation and characterization of Fenozymes, stability analysis of Fenozymes, the protection effects of Fenozyme in ECM with different doses, quantification analysis of the RBCs and pRBCs phagocytized in BMDM, *ex vivo* imaging of Fenozymes in mice brains, immunoblotting analyses of Raw264.7 cells and primary Kupffer cells, and ROS detection in brain endothelial cells of ECM mice (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: yanxy@ibp.ac.cn. Tel: +86 10-64888583. Fax: +86-10-64888584.

*E-mail: fankelong@ibp.ac.cn. Tel: +86-10-64888280. Fax: +86-10-64888584.

ORCID

Xiyun Yan: 0000-0002-7290-352X

Kelong Fan: 0000-0001-6285-1933

Author Contributions

K.L.F. and S.Z. designed the experiments. X.Y.Y. supervised this study. S.Z, H.X.D., and K.L.F. performed the experiments and analyzed the data. KL.F., YL.Y., and S.Z. prepared figures and wrote the paper.

Notes

The authors declare no competing financial interest.

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