ELF4 facilitates innate host defenses against Plasmodium by activating transcription of Pf4 and Ppbp

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ABSTRACT

Platelet factor 4 (PF4) is an anti-Plasmodium component of platelets. It is expressed in megakaryocytes and released from platelets following infection with Plasmodium. Innate immunity is crucial for the host anti-Plasmodium response, in which type I interferon plays an important role. Whether there is a crosstalk between innate immune signaling and the production of anti-Plasmodium defense peptides is unknown. Here, we demonstrate that E74 like ETS transcription factor 4 (ELF4), a type I interferon activator, can help protect the host from Plasmodium yoelii infection. Mechanically, ELF4 binds to the promoter of genes of two C-X-C chemokines, Pf4 and pro-platelet basic protein (Ppbp), initiating the transcription of these two genes, thereby enhancing PF4-mediated killing of parasites from infected erythrocytes. Elf4−/− mice are much more susceptible to Plasmodium infection than wild-type littermates. Expression level of Pf4 and Ppbp in
megakaryocytes from *Elf4*−/− mice is much lower than that of control animals, resulting in increased parasitemia. In conclusion, our study uncovered a distinct role of ELF4, an innate immune molecule, in host defense against malaria.

Malaria is a major public health problem, especially in Africa and Southeast Asia (1). Drug resistance of *Plasmodium* parasites remains a pressing concern (2). Nowadays, artemisinin combination therapy (ACT) which contains an artemisinin derivative is the standard treatment for *Plasmodium falciparum* infection according to guidelines for the treatment of malaria (3). However, artemisinin-resistant parasitod have been found in Western Cambodia (4, 5). There is a great need for new and effective approaches to fight malaria.

The innate immune system acts as the first defense against pathogens, relying on the recognition of pathogen-associated molecular patterns (PAMPs) by various kinds of pattern-recognition receptors (PRRs) (6, 7). Well-known PRRs include Toll-like receptors (TLRs) (8), RIG-like receptors (RLRs) (9) and several nucleic acid sensors such as cGMP-AMP synthesis (cGAS) (10). Of these receptors, TLR7, MDA5 and cGAS sense components of *Plasmodium*, either during the liver stage or blood stage of *Plasmodium* infection (11, 12, 13). Upon recognizing PAMPs, PRRs trigger downstream signaling activation, thus inducing the transcription of NF-κB and interferon regulatory factor 3 (IRF3) target genes. Many human cells in blood, including monocytes, neutrophils, and platelets take part in fighting malaria parasites (14, 15). Recent studies have shown that the human defense peptide (HDP) platelet factor 4 (PF4) can directly kill *Plasmodium falciparum* living in infected erythrocytes with the assistance of the erythrocyte Duffy-antigen receptor (16, 17). Another platelet-secreted chemokine, pro-platelet basic protein (PPBP), a platelet activation marker, also takes part in the process of clearing the parasites by inducing macrophage chemotaxis and mediating neutrophil accumulation (18, 19, 20). Nevertheless, how these chemokines are regulated by upstream signaling pathways, especially innate immune signaling molecules, remains elusive.

The ETS family transcription factors (TFs) are a family of molecules that participate in numerous signaling pathways (21). E74 like ETS transcription factor 4 (ELF4) is one of these TFs, which functions in tumorigenesis, DNA damage response, cell cycle regulation and innate immunity (22, 23, 24, 25). In the innate immune system, ELF4 functions as a novel and critical transcription factor of type I interferon (IFN), which is of vital importance for host defense against viral infection (25). However, the role of ELF4 in host defense against *Plasmodium* is unclear.

Here, we demonstrate that ELF4 plays a critical role in host defense against *Plasmodium yoelii* 17XNL. *Elf4*−/− mice exhibit increased parasitemia compared with WT mice. Expression of *Pf4* and *Ppbp* in *Elf4*−/− mice is much lower than that of WT mice during infection, without any difference in the number of megakaryocytes or platelets. Our *in vitro* experiments further reveal that ELF4 can directly bind to the promoter of *Pf4* and *Ppbp*, initiating their transcription, and modulating the overall levels of these two chemokines during *Plasmodium* infection.
Results

Elf4 deficient mice exhibit severe malarial anemia

We previously demonstrated that E74 like ETS transcription factor 4 (ELF4) is an important transcription factor of type I interferon (IFN) and is indispensable for host defense against virus infection (25). However, its function in defense against other pathogens has not been characterized. We sought to determine if ELF4 plays a role in defense against Plasmodium. Here, we used Plasmodium yoelii (P. yoelii) 17XNL, a non-lethal species of Plasmodium yoelii to investigate the function of ELF4 during murine infection with Plasmodium. Elf4+/+ and Elf4−/− mice were intraperitoneally injected with P. yoelii 17XNL infected red blood cells. Parasitemia peaked at day 18 post infection, when significant increased parasitemia was observed in Elf4−/− mice and lasted until day 25 (end of the parasitemia analysis) (Fig. 1A). Red blood cells (RBC), hemoglobin (Hb) and the percentage of hematocrit (HCT) during infection indicated the severity of malarial anemia (26), while the elevated level of mean corpuscular hemoglobin (MCH) was a marker of hemolytic anemia. Decreased levels of RBC and Hb, together with lower HCT and higher MCH levels were observed in Elf4−/− mice (Fig. 1B). In addition, Elf4−/− mice had elevated neutrophil (NE) counts (Fig. S1A) and lower level of lymphocytes (LY) (Fig. S1B). We also observed a higher level of mean corpuscular volume (MCV) (Fig. S1C). However, there was no difference in platelet counts (PLT) or mean platelet volume (MPV) between Elf4+/+ and Elf4−/− mice during infection, indicating the comparable level of thrombocytopenia (Fig. 1C). These results showed that Elf4−/− mice exhibited more severe malarial anemia and increased parasitemia compared with wild-type littermates. These findings establish ELF4 as an indispensable regulator for defense against Plasmodium.

Elf4 protects hosts from Plasmodium infection in a type I IFN independent manner

ELF4 is a transcription factor of type I IFN, which is essential for host defense against Plasmodium (27). To investigate the mechanism by which ELF4 mediates anti-Plasmodium defense, we examined if ELF4 functions through type I IFN. Surprisingly, we did not observe dramatic differences of Ifnb1 expression as well as Ifng post P. yoelii infection (Fig. 2). These data suggest that type I IFN is dispensable for ELF4 mediated host defense against Plasmodium.

The expression of Pf4 and Ppbp is impaired in Elf4 deficient mice

To determine whether anti-Plasmodium effectors are regulated by ELF4, we carried out RNA-seq to analyze gene expression in spleen and bone marrow after 2 days of P. yoelii 17XNL infection. Up- and down- regulated genes between Elf4+/+ and Elf4−/− mice were gated via a strategy that genes of Elf4−/− samples with more than 1.4 fold FPKM (up-regulated) or less than 0.67 fold FPKM (down-regulated) compared with Elf4+/+ samples were selected to draw the heatmaps (Fig. S2, A and B). GO (Gene Ontology) analysis and KEGG pathway analysis of up- or down- regulated genes suggest major differences in immune response and cytokine-cytokine receptor interaction process (Fig. S2, C-J). The expression level of Pf4 and Ppbp significantly decreased in the bone marrow of Elf4−/− mice (Fig. 3A). Previous studies have demonstrated that platelets are a critical platform of anti-
Plasmodium defense and PF4 secreted by platelets helps protect against malaria via direct killing of *Plasmodium falciparum* parasite (16). PPBP is known as a platelet activation marker and the *Ppbp* gene shares a common megakaryocyte-specific gene locus with *Pf4* (28). Consistent with the RNA-seq results, microarray analysis of whole blood samples confirmed the down regulation of *Pf4* and *Ppbp* in *Elf4*−/− mice (Fig. 3B). In order to validate the microarray results, qPCR of total blood RNA was used to assess *Pf4* and *Ppbp* mRNA levels (Fig. 3C). Therefore, ELF4 is essential for the production of PF4 and PPBP that mediate the clearance of *Plasmodium*.

**ELF4 binds to the promoter of *Pf4* and *Ppbp***

The ETS family TFs have highly similar DNA-binding domains, named ETS domains. It is known that ETS family TFs are able to initiate the transcription of megakaryocyte-specific genes (29, 30). We here reasoned that ELF4 might function through direct binding to promoters of *Pf4* and *Ppbp*. We constructed luciferase plasmids containing the mouse *Pf4* or *Ppbp* promoter region (-1-1000) (Fig. S3A). Remarkably, ELF4, but not other type I IFN signaling molecules significantly upregulated the luciferase activity of both mouse *Pf4* and *Ppbp* promoters (Fig. 4A) in a dose dependent manner (Fig. 4B). We also constructed luciferase plasmids containing the human *PF4* or *PPBP* promoter. ELF4 can also upregulate human *PF4* and *PPBP* luciferase activity in a dose dependent manner (Fig. 4C). ELF4 recognizes the target element by its ETS domain, which relies on the DNA binding activity motif RALR (267-270 aa for human ELF4 and 266-269 aa for mouse ELF4) (31). Mutation of RALR to AALA (herein as ELF4(AALA)) abolished ELF4 activation on both mouse *Pf4* and *Ppbp* promoters (Fig. 4D) and human *PF4* and *PPBP* promoters (Fig. 4E). These results indicate that ELF4 targets the promoters of *Pf4* and *Ppbp*.

We next intended to determine the exact ELF4 binding sites of *Pf4* and *Ppbp* promoters. ELF4 was characterized as a transcription factor mainly binding to the target sequence containing a core purine-rich site, GGAA (31). We identified four GGAA sites in both mouse *Pf4* and *Ppbp* promoters (Fig. S3C). None of the four GGAA sites which were replaced by GATC changed luciferase activity of *Pf4* promoter (Fig. 4F and 4G). Former investigations have found that multiple ETS TFs bind to the GGAA antisense sequence to activate *Pf4* expression (30). We thus hypothesize that ELF4 binds to the GGAA antisense sequence (TTCC) of *Pf4* promoter. Since multiple TTCC sites were found in the 1kb region of *Pf4* promoter, we firstly built deletion constructs containing 500 bp of the upstream promoter region (-1-500) or further 500 bp region (-500-1000) to narrow the options of potential sites. Only the -1-500 bp promoter but not the -500-1000 bp promoter luciferase was comparably activated with the full-length promoter by ELF4 (Fig. 4H). We then analyzed the 500 bp *Pf4* promoter and found four TTCC sites. Mutation of the -5 TTCC site but not the other three TTCC sites resulted in the disability of luciferase activation by ELF4 (Fig. 4I), suggesting that the -5 GGAA antisense TTCC site was the ELF4 binding site of *Pf4* promoter. Compared with *Pf4* promoter, activation of the *Ppbp* promoter luciferase was impaired when the first GGAA (-702) was replaced by GATC (Fig. 4J). We also intended to find the ELF4 binding sites on human PF4 and PPBP promoters. We constructed human *PF4* and *PPBP* promoter luciferase plasmids.
Following the same strategy as we found the binding sites on mouse promoters, we identified the -305 GGAA of PF4 promoter (Fig. S4B) and -57 TTCC of PPBP promoter (Fig. S4C) as ELF4 binding sites.

Although predicted promoter of genes usually locates at the upstream region of the transcriptional start site (TSS), there remains the possibility that some non-coding exons localized upstream of already known TSS could also be transcribed in specific tissues or cells (e.g. in megakaryocytes). Given that the -1.1 kb region upstream of rat PF4 was the promoter of rat PF4 in megakaryocytes (32), we wanted to make sure that the 1 kb region we used to build luciferase constructs contained the true promoters of mouse Pf4 and Ppbp in megakaryocytes. We designed primers targeting the -200, -400 or -1000 region upstream the TSS of Pf4 or Ppbp. We also designed +1-93 primer or +1-249 primer of Pf4 or Ppbp. Quantitative-PCR (qPCR) (Fig. S3, D and E) or RT-PCR (Fig. S3, F and G) using cDNA or genomic DNA of megakaryocytes isolated from wild type C57BL/6 mouse demonstrated that the upstream region of TSS was not included in the hnRNA of Pf4 or Ppbp. Taken together, our results demonstrate that ELF4 binds to Pf4 and Ppbp promoters.

**ELF4 promotes the expression of Pf4 and Ppbp**

We have already discovered that ELF4 binds to the promoter of Pf4 and Ppbp. Next we wanted to determine the effects of ELF4 on the expression of Pf4 and Ppbp. To evaluate the effects of ELF4 expression on PF4 and PPBP transcriptional activation, we used megakaryocytic HEL cells. We generated ELF4-KO HEL cells using CRISPR-Cas9 system. Using lentivirus packaging system, we also generated ELF4 or ELF4(AALA) expressing HEL cells. ELF4 stably expressing HEL cells expressed higher levels of PF4 and PPBP (Fig. 5A), while endogenous expression of PF4 and PPBP were much lower in ELF4-KO HEL cells (Fig. 5B). To prove that ELF4 directly binds to the promoter of PF4 and PPBP, we performed chromatin immunoprecipitation (ChIP) assay in HEL cells with or without ELF4 or ELF4(AALA) expression. ELF4 but not the ELF4(AALA) mutant directly bound PF4 and PPBP promoter (Fig. 5C). We also used another megakaryocytic cell line Dami cells to further verify our hypothesis. Overexpressed ELF4 but not ELF4(AALA) mutant upregulated PF4 and PPBP expression (Fig. S4A) and bound to the promoter region of both PF4 and PPBP (Fig. S4B) in Dami cells. Taken together, these results suggest that ELF4 directly binds to PF4 and PPBP promoter and promotes the expression of PF4 and PPBP.

**ELF4 is essential for controlling Pf4 and Ppbp expression during P. yoelii infection**

Bone marrow megakaryocytes are the main source of PF4 and PPBP in peripheral platelets. Although malarial anemia caused by *P. yoelii* infection could result in increased number of megakaryocytes and decreased number of thromocytogenic megakaryocytes, we observed no obvious difference in the quantity of bone marrow megakaryocytes (Fig. 6A) or peripheral platelets (Fig. 1C) in Elf4+/− and Elf4−/− mice. Since platelet secreted PF4 and PPBP mediate clearance of *Plasmodium* and ELF4 activates Pf4 and Ppbp, we hypothesize that ELF4 is essential for controlling expression of Pf4 and Ppbp in megakaryocytes. In order to determine the ongoing ability of ELF4 to activate Pf4 and Ppbp during *Plasmodium* infection, we
analyzed expression of Pf4 and Ppbp in bone marrow megakaryocytes and peripheral platelets, respectively. We isolated bone marrow megakaryocytes and peripheral platelets to assess the expression of Pf4 and Ppbp during P. yoelii infection. Gene expression of Pf4 first increased then reduced and was not significantly upregulated in megakaryocytes until day 9 post infection, when Pf4 expressed at a lower level in Elf4−/− mice. During *Plasmodium* infection, expression of Ppbp exhibited similar tendency with Pf4 (Fig. 6B). Immunoblot analysis indicated that protein level of both PF4 (Fig. 6C) and PPBP (Fig. 6D) decreased in peripheral platelets of Elf4−/− mice. These data indicated that lower level of Pf4 and Ppbp expression in Elf4−/− mice was not due to the decreased number of megakaryocytes or platelets. Taken together, these results suggest that ELF4 plays an important role in modulating the expression of Pf4 and Ppbp during *Plasmodium* infection.

**Discussion**

Diverse studies have examined how the immune system helps to defend against malaria (33). Host defense against malaria mainly includes innate immunity, adaptive immunity and other mechanisms to kill parasites. Recent studies have found that certain innate immune molecules, such as MAVS, STING and cGAS, suppressed host defense against *Plasmodium*, resulting in unexpectedly severe malaria. Different from their role in antiviral immunity, *Mb21d1* (cGAS)-, *Sting*- , *Mda5*- , *Mavs*- , or transcription factor Irf3-deficient mice produced high amounts of type I interferon in the serum and were resistant to lethal *Plasmodium yoelii* YM infection (13). Although innate immune pathway involved in anti-*plasmodium* defense has been well studied, there is no explicit innate signaling molecule that can function against *Plasmodium* infection independently of type I interferon. ELF4 is an ETS domain transcription factor that is essential for the production of type I interferon and antiviral immunity during virus infection. We here demonstrated that ELF4 mediated anti-*plasmodium* immunity in a type I interferon independent fashion. ELF4 directly binds to the promoter and facilitates the transcription of Pf4 and Ppbp, which help to kill *plasmodium* directly.

Accumulating evidence demonstrated that ELF4 regulates blood cell proliferation (21). When we analyzed cells in blood, we found elevated numbers of reticulocytes in Elf4−/− mice (data not shown). Since *P. yoelii* 17XNL prefers to invade reticulocytes but not erythrocytes (34), Elf4 deficiency provides an ideal environment for parasite infection. Because of the preference of *Plasmodium* to invade reticulocytes, more uninfected erythrocytes presenting phosphatidylserine will be opsonized by macrophages (35), resulting in aggravating anemia. Elevated reticulocytes can also result in a higher relative HCT percentage, which may explain the unexpected higher level of MCV.

Megakaryocyte-secreted PF4 can regulate hematopoietic stem cell quiescence (36). Previous studies have shown that PF4 promoted monocyte activation and subsequent cytokine production via increasing Klf4 expression in monocytes, resulting in severe cerebral inflammation in the experimental cerebral malaria (ECM) (17), which may be avoided due to ELF4 deficiency. These studies demonstrate that ELF4 may function via PF4 to regulate various kinds of blood cells. Ppbp shares a common proximal location in genome with Pf4. These two
cytokines are expressed in a similar period during megakaryocyte development (28). PPBP functions through two steps of N-terminal cleavage, forming neutrophil-activating peptide-2 (NAP-2), an antibacterial protein functioning in innate immune system (37). However, we observed equal level of thrombocytopoiesis in Elf4+/+ and Elf4−/− mice during P. yoelii infection in our study, indicating the need for detailed examination of changes in different cell types in uncovering the more complicated mechanism in which ELF4 plays a role in the P. yoelii infection model. In summary, although ELF4 directly promotes transcription of Pf4 and Ppbp, how ELF4 fine tunes the anti-plasmodium immunity remains to be further investigated.

We previously found that ELF4 was involved in innate immune signaling. ELF4 was recruited by STING and activated by TANK-binding kinase 1 (TBK1). A recent study showed that STING and TBK1 suppressed the anti-plasmodium immunity (13). It is interesting to explore if these two molecules may regulate the expression of PF4 and PPBP. In conclusion, our study showed that the innate immune signaling molecule ELF4 functions in defending against P. yoelii infection via activating transcription Pf4 and Ppbp.

Experimental procedures

Ethics statement

This study was carried out in accordance with the recommendations of National Regulations for the Administration of Affairs Concerning Experimental Animals. The protocol was approved by the Peking University animal care and use committee. The project license number is LA2016239.

Malaria parasites and mice

The rodent parasite Plasmodium yoelii 17XNL strain used in this study was kindly provided by Dr. Yuan J (School of Life Science, Xiamen University). GFP-expressing Plasmodium yoelii 17XNL strain used in this study was initially obtained from the Malaria Research and Reference Reagent Resource Center. P. yoelii parasites were thawed from frozen stocks and maintained alive by continuous intraperitoneal passage in mice after every 6 days. Elf4−/− mice on a C57BL/6J background have been described previously (24). All mice were housed under specific pathogen-free (SPF) conditions, and all mice were analyzed at 6-8 weeks of age (unless otherwise specified).

Infection of mice with P. yoelii parasites

An inoculum containing appropriate numbers of iRBC (1×10⁵) suspended in 100 μl phosphate buffered saline (PBS), pH 7.4, from donor mice was injected intraperitoneally (i.p.) into experimental C57BL/6J knockout mice or littermates. Mice blood was collected at indicated day to monitor parasitaemia. Parasitaemia was analyzed by flow cytometry (FCM). For FCM analysis, using the FACSVerse (BD Biosciences), a drop of blood in PBS was used to measure the blood parasitemia. GFP-expressing parasites were detected in the green fluorescent channel FL1. The gated amount related to all detected live cells corresponds to the blood parasitemia in percent.

Blood indices

Blood was obtained at indicated day after infection by a tail snip. Twenty microliters blood was collected to heparin-coated tube and then was analyzed with HEMAVET 950 Analyzer (Drew Scientific Inc.).

Platelet isolation

Whole blood of mice was collected from the retro-orbital sinus into EDTA-containing microtubes. Platelet-rich plasma was obtained by centrifugation of whole blood at 110 g for 10 min. Platelets were isolated by further centrifugation at 800 g for 10 min and lysed using RIPA lysis buffer to perform the subsequent immunoblotting assay.

Bone marrow collection and megakaryocytes
**purification**

Bone marrow was collected from the tibias and femurs of mice at indicated days post *P. yoelii* infection. Megakaryocytes were purified using the mouse megakaryocytes isolation kit (MEG2014M, TBD sciences). Briefly, bone marrow was resuspended and passed through a 70 μm nylon cell strainer, followed by Percoll gradient (63/30%) centrifugation. Megakaryocytes were harvested from the top interphase. Collected megakaryocytes were washed with PBS twice and used to purify total RNA by RNeasy Mini kit (QIAGEN).

**Determination of megakaryocyte number**

Quantification of megakaryocytes were determined via bone marrow megakaryocytes isolation and counting. Briefly, the bone marrow was flushed out using PBS, resuspended and passed through a 70 μm nylon cell strainer, followed by total bone marrow cell counting. After the megakaryocytes were isolated, the number of collected cells were counted and the percentage of megakaryocytes in total bone marrow cells was calculated.

**Cell culture**

HEK293T (human embryonic kidney 293T) cells were maintained in DMEM medium (Gibco) with 10% FBS and 1% glutamine (Gibco). HEL and Dami cells were cultured in RPMI1640 medium (Gibco) with 10% FBS and 1% glutamine (Gibco). All cells were incubated at 37°C with 5% CO₂. Cells tested negative for mycoplasma contamination.

**Generation of stable cell lines**

ELF4 KO HEL cells and Dami or HEL cells stably expressing Flag-ELF4 or ELF4 (AALR) were generated as follows. For ELF4-KO cells, sgRNA oligos (targeting sequence: 5'-GAGTTGGAGCGAGTTACAA-3') were cloned into lentiCRISPRv2 following the lentiCRISPRv2 oligo cloning protocol. For ELF4 overexpression, Flag-ELF4 (or ELF4(AALR)) was amplified by PCR and cloned into pCDH-CMV-MCS-EF1-puro vector. Lentiviral vectors (lentiCRISPRv2 or pCDH-CMV-MCS-EF1-puro) were co-transfected with packaging plasmids pVSVg and psPAX2 into HEK293T cells for 48 h. Culture supernatants containing viral particles were collected and filtered through 0.45 μm nitrocellulose filter (Millipore). Cells were infected in the presence of 4μg/ml polybrene and selected with 10 μg/ml puromycin. All stable cell lines were used at early passages in experiments.

**Reporter assay**

HEK293T (2×10⁵) cells were plated in 24-well plates and transfected using polyethylenimine (PEI) (Polysciences), with plasmids encoding *Pf4* or *Pphp* luciferase reporter (firefly luciferase; 100 ng) and pRL-TK (renilla luciferase plasmid; 10 ng) together with 100 ng plasmid encoding Flag-ELF4, Flag-ELF4(AALR), Flag-MAVS, Flag-STING, Flag-IRF3, or Flag-IRF7. Deletion or GGAA (TTCC) mutation of mouse *Pf4* and *Pphp* (or human *PF4* and *PPBP*) promoter region containing luciferase plasmids was carried out using standard molecular biology methods. Empty p3×FLAG-CMV-7.1 vector was used to maintain equal amounts of DNA among wells. After transfection for 24 h, cells were lysed and luciferase activity was measured with the Dual-Luciferase Assay System (Promega) according to the manufacturer’s instructions. Reporter gene activity was determined by normalization of the firefly luciferase activity to renilla luciferase activity.

**RT-PCR and Quantitative Real-Time PCR**

Total RNA was extracted from tissue or cells using RNeasy Mini kit (Qiagen), and the complimentary cDNA was generated using reverse transcriptase III (Invitrogen). For reverse transcription-PCR, 1 μl of RT reaction was used for each PCR whose number of cycles was optimized to avoid saturation (32 for all the primer pairs). 3 μl of reaction were loaded on agarose gels. Real-time PCR was carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems) using the SYBR GreenER qPCR Super Mix Universal (Invitrogen) and specific primers. The relative RNA expression level was normalized to *Hprt*, *Actb* (for mouse) or *GAPDH* (for human) according to the 2^ΔΔCt_ calculation method. Primer pairs used to detect target gene transcripts are listed in Supplementary Table 1.
**Transcriptomic analysis**

The total RNA of blood samples was purified using RNeasy Mini kit (QIAGEN). RNA quantity was evaluated spectrophotometrically, and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies). All samples showed RNA integrity number >8. RNA sequencing libraries were generated using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB), and sequenced on an Illumina Hiseq platform. Sequencing was performed at Beijing Novogene Bioinformatics Technology Co. Ltd and Nanjing Vazyme Biotech Co. Ltd. The filtered reads were mapped to the mouse genome reference sequence (GRCm38/mm10 Ensemble release 83) using HISAT2. Gene expression was quantified as fragments per kilobase of transcript per million fragments mapped (FPKM) algorithm. Genes with an FPKM value >1.4 fold or <0.67 fold were assigned as differentially expressed. Network visualization of Gene Ontology Annotation (GO Annotation) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping was performed with DAVID 6.7. Heatmaps were generated using HEML 1.0 (Heatmap Illustrator, version 1.0 (38)). See Supplementary Table 2 and 3 for more details of gene list and analysis results.

**Microarray analysis**

Peripheral blood samples were collected into heparin-coated tubes. RNA was extracted using RNeasy Mini kit (QIAGEN) and performed DNase-treatment with DNase I (sigma). The complementary DNA was subsequently synthesized. Labeled cDNA (using Cyanine-3) was synthesized, purified, quantified and prepared for hybridization following the Agilent protocol. The microarray experiment was performed on a one-color 4×180K oligonucleotide array (Agilent Technologies: G4839A) according to the manufacturer’s protocol. The microarray slides were pre-processed (signal background corrections) and scanned using Agilent’s High-Resolution C Scanner. Following normalization, the probe intensity of all probes in a probe set was summarized to a single value. The bulk of the analyses were done on the RMA normalized data in log2 scale.

**Immunoblotting**

Whole-cell extracts were prepared using RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% deoxycholate, supplemented with a protease inhibitor tablet (Roche)) and centrifuged at 10,000 g for 10 min at 4°C. The protein concentration was measured using Pierce BCA protein assay kit (Thermo Fisher Scientific). Proteins were resolved on 15% SDS-PAGE and transferred onto nitrocellulose membranes (Millipore). Blocked membranes were incubated with the primary antibodies (rabbit anti-PF4 (Proteintech), rabbit anti-PPBP (Sigma-Aldrich), and mouse anti-GAPDH (Proteintech)). Secondary antibodies conjugated to HRP and Western Chemiluminescent HRP Substrate (Millipore) were used for detection.

**ChIP assay**

HEL or Dami cells (1.5×10^7) in 10 cm dishes were cross-linked with 1% formaldehyde for 10 min at room temperature. The reaction was quenched by adding glycine solution (final concentration, 0.1 M) then incubating for 5 min. Cells were then washed with ice cold PBS with PMSF, and resuspended in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8.1), supplemented with a protease inhibitor tablet (Roche)). Chromatin was sheared by sonication on ice to generate 200-1000 bp chromatin fragments, and the insoluble fraction was removed by centrifugation. The lysate was precleared with Protein A/G beads (Invitrogen) and then incubated with Anti-Flag Affinity Gel (B23102, Bimake Biotech) overnight at 4°C. Immunocomplexes were collected and washed with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 7.9), and 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 7.9), and 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 0.5 mM Sodium deoxycholate, 1 mM EDTA, 0.5% NP40 and 10mM Tris-HCl (pH 8.0)), and TE buffer (10 mM Tris-HCl (pH 7.9) and 1 mM EDTA). Precipitated
chromatin fragments were eluted using elution buffer (1% SDS and 0.1 M NaHCO₃). After adding NaCl (final concentration, 0.2 M), samples were reverse cross-linked at 65°C overnight. After proteinase K (GE201, TransGen Biotech) digestion, DNA was extracted using phenol/chloroform, and purified for quantitative real-time PCR analyses using specific primers (See Supplementary Table 1 for primer sequences).

Statistical analysis
All analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA). Data are presented as means ± s.d., unless otherwise stated. Statistical significance of differences between two groups was assessed by unpaired Student’s t tests and a p value of < 0.05 was considered significant.

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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

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FOOTNOTES

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The abbreviations used are: ACT, artemisinin combination therapy; PAMPs, pathogen-associated molecular patterns; PRRs, pattern-recognition receptors; TLRs, Toll-like receptors; RLRs, RIG-like receptors; cGAS, cGMP-AMP synthesis; HDP, human defense peptide; PF4, Platelet Factor 4; PPBP, Pro-Platelet Basic Protein; RBC, Red blood cells; Hb, hemoglobin; HCT, hematocrit; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; NE, neutrophil; LY, lymphocyte; Klf4, Kruppel-like factor 4; ECM, experimental cerebral malaria; NAP-2, neutrophil-activating peptide-2; PEI, polyethylenimine; qPCR, quantitative real time PCR.
Fig. 1. *Elf4* deficient mice suffer from severe malarial anemia.

(A) Parasitemia of *Elf4*+/+ and *Elf4*−/− mice infected with GFP- *P. yoelii* 17XNL (1×10^5 infected red blood cells) at indicated days post infection (P.I.) was assessed by analyzing percentage of GFP-positive red blood cells (infected) via flow-cytometry. (B) Severity of malarial anemia was assessed by detecting red blood cell (RBC), hemoglobin (Hb), hematocrit (HCT) and mean corpuscular hemoglobin (MCH) levels at indicated days post infection (P.I.). (C) Level of thrombocytopenia was assessed by peripheral platelet counts (PLT) and mean platelet volume (MPV). Data in (A) is means ± s.e.m. (n=8 for *Elf4*+/+, n=7 for *Elf4*−/−). Data in (B) and (C) are means ± s.d.. All data are from three independent experiments; *t*-test, *P* < 0.05; **P* < 0.01; NS, not significant.
Fig. 2. Type I IFN is irrelevant to mediate protection of mice from *P. yoelii* 17XNL infection.

Quantitative PCR analysis of *Ifnb1* and *Ifng* mRNA from the blood sample of *Elf4*+/+ and *Elf4*−/− mice infected with *P. yoelii* 17XNL at indicated days post infection. Gene expression was relative to mouse *Hprt*. All data are means ± s.d. from three independent experiments; *t*-test, NS, not significant.
Fig. 3. *Pf4* and *Ppbp* expression was lower in *Elf4*−/− mice after *P. yoelii* 17XNL infection.

(A) List of the very top enriched genes from RNA-seq data that were induced more than 1.4-fold in the bone marrow of *Elf4*+/+ mice compared with *Elf4*−/− mice. (B) Microarray analysis of diverse genes expression between *Elf4*+/+ and *Elf4*−/− mice in response to infection with *P. yoelii* 17XNL. (C) Quantitative PCR analysis of *Pf4* and *Ppbp* mRNA in the blood of *Elf4*+/+ and *Elf4*−/− mice at indicated days post infection with *P. yoelii* 17XNL. Gene expression in (C) was relative to mouse *Hprt*. For (C), data is means ± s.d. from three independent experiments; *t*-test, *P* < 0.05; ****P < 0.0001; NS, not significant.
Fig. 4. ELF4 activates the promoter of Pf4 and Ppbp.

(A-B) Luciferase activity in HEK293T cells transfected with mouse Pf4 or Ppbp promoter-driven luciferase reporters, together with plasmids encoding Flag-ELF4, Flag-MAVS, Flag-STING, Flag-IRF3, or Flag-IRF7 (A) and increasing amount of plasmids expressing ELF4 (B). (C) Luciferase activity in HEK293T cells transfected with human PF4 or PPBP promoter-driven luciferase reporters, together with increasing amount of plasmids expressing ELF4. (D-E) Luciferase activity in HEK293T cells transfected with mouse Pf4 or Ppbp promoter- (D) or human Pf4 or PPBP promoter- (E) driven luciferase reporters together with plasmids encoding ELF4 or its mutant ELF4(AALA). (F) Luciferase activity in HEK293T cells transfected with mouse Pf4 promoter or
the different GGAA-mutated promoters-driven luciferase reporters, together with Elf4 expression plasmid or empty vector. (G) Luciferase activity in HEK293T cells transfected with different combination of Pf4 promoter-driven luciferase reporters, together with Elf4 expression plasmid or empty vector. (H-I) Luciferase activity in HEK293T cells transfected with truncation (H) or TTCC-mutation (I) of Pf4 promoter-driven luciferase reporters, together with Elf4 expression plasmid or empty vector. (J) Luciferase activity in HEK293T cells transfected with mouse Pphp promoter or the different GGAA-mutated promoters-driven luciferase reporters, together with Elf4 expression plasmid or empty vector. All data are means ± s.d. from three independent experiments; t-test, *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 5. ELF4 mediates transcriptional activation of Pf4 and Pphp.

(A) Expression of PF4 and PPBP in wild-type (WT) HEL cells or HEL cells stably transfected with ELF4 was assessed by quantitative PCR (qPCR) analysis. (B) Expression of PF4 and PPBP in
WT or ELF4-KO HEL cells was assessed by quantitative PCR (qPCR) analysis. (C) Binding ability of ELF4 on PF4 and PPBP promoters was assessed by chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) analysis in WT HEL cells or HEL cells stably transfected with ELF4 or ELF4 (AALA). For (A-B), gene expression was relative to human GAPDH. All data are means ± s.d. from three independent experiments; t-test, *P < 0.05; **P < 0.01.

Fig. 6. ELF4 modulates the expression of Pf4 and Ppbp during P. yoelii infection.

(A) Quantification of bone marrow megakaryocytes was assessed by calculating the percentage of megakaryocytes in total bone marrow cells at indicated days post infection. (B) Expression of Pf4 and Ppbp in isolated megakaryocytes from Elf4+/+ or Elf4−/− mice at day 0, 6, 9 or 18 after infection. (C) Protein level of Pf4 in peripheral platelets at day 0, 7 or 14 after P. yoelii infection from individual Elf4+/+ or Elf4−/− mice. (D) Protein level of Ppbp in peripheral platelets at day 0, 7 or 14 after P. yoelii infection from the same individual Elf4+/+ or Elf4−/− mice as in (C). For (B), gene expression was relative to Actb. For (C) and (D), GAPDH was used as control. Data in (A-B) are means ± s.d. (n=3). All data are from two independent experiments; t-test, *P < 0.05; **P < 0.01; NS, not significant.
ELF4 facilitates innate host defenses against *Plasmodium* by activating transcription of *Pf4* and *Ppbp*

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