Title:

Neutrophil extracellular traps drive inflammatory pathogenesis in malaria

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Abstract

Neutrophils are essential innate immune cells that extrude chromatin in the form of neutrophil extracellular traps (NETs). This form of pro-inflammatory cell death has potent immunostimulatory activity. We show that heme-induced NETs are essential for malaria pathogenesis. Using patient samples and a mouse model, we define two mechanisms of NET-mediated inflammation of the vasculature: activation of emergency granulopoiesis via GCSF production, and induction of the endothelial cytoadhesion receptor ICAM-1. Soluble NET components facilitate *Plasmodium* sequestration and mediate tissue destruction. We demonstrate that neutrophils have a key role in triggering malaria immunopathology and propose inhibition of NETs as a treatment strategy in vascular infections.

Introduction

Neutrophils are essential innate immune cells that circulate in the blood and respond to pathogens by phagocytosis, generation of oxidants and externalization of microbicidal peptides and proteases [1]. The release of these compartmentalized antimicrobials is achieved by either degranulation or the release of neutrophil extracellular traps (NETs). NETs consist of decondensed chromatin decorated with microbicidal and immunostimulatory molecules [2, 3]. NETs are released by a cell death program termed 'NETosis' and they ensure high local concentrations of active antimicrobials. Eventually, Deoxyribonuclease I (DNase 1), a constitutive plasma endonuclease, degrades NETs and facilitates their removal [4].

NETosis is an active process that requires microbial or mitogenic signaling [5, 6], the production of reactive oxygen species (ROS) [7], the activity of two serine proteases: neutrophil elastase (NE) and proteinase 3 (PR3) [8, 9] and the activation of the pore forming protein gasdermin D [10]. NE translocates from the granules to the nucleus during NET induction, where it cleaves histones to allow chromatin decondensation prior to plasma membrane breakdown [8]. NE and PR3 have partially overlapping substrates [11] and are both required for maximal NET induction *in vivo* [9].

Triggering of NETosis by various microbes in tissues or the mucosa is thought to limit pathogen proliferation and dissemination. NET release inside the vasculature, however, can be pathogenic by triggering autoimmunity [12] and vasculitis [13], as well as directly damaging endothelia [14] and inducing thrombosis [15].

To understand the role of neutrophils in intravascular infections, we investigated malaria, a disease caused by protozoan parasites that invade red blood cells (RBCs) and trigger systemic neutrophil activation [16, 17]. *Plasmodium falciparum* is the most

important and virulent species, causing over 200 million malaria episodes and close to 500,000 deaths annually [18] . It encompasses diverse pathological manifestations that can range from mild unspecific symptoms, fever and mild anemia to organ failure, acidosis, coma and death. Complications of severe malaria include cerebral malaria, acute respiratory distress syndrome, renal failure, placental malaria, liver damage and severe anemia [19].

Pathogenesis of *P. falciparum* malaria is precipitated by its interaction with the vascular endothelium. In the second half of the asexual erythrocytic lifecycle, parasites express cytoadhesion factors on the surface of infected RBCs (iRBCs), allowing binding and sequestration in postcapillary venules. Attachment and withdrawal from circulation is thought to aid in preventing clearance of iRBCs by splenic macrophages [20]. Disease severity is synergistically determined by sequestration patterns and host inflammatory responses [21, 22]. Cytoadhesion of iRBCs leads to endothelial activation and vascular occlusion [22], while release of pathogen- or danger-associated molecular pattern (PAMP or DAMP) molecules leads to pathological inflammatory responses mediated by cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-1β [23]. Organ-specific iRBC sequestration is associated with corresponding pathology [21, 22].

Despite the important inflammatory component of the disease, the role of neutrophils in *P. falciparum* malaria remains unclear. Neutrophils isolated from malaria patients have a reduced capacity to mount an oxidative burst [24]. On the other hand, several studies have linked activation of these cells to pathogenesis and severe disease [16, 17, 25]. For instance, a recent blood transcriptomic analysis comparing severe and uncomplicated malaria identified a granulocyte colony stimulating factor (GCSF)-regulated neutrophil granulopoiesis signature as a specific feature of severe malaria [26]. Granulopoiesis refers to production of neutrophils from progenitor cells in

the bone marrow; this blood signature therefore identifies increased neutrophil abundance as a pathogenic factor in malaria. Furthermore, genes encoding neutrophil granule proteins, such as NE and matrix metalloproteinase-8 (MMP-8), showed the highest log-fold upregulation between severe and uncomplicated malaria [17]. Similarly, a study in Malawi by Feintuch et al. demonstrated that retinopathy-positive cerebral malaria is specifically associated with accumulation of externalized neutrophil proteins such as NE and PR3 [16]. Several studies in mouse models have also linked neutrophils to severe malaria [27-30].

In addition to the accumulation of soluble neutrophil proteases, severe disease is associated with an increase in extracellular human nucleosomes in patients' plasma [31], which could indicate NET release. NETs are a platform for externalizing both nucleosomes and neutrophil proteases and could thus be an important pathogenic factor in malaria. Indeed, NETs were reported in mouse malaria [30] and NET-like structures were observed on patient blood smears [32].

We show, using patient samples, that NETs are triggered by heme in malaria. We found NETs to be a source of immunostimulatory molecules - alarmins - that activate emergency hematopoiesis via GCSF induction. In a *Plasmodium chabaudi chabaudi* malaria model, host DNase 1 liberated neutrophil proteins from NETs and this release was required for neutrophilia and neutrophil infiltration in the liver. Soluble NET components were also required for parasite organ sequestration. Genetic depletion of NETs, or NET-processing DNase 1, reduced organ damage. We demonstrate an undescribed physiological role for NETs in circulation, as well as identify a potential target for adjunctive malaria therapy.

Results

Intravascular NET formation in *P. falciparum* malaria

To test if *P. falciparum* malaria is accompanied by *bona fide* NET induction, we analyzed plasma samples from forty-three parasitologically confirmed pediatric and adult patients, treated at the Albert Schweitzer Hospital, in Lambaréné, Gabon, a highly malaria-endemic region in Central Africa. The patients presented with variable disease severity, spanning from uncomplicated malaria to hyperparasitemia and severe anemia (Table S1). All patients recovered upon antimalarial treatment. NETs are defined as complexes of chromatin and neutrophil granule proteins; hence, we designed an ELISA that detects NETs with an anti-DNA detection antibody, preceded by a capture antibody against NE. Notably, malaria patients had significantly elevated levels of NETs compared to healthy controls from the same region (n = 9) (Fig. 1A). NET levels did not correlate with parasitemia or body temperature (data not shown).

We also isolated peripheral blood neutrophils from hospitalized adult patients and monitored NET formation. Neutrophils from malaria patients (n = 8) released twofold more NETs than healthy controls (n = 6) (Fig. 1B). Importantly, NETs were released without the addition of exogenous stimuli, indicating that NETosis in malaria is activated *in vivo*.

To confirm NET formation in the vasculature of organs affected by malaria, we detected NETs in a brain autopsy sample from a previously unexposed traveler with cerebral malaria. Using confocal imaging, we observed neutrophils in brain blood vessels where NE and chromatin colocalized (Fig. 1C), a reliable marker for early NETosis [8]. Together, these data indicate that NETs are induced in the bloodstream of malaria patients.

NETs in malaria are induced by heme and TNF

To identify factors that trigger NET formation in malaria, we co-incubated neutrophils from healthy donors with *P. falciparum* cultures. Neutrophils were either primed with TNF, a major malaria-associated proinflammatory cytokine [22], or left unprimed. We exposed neutrophils to iRBCs, free merozoites, parasite digestive vacuoles, which are released upon RBC rupture and contain the hemozoin crystal, as well as heme, a known malaria DAMP that is released during parasite egress, as well as during 'bystander hemolysis' – the inflammatory destruction of uninfected RBCs [23, 24]. Interestingly, only heme robustly induced NETs in combination with TNF priming (Fig. 1D & S1A), as previously reported in sickle cell disease [33].

To verify that NET formation is linked to hemolysis *in vivo*, we determined the heme concentrations in our patient cohort. Free heme correlated with NETs in plasma, further validating heme as the NET-inducing factor in malaria (Fig. 1E).

Heme-induced NETs require oxidants and NE/PR3 mediated proteolysis

There are different pathways for NET formation [34]. We tested the involvement of host factors previously implicated in NETosis, starting with the ROS-producing enzyme NOX2 [7]. We isolated neutrophils from patients with chronic granulomatous disease (CGD) (n=3), who carry NOX2 mutations, rendering them completely deficient in ROS production (Fig S1B). Heme induced similar levels of NETs in CGD and control neutrophils, unlike PMA, which failed to induce NETs in CGD cells (Fig 2A). Although this oxidase was not involved, heme-induced NETs required ROS signaling since treatment with the ROS scavenger pyrocatechol (Fig S1C) completely abolished NETosis (Fig 2B), suggesting that heme itself might be the oxidizing agent. In addition to oxidants, heme-triggered NETs required activity of protein kinase C (PKC) [35], cyclin dependent kinase 6 (CDK6) [5], and NE/PR3 [9] but were independent of

peptidyl arginine deiminase 4 (PAD4)-mediated citrullination [36] (Fig 2B). We also tested the requirement for *de novo* protein synthesis using the translational inhibitor cycloheximide. This drug, at a concentration that fully blocked synthesis of the cytokine IL-8 (Fig. S1D), had no effect on NET formation (Fig 2B), as previously reported for other stimuli [37].

To genetically confirm the role of proteases in heme NET induction, we purified peritoneal neutrophils from NE single and NE/PR3 double knockout mice. NE/PR3 -/- neutrophils failed to release extracellular chromatin, while NE -/- cells displayed a partial deficiency (Fig. 2C & D), demonstrating that these proteases have an essential non-redundant function in decondensing chromatin. In contrast, there was no difference in NET formation between PAD4 -/- and control neutrophils (Fig 2C & D).

Soluble NET components drive malaria pathology *in vivo*

To address the function of NETs in *Plasmodium* infections *in vivo*, we used NE/PR3 -/- mice as a NET deficient model. Additionally, to investigate the effect of a failure to degrade NETs extracellularly we used DNase 1 -/- mice. In the absence of DNase 1, NETs are made normally (Fig. 2C & D) but they persist at sites of release because they are not processed into soluble components [4]. DNase 1 -/- animals are deficient in dispersal of NET components and are a model to study the systemic effects of NET-associated alarmins.

We infected mice with the erythrocytic stages of *P. chabaudi*, a rodent malaria parasite that causes a non-lethal, two-week acute infection. Similar to *P. falciparum, P. chabaudi* iRBCs synchronously sequester in organs and induce pathology [38, 39]. We quantified NETs in plasma by detecting soluble complexes of DNA and the granular protein myeloperoxidase (MPO). We chose this granular component over NE in order to enable us to analyze NETs in NE deficient mice. NET components (Fig 3A)

and extracellular nucleosomes (Fig. 3B) increased in infected WT mice but were completely absent in NE/PR3 and DNase 1 -/- mice. This result is consistent with a failure to produce NETs in the case of NE/PR3 -/- animals and with a failure to solubilize the NET macrostructure in the case of DNase 1 -/-. Notably, parasitemia was similar in all three mouse strains (Fig. 3C), showing that NETs are not antiparasitic. As previously described [27, 38], parasitemia peaked between day 9 and 11 and was suppressed by day 13 post infection.

P. chabaudi sequesters in the liver and lungs where it induces tissue damage and immunopathology [38]. Livers from WT mice were severely darkened and discolored because of the accumulation of hemozoin and hepatocyte death (Fig. 3D). Remarkably, livers of infected NE/PR3 -/- and DNase 1 -/- mice were completely unaffected and indistinguishable from uninfected controls (Fig. 3D). Livers of WT, but not NE/PR3 or DNase1 -/- mice, showed necrosis and immune infiltration, characteristic malaria pathology, upon histological analysis of haematoxylin and eosin (H&E) stained sections (Fig. 3E and Fig. S2A). We confirmed the liver pathology in wild type, but not mutant mice, with the hepatic damage marker asparate aminotransferase (AST) in plasma (Fig. 3F). Tissue damage was also significantly reduced in lungs of NE/PR3 knockout mice (Fig. S3) compared to WT controls, although *P. chabaudi* causes only mild lung pathology [38]. These data demonstrate that release of components from NETs promotes organ pathology in malaria.

Exogenous NET components restore pathology in NET-deficient mice

To confirm that NETs are pathogenic in malaria, we injected mice with *in vitro* generated NET fragments. We chose NE/PR3 -/- as the NET-deficient strain in which to carry out this complementation experiment. We first purified peritoneal neutrophils from WT mice and induced them to form NETs. After washing, NETs were dislodged

by scraping and sonicated to obtain fragments, which were quantified and injected into the tail vein of control and *P. chabaudi* parasitized mice. Injection of NET fragments did not cause liver pathology in uninfected mice (Fig 3G) nor affect parasitemia in any of the infected genotypes (Fig. S4). Strikingly, restoring NET fragments in parasitized NE/PR3 -/- mice fully recapitulated the liver damage observed in WT mice (Fig 3G). This result demonstrates the direct pathogenicity of NETs and rules out a cell-autonomous effect of proteases as the cause of the protective effect in the knockout animals.

NETs contain multiple components with inflammatory activity [40]. These include the DNA backbone, as well as the protein fraction that contains many alarmins. Furthermore, extracellular nucleosomes and histones, which form a major portion of NETs, are inflammatory when found in the blood stream [41]. To identify which NET components are responsible for inducing pathology, we used recombinant DNase 1 to fully digest the DNA of the *in vitro* NET preparation, leaving only the protein components. Notably, the NET protein fraction was sufficient to induce liver damage in NE/PR3 null mice (Fig 3G). As a control, we also injected mouse nucleosomes purified from bone marrow derived macrophages, which failed to induce AST release after injection (Fig 3G). These data show that the pathogenic activity derives from either a NET-specific protein or a NET-specific protein modification.

NETs induce emergency granulopoiesis via GCSF induction

Neutrophils cause tissue destruction due to the cytotoxic molecules they carry. We quantified neutrophil infiltration into the livers of parasitized mice using immunofluorescence staining of the intracellular neutrophil marker calgranulin. Neutrophils accumulated in the livers of WT, but not NE/PR3 and DNase 1 -/- animals (Fig4 A and Fig. S5), consistent with neutrophils being initiators of hepatic pathology.

We quantified systemic neutrophil numbers to determine why both deficient mice genotypes failed to recruit neutrophils into the liver. In malaria, like other infections, the number of circulating neutrophils increases due to emergency granulopoiesis in the bone marrow [42-44]. We observed that *P. chabaudi* infection leads to neutrophilia in WT mice but not in NE/PR3 and DNase1 knockouts (Fig 4B).

The major mediator of emergency granulopoiesis is GCSF [45]. We speculated that NETs directly induce GCSF. To test this, we stimulated macrophages - a significant physiological source of this cytokine - with NETs *in vitro*. NETs robustly induced production of GCSF in monocyte-derived human macrophages (Fig 4C).

In *P. chabaudi* infected WT mice, the concentration of GCSF in plasma increased with rising parasitemia; however, there was no increase in either NE/PR3 or DNase 1 -/- mice (Fig 4D). To directly demonstrate that NETs induce GCSF *in vivo*, we showed that injection of NET fragments fully restored GCSF production in NE/PR3 mice to levels seen in WT mice (Fig 4E). As with the liver damage marker AST, GCSF production was induced by the protein component of NETs, as complete removal of DNA prior to injection did not abrogate the effect. These data show that NET-associated alarmins drive emergency hematopoiesis by inducing GCSF release.

NETs promote parasite sequestration in organs

Malaria tissue pathology is linked to parasite sequestration in the microvasculature of afflicted organs. The lack of discoloration in livers of infected NE/PR3 and DNase 1 -/- mice (Fig 3D) suggests a lack of parasite adhesion. To directly analyze sequestration, we infected mice with a luciferase-expressing strain of *P. chabaudi* [38] and quantified parasite load in organs after perfusing animals to remove unbound, freely-circulating parasites.

As reported, *P. chabaudi* sequestered most prominently in the liver and the lung and, to a lesser degree, in the kidneys [38]. Remarkably, there were tenfold fewer parasites sequestered in the livers and lungs of NE/PR3 and DNAse 1 -/- mice compared to WT controls (Fig 5A). We confirmed this sequestration pattern by histological enumeration of schizonts in the liver microvasculature (Fig. 5B and Fig. S2B) as well as by electron microscopy (Fig. S2C).

NETs induce upregulation of ICAM-1

The difference in abundance of neutrophils in livers of WT and knockout animals was greater than the difference observed in peripheral blood, indicating that, in addition to emergency granulopoiesis, NETs regulate neutrophil trafficking. Interestingly, both neutrophils [46] and parasites [47, 48] use the same receptor to dock to endothelial cells: intercellular adhesion molecule 1 (ICAM-1). We analyzed entire liver sections by immunofluorescence and observed upregulation of ICAM-1 in infected WT but not NE/PR3 or DNase1 -/- mice, coinciding with the onset of liver damage (Fig. 5C). We also measured soluble ICAM-1 in plasma by ELISA and found no induction of this adhesion receptor in NE/PR3 -/- compared to WT animals (Fig. 5D), confirming our microscopy results. Injecting *in vitro* generated NET fragments into parasitized NE/PR3 -/- mice restored the expression of ICAM-1 (Fig. 5D), demonstrating that NET components control ICAM-1 expression.

Neutralizing GCSF antibodies decrease liver damage

To test whether GCSF-induced neutrophilia is pathogenic, we injected parasitized animals with an anti-GCSF antibody two days before the parasitemia peak. The neutralizing antibody significantly decreased liver damage compared to the isotype control (Fig. 6B). As expected, the treatment did not impact parasite burden (Fig. 6A).

Notably, the GCSF concentration in plasma of malaria patients is significantly increased in infected individuals (Fig 6C), as previously reported [49]. Interfering with GCSF signaling can therefore alleviate malaria pathology.

Discussion

Malaria pathophysiology is based on an interplay of parasite proliferation, host inflammatory response and microvascular obstruction due to binding of iRBCs to activated endothelia. Despite important recent advances [16, 17, 24, 27, 30], the contribution of neutrophils to these processes remains poorly characterized. Here we demonstrate that neutrophils play an essential role in both propagation of inflammation and facilitation of parasite cytoadherence.

Firstly, we showed that in malaria, as in sickle-cell disease [33], extracellular heme triggers NETosis in TNF-primed neutrophils. Heme-induced NETs require some of the same signaling intermediates demonstrated for other NET inducers, including neutrophil proteases [8], CDK6 [5] and PKC [35]. Heme is known to activate PKC in neutrophils, initiating chemotaxis and IL-8 production [50]. TNF priming thus provides a synergistic signal required for NETosis; this signal is posttranslational, since it is not blocked by the translational inhibitor cycloheximide. Heme-induced NETs are independent of the citrullinating enzyme PAD4, which is implicated in ionophore-induced NETs [36] and of the oxidant-generating enzyme NOX2 [34], which is known to be suppressed in neutrophils from malaria patients [24]. ROS signaling is nevertheless required for heme NETs, as the response is blocked by the ROS scavenger pyrocathecol. This may be due to the fact that heme is itself a redox-active molecule, with multiple mechanisms for initiating and propagating free radicals

[51]. In summary, heme utilizes a unique pathway for NET induction that nevertheless requires both protease activity and ROS signaling.

NETs are essential for malaria pathology in the *P. chabaudi* model. We showed that solubilization of NETs by serum Dnase 1 liberates immunostimulatory components that diffuse systemically and are pathogenic via two mechanisms. The first is induction of GCSF in macrophages, which initiates emergency granulopoiesis. This corresponds to what is seen in patients, where GCSF is elevated in both *P. falciparum* [49] and *P. vivax* infections [52]. Moreover, neutrophil turnover is often higher in malaria [42-44] and, in children living in endemic areas, increased neutrophil counts correlate with symptoms of severe disease, such as prostration, coma and respiratory distress [53-55]. In a second mechanism, NET components upregulate ICAM-1, a key cytoadhesion receptor that sequesters parasitized RBCs in the microvasculature [47, 48]. Interestingly, in *P. falciparum* malaria, ICAM-1 mediates cytoadhesion in the brain and is a key mediator of cerebral malaria [48, 56]. Antibodies against ICAM-1 binding variants of *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1), the main parasite cytoadhesion factor, protect against clinical disease [57, 58]. NET induction of ICAM-1, which facilitates *P. chabaudi* adhesion in the liver, may thus operate in human malaria to recruit *P. falciparum* iRBCs to the brain, a far more dangerous sequestration site. This is consistent with recent reports showing an association between neutrophil proteins and cerebral malaria [16, 26].

NET components are also required for pathology and *P. chabaudi* sequestration in the lungs, demonstrating that this mechanism may be broadly generalizable to different vascular beds. Respiratory distress significantly contributes to morbidity and mortality of malaria. Furthermore, in the *P. chabaudi* model, lung sequestration is mediated by an unidentified receptor other than ICAM-1 [47], indicating that NET components may be upregulating more than one cytoadhesion molecule.

Our data demonstrate the essential role of DNAse 1 in releasing pathogenic NET fragments in malaria. NETs are thought to be anchored to the endothelium after release [59, 60], through von Willebrand factor [59] and probably other mediators. Serum DNAse 1 allows systemic diffusion of NET components, as demonstrated by absence of NET fragments in DNase 1 knockouts. A similar pathogenic function of DNAse 1 was shown in a polymicrobial sepsis model, where injection of recombinant DNase 1 promotes liver damage and neutrophil accumulation in liver and lung [61]. However, DNase 1 has contradictory roles in inflammation; in thrombosis [62, 63], cancer [64] and SLE [4], this endonuclease is protective rather than pathogenic. In non-microbial inflammation it is therefore the unprocessed NET 'macrostructure' that is detrimental, while in infections such as malaria and sepsis, it is the discrete molecular components of NETs that cause disease.

The NET proteins that induce GCSF and ICAM-1 remain unknown. Many proteins found on NETs are classified as alarmins [65]; including α-defensins, cathelicidin, calgranulin and lactoferrin [66]. Once released, these alarmins can induce maturation and activation of other immune cells such as dendritic cells, T cells, macrophages and endothelial cells [65]. Additional experiments will determine which molecules are responsible for triggering granulopoiesis and endothelial activation.

NET-associated molecules are necessary but not sufficient to drive inflammation in malaria, as shown by the injection of NET fragments into uninfected mice. Additional stimuli, most likely *Plasmodium* PAMPs or DAMPs [23] are required to initiate emergency hematopoiesis and liver damage. Furthermore, inflammation in malaria is complex with type-I interferons [27], CD8+ T cells [38, 67] and hemostasis [68] contributing to immunopathogenesis. Further work is required to understand how neutrophils crosstalk with other cell types in initiating disease.

Malaria exerts an evolutionary selective pressure on populations living in endemic areas, selecting for gene variants that promote tolerance [69]. Interestingly, people of African descent and some ethnic groups from the Middle East, have low neutrophil counts [70]. This “ethnic or benign neutropenia” could be the result of selective pressure to suppress neutrophil counts since they are detrimental in this disease. Strikingly, in addition to Duffy antigen, the loci linked to ethnic neutropenias include *CDK6* and *GCSF [71]*, both of which are directly involved in the NET-mediated pathogenic mechanism described here.

Recent studies confirmed the central role of heme in the pathophysiology of malaria [72]. Moreover, extracellular heme accumulation is not limited to malaria; it is a confirmed pathogenic factor in sepsis, sickle cell disease, intracerebral hemorrhage and atherosclerosis [51], all of which also have reported neutrophil involvement. It would therefore be interesting to examine if emergency hematopoiesis and endothelial activation triggered by NET fragments are universal outcomes in intravascular hemolytic diseases.

Adjunctive therapies that treat the life-threatening complications of malaria are urgently needed. We show that NETs control inflammation and parasite cytoadherence, placing neutrophils at the nexus of malaria pathophysiology and identifying them as a potential target for adjunctive therapy.

Materials and Methods

Antibodies, staining reagents, stimuli and inhibitors

Anti-human-NE-Biotin (Ab79962, abcam), anti-mouse-MPO-Biotin (HM1051BT, Hycult), anti-DNA-POD (Cell Death Detection ELISA Kit, Roche), anti-mouse-ICAM (AF796, Novus Biologicals), anti-mouse-Calgranulin (MPIIB, in house), anti-chromatin (PL2-3, [73]), Hoechst (639, Immunochemistry), PMA (P8139, Sigma), Heme (H651-9, Frontier Scientific), murine TNF (315-01A, Peprotech), human TNF (300-01A, Peprotech), PKC inhibitor (Go6976, Tocris), pyrocatechol (C9510, Sigma), Cycloheximide (Sigma-Aldrich), Cdk4/6 inhibitor (LY2835219, Selleck), PAD4 inhibitor (TDFA, Tocris), NE inhibitor (GW311616A, Sigma-Aldrich), Luminol (11050, AAT-Bioquest), horse radish peroxidase (HRP, 31941, Serva), Bright-Glo Luciferase Substrate (E2610, Promega)

ELISA Kits

Cell Death Detection ELISA Plus (11920685001, Roche Diagnostics), Human Interleukin 8 Quantikine ELISA (S8000C, R&D Systems), mouse GCSF Quantikine ELISA (MCS00, R&D Systems), human GCSF Quantikine ELISA (SCS50, R&D Systems), mouse ICAM-1 ELISA (DY796, R&D Systems)

Human samples

Our study was conducted in accordance with the Helsinki Declaration. Blood donations from healthy donors and CGD patients were collected after obtaining informed consent. Histopathological brain samples were collected anonymously at Charité University Hospital, Berlin, from autopsies of travellers returning from malaria endemic areas. Both blood and pathology sample collection was approved by the ethical committee of Charité University Hospital, Berlin, Germany.

Collection of blood from malaria patients was approved by the Comité d'Ethique Régional Indépendent de Lambaréné in Gabon. All study participants provided written informed consent before being enrolled in the study.

Mice

Mouse breeding, infections and isolation of peritoneal neutrophils were approved by the Berlin state authority *Landesamt für Gesundheit und Soziales*. Animals were bred at the Max Planck Institute for Infection Biology. Mice were housed in specific pathogen free (SPF) conditions, maintained on a 12-hour light/dark cycle and fed *ad libitum*. NE -/- were decribed here [74] and NE/PR3 -/- mice here [75] PAD4 -/- [76] were a kind gift of Denisa Wagner. Production of Dnase 1 -/- mice will be described shortly (Kenny et al, submitted).

Human Neutrophil isolation and stimulation

Cells were purified by a first centrifugation of whole blood over Histopaque-1119 (Sigma) followed by a discontinuous Percoll gradient (20). All experiments were done in RPMI-1640 (w/o phenol red, Gibco) supplemented with 10mM HEPES and 0.05% human serum albumin. 10^5 Cells were seeded onto glass coverslips in a 24 well plate and incubated with inhibitors for 30 min, followed by 15 min priming with TNF and addition of the stimuli.

Luminol assay

To assess ROS production, $1x10^6$ neutrophils were activated (after treatment with inhibitors/ ROS scavengers) with 100 nM PMA. ROS production was measured by monitoring luminol (50 µM) luminescence in the presence of 1.2U/ml horseradish peroxidase.

Mouse neutrophil isolation and stimulation

Murine neutrophils were isolated from peritoneal cavities of WT, NE -/-, NE/PR3 -/-, PAD4 -/- and DNase1 -/- mutant animals after injection of casein (Sigma) intraperitoneally by centrifugation over Percoll [77].

Cells were seeded onto glass coverslips at 10^5/well in 24 well plates in RPMI (Gibco) containing penicillin/streptomycin (Gibco) and glutamine (Gibco), 1% murine DNase 1 -/- serum and 100 ng/ml murine G-CSF (Peprotech). After 30 min settling and 15 min TNF priming, cells were stimulated with 100 nM PMA or 20 µM heme. NETs were quantified after 18 hours stimulation

Quantification of NET formation

The quantification of NETosis was carried out as previously described [78]. Briefly, cells were fixed for 30 minutes at room temperature in 2 % paraformaldehyde (PFA), permeabilized with 0.5 % Triton-X100 and blocked for 30 minutes in blocking buffer. Cells were then stained with the anti-neutrophil elastase antibody (see above) and antibody directed against the subnucleosomal complex of Histone 2A, Histone 2B and chromatin [73], as well as secondary antibodies goat anti-mouse Alexa Fluor 568, goat anti-rabbit Alexa Fluor 488 and Hoechst 33342 (Sigma-Aldrich). Samples were mounted on coverslips with Mowiol. Image acquisition was done using a Leica DMR upright fluorescence microscope equipped with a Jenoptic B/W digital microscope camera and analyzed using ImageJ/FIJI software.

Heme preparation

Heme for *in vitro* stimulation of neutrophils and for standard curve was prepared fresh on the day of the experiment. 10 mM stock solution was prepared by dissolving 0.0325 g Hemin (H651-9, Frontier Scientific) in 5 ml DMSO.

Quantification of plasma heme

Heme was quantified using the formic acid assay [79]. Briefly, samples were diluted 1:50 in H_2O in white 96 well plates. The heme concentration was determined after the addition of formic acid (150 µL/well, 100 %, Merck) to all samples and absorbance measurement at 405nm using a microplate reader. Measurements were compared to a hemin standard curve in the range of 0.25 – 16 µM in H_2O.

***P. falciparum* culture**

P. falciparum parasites were cultured using standard procedures as described previously [80] . Parasites were grown at 5% hematocrit in RPMI 1640 medium, 0.5% AlbuMax II (Invitrogen), 0.25% sodium bicarbonate, and 0.1 mg/ml gentamicin. Cultures were incubated at 37°C in an atmosphere of 5% oxygen, 5% carbon dioxide, and 90% nitrogen.

Trophozoite and merozoite preparation

The mature-stage *P. falciparum* culture was washed and taken up in 2 ml of RPMI and layered onto 5 ml of a 60% Percoll solution. The mixture was centrifuged and trophozoites were collected at the interphase between RPMI and Percoll, while uninfected RBCs and ring stage infected RBCs were pelleted. Parasites were washed three times with RPMI and iRBCs were pelleted. For merozoite isolation iRBCs were lysed with 0.03 % saponin solution. Subsequently the sample was centrifuged, washed

three times with PBS and taken up in RPMI. Concentration of merozoites was determined by use of a Neubauer chamber.

Isolation of digestive vacuoles from *P. falciparum*

Late trophozoite cultures with 10 % parasitemia were first exposed to saponin (0.5 µg/ml) lysis, washed in PBS and parasites were then lysed in ice-cold H_2O and by passage through a 27 G needle. This preparation was spun down, resuspended in uptake buffer (pH 7.4, 2mM $MgSO_4$, 100 mM KCl, 25 mM HEPES, 25 mM $NaHCO_3$ and 5mM Na_3PO_4) and separated by density using 42 % Percoll. The DV could be collected at the bottom, washed and used in subsequent experiments.

***P. chabaudi* infection, plasma and tissue preparation**

Male mice aged 8-15 weeks of all genotypes were infected by intravenous injection of viable *P. chabaudi* AS parasites (WT) or PccASluc (luciferase-expressing; [38]). To ensure viability of the parasites, a frozen aliquot was thawed and injected intraperitoneally (i.p.) into a transfer mouse. The number injected into each mouse was adjusted according to body weight so that every animal received $1x10^4$ iRBCs per 20 g.

Parasitemia of infected mice was monitored from day 5 post infection onward every 48 hours by Giemsa-stained thin blood smear.

Mice were bled by cardiac puncture under non-recovery deep anesthesia. Blood was kept from coagulating by addition of 50 µM final concentration of EDTA. Plasma was generated by centrifugation at 10,000 x g at 4°C for 10 minutes. Plasma was aliquoted, snap frozen in liquid nitrogen and stored at -80°C until further use. Plasma was always thawed on ice.

Organs were harvested without additional perfusion (except in parasite sequestration experiments) as blood was removed by terminal bleeding of the animals. The organs were fixed for 20h at room temperature in 2% PFA.

Immunohistochemistry

The blinded scoring of liver pathology as well as the counting of parasites sequestered in the microvasculature of the livers was performed by trained pathologists at the iPATH-Berlin Core Unit for immunopathology of experimental model organisms from H&E stained Paraffin sections of 1 µm thickness.

The scores were defined as follows:

Hepatitis (Malaria)
Modified according to [81].

Histopathologic changes	Histopathologic grading			
	0	1	2	3
Fatty change	No fatty change	< 10%	10-50%	> 50%
Kupffer cells/HPF	< 20/HPF	20-35/HPF	36-50/HPF	> 50/HPF
Portal tract inflammation	< 5% of portal tract area	5-15% of portal tract area	16-30% of portal tract area	> 30% of portal tract area
Bile duct proliferation	No proliferation	Mild proliferation	Moderate proliferation	Severe proliferation
Sinusoid congestion	No congestion	Mild congestion	Moderate congestion	Severe congestion
Haemozoin deposition	No deposition	Mild deposition	Moderate deposition	Severe deposition
Necrosis	none	<10%	11-25%	>25%

Acute Lung injury
Modified according to [82].

0, thin and delicate alveolar septae, no intra-alveolar fibrin strands or hyaline membranes and <5 intra-alveolar cells, no perivascular or peribronchial infiltrates

1, mildly congested alveolar septae, few fibrin strands or hyaline membranes and <10 intra-alveolar cells with mild perivascular and/or peribronchial infiltration
2, moderately congested alveolar septae, some fibrin strands or hyaline membranes, <20 intra-alveolar cells with moderate perivascular and/or peribronchial infiltration
3, severely congested alveolar septae, many fibrin strands and presence of hyaline membranes, >20 intra-alveolar cells with severe perivascular and/or peribronchial infiltration

Immunofluorescence of tissue sections

Mouse tissue was fixed in 2% paraformaldehyde solution in Tris-buffered saline (TBS, pH 7.4) for 20 hours at room temperature. The tissue was then dehydrated and paraffin-embedded (60°C) using a Leica TP 1020 tissue processor.

Paraffin blocks of both mouse and human samples were cut to 3 µm and sections were mounted and dried on Superfrost Plus slides (Thermo Scientific) avoiding temperatures above 37°C. After dewaxing and rehydration, sections were incubated in HIER buffer pH6 (citrate buffer) [20 minutes at 96°C in a steam cooker (Braun)].

After antigen retrieval, sections were left in the respective HIER buffer at room temperature to cool below 30°C, rinsed three times with deionized water and once with PBS pH 7.4, and permeabilized for five minutes with 0.5% Triton-X100 in PBS at room temperature, followed by three rinsing steps with PBS.

Sections were surrounded with PAP-pen and treated with blocking buffer for 30 minutes to prevent non-specific binding. Primary antibodies were diluted in blocking buffer and incubated on the sections over night at room temperature. We used secondary antibodies raised in donkey and pre-absorbed against serum proteins from multiple host species (Jackson Immuno Research). Dilution and blocking was done in

PBS supplemented with 1% BSA, 2% donkey normal serum, 5% cold water fish gelatin, 0.05% Tween 20 and 0.05%Triton X100.

Slides were mounted using Mowiol and digitized with a ZEISS AxiosScan.Z1. This is an automated microscope that generates a series of overlapping photographs which are assembled to a single image of a complete organ section. Using the software package Volocity 6.3 the tissue area positive for Calgranulin A and ICAM-1 was determined and expressed as percentage of the total tissue area of the respective section.

NET ELISA

NETs in circulation were determined as NE/DNA complexes in human samples and MPO/DNA complexes in mouse samples. For the mouse ELISA; the biotinylated primary AB mouse-MPO (1µg/ml final concentration) was coated onto a streptavidin coated plate at 4°C over night and the plates were blocked for 2h with 1 % BSA in PBS. For the human ELISA we used the precoated and blocked plates of the Hycult human NE ELISA (HK319-02). Samples were incubated for 2h at room temperature with 350 rpm agitation. The anti-DNA-POD antibody was diluted 1:100; plate was incubated for 2h at room temperature, followed by signal development using TMB substrate and H_2SO_4 stopping. Signal was acquired at 450 nm.

Determination of liver enzyme concentration in mouse plasma

The concentration of the hepatocyte specific enzyme aspartate-aminotransferase in the plasma of experimental animals were determined by the routine veterinarian service laboratory at SYNLAB.vet GmbH (Turmstraße 21, 10559 Berlin).

FACS analysis of mouse whole blood

100 µl of mouse whole blood were stained directly by addition of 100 µl of FACS antibodies diluted 1:100 in PBS (2.5 % FCS, 0.1 % NaN_3) for 30 minutes. Cells were treated with 3 ml 1-Step Fix/Lyse (00-5333-54) for 60 min at room temperature, spun down, washed and taken up in 250 µl FACS buffer prior to analysis using a MACSQuant Analyser.

Antibodies all from BD: V500 anti-CD45 (561487), FITC anti-CD3(561798), APC anti-CD8a (561093), PECy7 anti-CD4 (552775), PE anti-CD115 (565249), PerCP-Cy5.5 anti-Ly6G/C (561103)

Assessment of sequestration of luciferase-expressing parasites

Mice were infected with a luciferase-expressing strain of *P. chabaudi* (PccASluc [38]) as described above but kept on a reverse light cycle, as sequestration occurs during the dark cycle [38]. At the time of maximum sequestration (12.00 – 14.00 h coordinated universal time (UCT), reverse light) mice were sacrificed and perfused systemically by injection of 10 ml PBS into the heart. Organs were harvested and 0.1 g of tissue was transferred to a Precellys homogenizer tube in PBS and dissociated for one cycle, 10 seconds at 4500 rpm in a Precellys Evolution Homogenizer. The sample was then diluted 1:10 in PBS, and an equal volume of Bright-Glo substrate (Promega, 100 µL) was added. Luciferase activity was measured after 2 min incubation using a Perkin Elmer VICTOR X Light Multilabel Plate Reader.

Injection of exogenous NETs

Murine NETs were prepared from WT peritoneal neutrophils as described above, washed twice with PBS to remove residual PMA, scraped from the plate and sonicated for 15 seconds at 70 % Power using a Bandelin SONOPLUS sonicator. DNA concentration was quantified by PicoGreen assay (P11496, Thermo Fisher Scientific)

and NanoDrop measurements . Where applicable sample was then treated with 2 U DNase 1 from TURBO DNA-free Kit (AM1907, ThermoFisher Scientific) overnight at 37°C. The kit was chosen because it contains a DNase inactivating agent, which was used according to manufacturers specifications to ensure that no DNase activity was introduce into injected mice. Complete digestion of DNA was confirmed both by agarose gel electrophoresis and PicoGreen measurement. Mice were injected with an amount of NETs and chromatin previously observed to have accumulated in infected WT mice, which was 300 ng/ml of blood. We assumed a blood volume of an adult male mouse of 1.5 ml and therefore injected 450 ng of either NETs or chromatin into each mouse.

Chromatin was isolated from bone marrow derived macrophages, which were prepared according to standard protocol [83] and chromatin was prepared as previously described [84]. Briefly, when cells were confluent they were harvested, washed and counted, per 5 x 10^6 cells 300 µl of hypotonic buffer A (10 mM HEPES, pH 7.5, 10 mM KCl, 3 mM NaCl, 3mM $MgCl_2$, 1 mM EDTA, mM EGTA and 2 mM dithiothreitol and a general protease inhibitor cocktail (78430, ThermoFisher Scientific)) was added and incubated on ice for 15 minutes. Subsequently 0.05 volumes of 10 % Nonidet P-40 were added, the cells were vortexed and centrifuged at 500 x g for 10 minutes at 4°C. The supernatant was discarded, the nuclei in the pellet washed in buffer A and subsequently resuspended in 50 µl of ice-cold buffer NE (20 mM HEPES, pH 7.5, 25 % glycerol, 0.8 mM KCl, 1mM $MgCl_2$, 1 % Nonidet P-40, 05. mM EDTA, 2 mM dithiothreitol). Following a 20 minutes incubation on ice with occasional mixing the samples were centrifuged at 14,000 x g for 15 minutes at 4°C. The supernatant was discarded and the pellet containing the chromatin resuspended

in ddH_2O. Chromatin concentration was determined by Picogreen assay (see above) and samples were stored at -80°C.

Macrophage stimulation with NETs

Monocytes were isolated by magnetic CD14 positive selection (130-050-201, Miltenyi Biotec) and differentiated for 7 days into macrophages in RPMI 1640 containing P/S, Glutamine and 5 ng/ml human MCSF. At the day of the experiment, $3x10^6$ neutrophils were stimulated for 4 h with 100 nM PMA. The resulting NETs were washed three times with PBS, harvested by scraping and sonicated. The NET concentration was determined by Picogreen assay. Macrophages were stimulated for 12 h with 1 µg/ml isolated NETs. Supernatants were collected and GCSF was quantified by ELISA.

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