Neutrophil extracellular traps drive inflammatory pathogenesis in malaria

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ABSTRACT

Malaria is the disease caused by an infection of a mammalian host by the mosquito borne eukaryotic parasite *Plasmodium*. The associated morbidity and mortality are a major burden on endemic regions and clearly related to poverty and instability. The symptoms of the disease are diverse, ranging from fever and rigor in most patients to severe damage in solid organs such as brain, lung, kidney and liver in a small fraction of the afflicted. Clinical symptoms of the disease only occur when the parasite undergoes asexual replication within the red blood cells of the host. Destruction of these cells and subsequent release of cytokines are responsible for the recurring fever cycles of mild malaria. The mechanism underlying the occurrence of tissue damage however, remain mostly elusive. The adhesion of infected red blood cells to the endothelial wall of the microvasculature in the affected organs is a necessary requirement and pathology is associated with the activation of specific immune cells residing within the blood stream. Severity of disease is linked to extracellular accumulation of neutrophil proteins. Neutrophils are abundant white blood cells, known to readily deploy an arsenal of weaponry either by degranulation or by externalization of chromatin.

In this study we report a direct causal relationship between the active inflammatory neutrophil cell death (NETosis) and the development of organ damage during a *Plasmodium* infection. We show that NETs are released in circulation, digested by extracellular DNase and thereby supply immune activation signals that drive inflammation. The systemic dissemination of these factors leads to the release of cytokines, emergency granulopoiesis and upregulation of cellular adhesion markers on endothelial cells thereby allowing for the binding of both infected red blood cells and immune cells to the microvasculature of specific organs. Furthermore we supply evidence, that repression of NETosis or inhibition of granulopoiesis abrogate these processes and present promising therapeutic strategies.

ZUSAMMENFASSUNG

Malaria ist die Erkrankung, die durch Infektion eines Säugetiers mit dem eukaryotischen Parasiten *Plasmodium* entsteht. Die hiermit einhergehende Morbidität und Mortalität sind eine enorme Belastung für die endemischen Gebiete und eindeutig mit Armut und Instabilität derselben assoziiert. Die Symptome dieser Erkrankungen reichen von Fieber und Gelenkschmerzen bis zu schweren Organschäden in Hirn, Lunge, Niere und Leber bei einem geringen Teil der Erkrankten. Diese klinischen Symptome treten nur auf, während der Parasit sich asexuell in roten Blutzellen vermehrt. Die Zerstörung von Erythrozyten und die daraus resultierende Freisetzung von Zytokinen sind die Verursacher der malariatypischen wiederkehrenden Fieberzyklen. Der Mechanismus, der zur Entstehung von Gewebsschäden führt, ist hingegen nur unzureichend bekannt. „Eine notwendige Bedingung für das Auftreten von Gewebeschäden ist, dass infizierte rote Blutzellen an das Endothel der Mikrovaskulatur bin. Des Weiteren ist Organpathologie mit der Aktivierung bestimmter Immunzellen innerhalb des Blutstroms korrelierbar. Die Schwere der Erkrankung ist direkt mit dem extrazellulären Auftreten von Stoffen verbunden, die normalerweise von neutrophilen Granulozyten im Zellinnern gespeichert werden. Neutrophile sind reichlich vorkommende weiße Blutzellen, die dafür bekannt sind, ein ganzes Arsenal an Waffen bereitwillig durch Degranulierung oder programmierten Zelltod einzusetzen.

In dieser Studie berichten wir von einem direkten kausalen Zusammenhang zwischen dem aktiven inflammatorischen Zelltod (NETose) von Neutrophilen und der Entstehung von Organschäden bei einer *Plasmodium*-Infektion. Wir zeigen, dass NETs in Zirkulation freigesetzt und von extrazellulären DNase verdaut werden. Dadurch werden systemisch Aktivierungssignale für eine weitere Immunantwort zur Verfügung gestellt und es kommt zur Freisetzung von Zytokinen, Notfallgranulopoese und der Hochregulierung von zellulären Adhäsionsmarkern auf Endothelzellen. Dies erlaubt das Binden von infizierten Erythrozyten und Immunzellen an die Mikrovaskulatur bestimmter Organe. Wir zeigen außerdem, dass eine Intervention mit der Entstehung von NETs oder der Freisetzung neuer Neutrophiler diesen Prozess unterbindet und einen vielversprechenden therapeutischen Ansatz darstellt.

ABBREVIATIONS

NE	Neutrophil Elastase
WHO	World Health Organization
DAMP	Danger associated molecular pattern
PAMP	Pathogen associated molecular pattern
DNA	Deoxyribonucleic acid
Pr3	Proteinase 3
CG	Cathepsin G
TLR	Toll-like receptor
RBC	Red blood cell
DV	Digestive vacuole
PfEMP	*Plasmodium falciparum* erythrocyte membrane protein
VCAM	Vascular cell adhesion molecule
ICAM	Intercellular adhesion molecule
EPCR	Endothelial protein C receptor
IL	Interleukin
TNF	Tumor necrosis factor
IFN	Interferon
CM	Cerebral malaria
SMA	Severe malarial anemia
GPI	Glycosylphosphatidylinositol
G-CSF	Granulocytes colony stimulating factor
CLR	C-type lectin receptors
ROS	Reactive oxygen species
NETs	Neutrophil extracellular trap
MPO	Myeloperoxidase
CGD	Chronic granulomatous disease
CDK	Cyclin dependent kinase
SVV	Small Vessel Vasculitis
ANCA	Anti-Neutrophil Cytoplasmic Antibodies
SLE	Systemic lupus erythematosus
DC	Dendritic cells
MMP	Metalloproteinase
TF	Tissue factor

TFIP	Tissue factor pathway inhibitor
APC	Activated protein C
ELISA	Enzyme linked immunosorbent assay
GBS	Group b *streptococcus*
PKC	Protein kinase C
MEK	Mitogen-activated protein kinase kinase
ERK	Extracellular signal activated kinase
PAD	Peptidylargininedeaminase
CHOP	C/EBP-homologous protein
IFA	Immunofluorescence analysis
PMA	Phorbol 12-myristate 13-acetate
LPS	Lipopolysaccharide
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
WT	Wild Type
BMM	Bone marrow derived macrophages
GPCR	G-protein coupled receptor
HO-1	Heme oxygenase 1
LTB4	Leukotriene B4
FCS	Fetal calf serum
NSP	Neutrophil serine protease
PBMCs	Peripheral blood mononuclear cells

INTRODUCTION

1. The parasite *Plasmodium*

Malaria is the disease resulting from infection of a mammalian host by parasites of the genus *Plasmodium*. While malaria was once endemic even in southern Europe it is now restricted to a broad band around the equator including wide areas in South America, Asia and Africa. Malaria remains a devastating disease that is very clearly associated with poverty and has a major negative impact on the economic, social and political stability of endemic regions.
Despite enormous efforts of humanitarian organizations such as the WHO and the Bill and Melinda Gates foundation the number of newly infected cases remain in the hundreds of millions (approximately 200 Mio. in 2017) each year, including up to 500.000 mortalities annually (Varo, Crowley et al. 2018). The highest risk of severe disease remains with children under the age of 15 years of age in sub-Saharan Africa. Pregnant women are also at very high risk of disease and pregnancy associated malaria can lead to infant death as well as impaired learning and cognitive abilities in children born from mothers infected with *Plasmodium falciparum.*

1.1 The plasmodium parasite and its life cycle

Of the more than 200 described species of *Plasmodium* the five that cause human disease are *Plasmodium knowlesi, ovale, vivax, malariae and falciparum*. The latter is responsible for the majority of infections (~ 75%) and nearly all deaths associated with malaria. *Plasmodia* are protists – a unicellular eukaryote – of the phylum Apicomplexa. These parasites display a complex life cycle in which a vertebrate host and a vector – mostly mosquitoes - are infected. *Plasmodium* infects several different cell types, develops different morphologies and affects different niches within the host (Fig. 1).

1.1.1 Entry and infection of the liver

The infection of a mammalian host by *Plasmodium* starts with the bite of a female infected mosquito as it takes a blood meal. Prior to transmission the parasite resides in the salivary glands of the mosquito and upon biting is injected into the wound along with the insect saliva that prevents blood clotting. The sporozoites find a blood vessel and travel to the liver where they establish a primary infection in hepatocytes. In this clinically silent liver stage the parasite undergoes asexual replication and eventually thousands of merozoites are released into the blood stream (Fig. 1).

1.1.2 The blood stage of *Plasmodium*

The merozoites released from the liver find and attach to red blood cells (RBCs). This initial contact is mediated by a range of surface molecules including members of the major surface protein (MSP) family, the reticulocytes binding protein homolog (PfRh) and PfAMA1 and leads to invasion of the RBC. Throughout this process, the parasite sheds most of its surface receptors and establishes the parasitophorous vacuole in which it grows under optimal conditions. Astonishingly, the process of invasion takes only around 60 seconds and it is the only time where parasites themselves are directly exposed to the immune system (Waldron 2015).

Once the parasite arrives within its niche it enters into young trophozoite stage which is also referred to as the ring stage because of its morphology (Fig. 1). The parasite then continuously grows within the infected red blood cell (iRBC) and eventually undergoes schizogonic division, meaning that DNA replication occurs first and cellular segmentation only occurs when merozoites are formed. In the case of *P. falciparum* this process takes 48 hours, whereas some of the commonly used murine parasites such as *Plasmodium chabaudi* display a 24 hour cycle (Brugat, Cunningham et al. 2014)

The parasite uses the main protein that is abundantly available in erythrocytes - hemoglobin - as a food source eventually digesting up to 80 % of what used to be stored in the RBC. The undigested hemoglobin is released into circulation as the RBC lyses. The breakdown of the hemoglobin protein structure by parasite aspartic acid proteases leads to the release of free heme,

which possesses an enormous redox potential and is therefore capable of generating oxygen radicals such as hydroxyl and hydroperoxyl radicals. It is highly cytotoxic when not regulated within the protein structure (Ferreira, Balla et al. 2008). The parasite detoxifies this prosthetic group by transferring it into a waste management vacuole (digestive vacuole, DV) where it is packaged into crystals called hemozoin and which is referred to as the malaria pigment (Wunderlich, Rohrbach et al. 2012). Hemozoin is easily recognizable by its black color when it accumulates in immune cells and organs of infected individuals.

Eventually 16 to 32 merozoites per iRBCs (Grüring, Heiber et al. 2011) are released into the blood stream. While this process is poorly understood, it is hypothesized that a sudden increase in intracellular pressure leads to the explosive lysis of the iRBCs which serves to disperse the non-motile merozoites and ensure optimal reinfection of RBCs. This process also releases the remaining contents of the iRBC and the parasitophorous vacuole, thereby providing ample opportunity for the activation of surrounding immune cells.

Before returning to the vector, a small portion of the parasites undergo sexual development and transform into male and female gametocytes, which can be taken up by a female mosquito taking a blood meal from an infected mammalian host (Fig. 1).

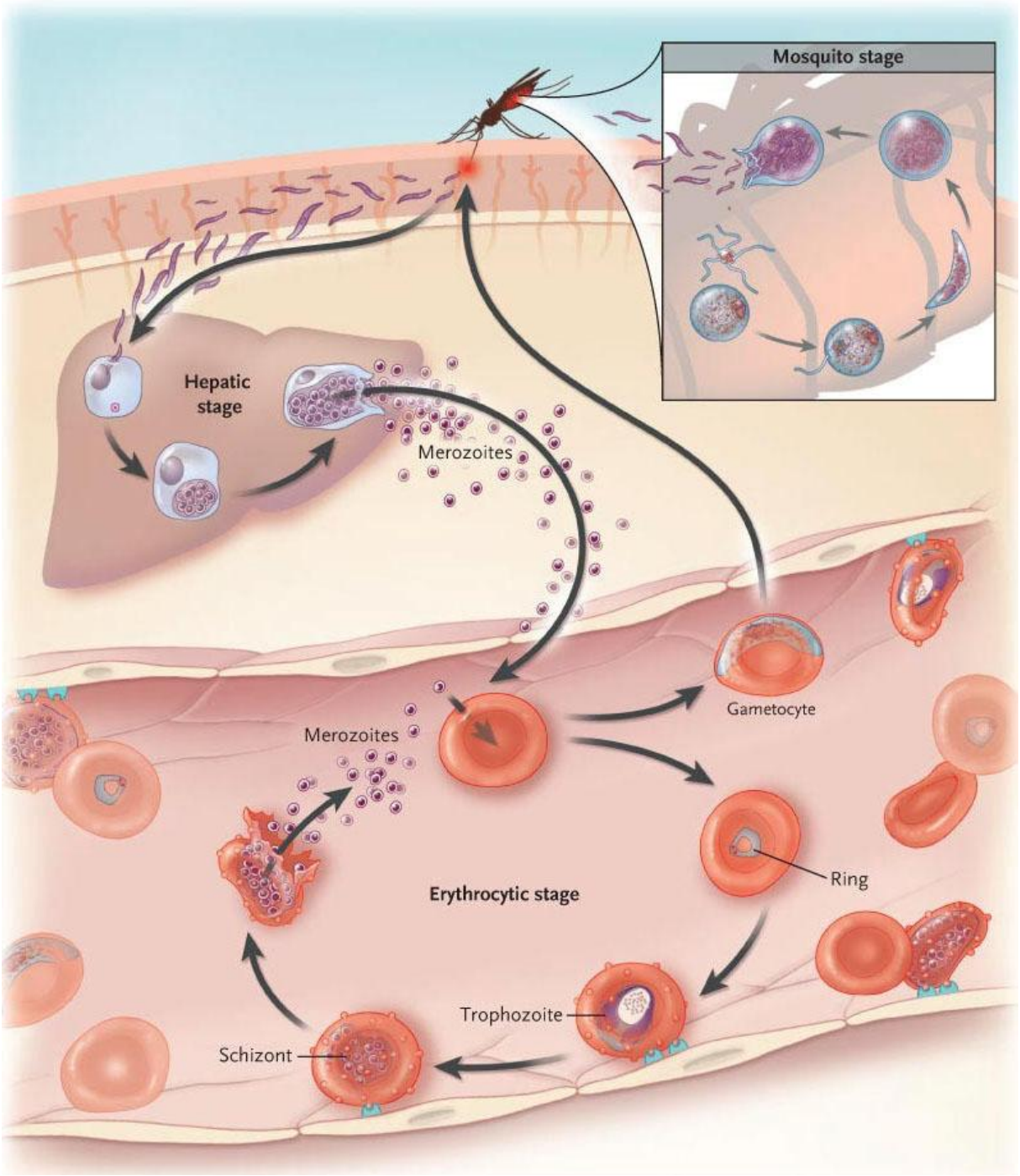

Figure 1: The lifecycle of plasmodium falciparum modified from (Rosenthal, 2008)

1.2 Modification of infected red blood cells

The parasite changes the iRBC drastically upon infection. Morphologically the once biconcave and flexible cell loses its elasticity and adopts a parachute-like shape with protrusions on the surface. These knobs on the surface serve the parasite to present a range of membrane-bound

proteins and receptors. Most prominent among these is the erythrocyte membrane protein family (PfEMP) (Chan, Fowkes et al. 2014), which mediates cell-cell contact between the iRBC and other cells. Two important mechanism are mediated by these interactions. To mask their presence from the immune system the iRBCs bind to uninfected RBCs, thus limiting the display of foreign molecules and avoiding recognition and clearance of iRBCs in the blood stream. Additionally, the PfEMP family mediates binding to endothelial adhesion markers such as VCAM, ICAM-1, CD36 and endothelial protein C receptor (EPCR) (Kessler, Dankwa et al. 2017) thus allowing for the iRBC to cytoadhere to the endothelium of the blood vessels. In a mechanism known as sequestration the parasite withdraws from the circulating blood stream during the late stages of its intracellular replication where the changes to RBC morphology are most drastic. By doing so the parasite avoids recognition and removal of misshaped RBCs by splenic macrophages (Del Portillo, Ferrer et al. 2012).

2. Malaria

2.1 Clinical manifestations of malaria

As mentioned above only the asexual blood stage of the parasite leads to clinical symptoms. The classical symptom of malaria is paroxysm, or reoccurring coldness, rigor and fever. These manifestations can be linked directly to the synchronous life cycle of the parasite. As the iRBCs rupture – in the case of *Plasmodium falciparum* every 48 hours and in the case of the rodent malaria *Plasmodium chabaudi* every 24 hours - both pathogen associated molecular patterns (PAMPs) in the form of merozoites, digestive vacuoles and hemozoin as well as danger associated molecular patters (DAMPs) in the form of undigested hemoglobin, free heme and other remnants of the red blood cell are released into the blood stream (Schofield and Grau 2005).

The immune system recognizes the appearance of such stimuli and responds by producing cytokines, such as interleukin (IL) 1, IL 6 and tumor necrosis factor (TNF), which act as endogenous pyrogens. They diffuse to the hypothalamus and influence body temperature and cause the fever cycles typically associated with malaria (Kwiatkowski 1990).

While that form of the disease is the most common some patients progress to severe disease, meaning that damage occurs in the tissue of the organs. The syndromes of this form of malaria can be discrete and overlapping and those afflicted can display single-organ or multi-organ damage and/or failure. The most life threatening of these are cerebral malaria (CM), acute respiratory distress and severe malarial anemia (SMA). These manifestations mainly occur in infants and small children in regions with high transmission (Schofield and Grau 2005). In regions with lower transmission severe disease more frequently involves additional disturbances such as renal failure, jaundice and pulmonary edema because primary infection can occur in adulthood thus triggering distinct pathogenic mechanisms (Schofield, Novakovic et al. 1996).

The diversity of syndromes impedes the identification of unifying mechanisms of disease. Nonetheless recent studies suggest a common scheme which requires an organ-specific localization of iRBCs in target organs, the systemic and local recognition of PAMPs and DAMPs and the subsequent production of proinflammatory cytokines eventually resulting in the activation, recruitment and infiltration of inflammatory cells into the afflicted organs. Thus, the occurring tissue damage is the end-stage manifestations of dysregulated inflammatory cascades that are initiated in the target organs by PAMPs and DAMPs but are maintained by infiltrating immune cells through positive feedback cycles (Schofield and Grau 2005).

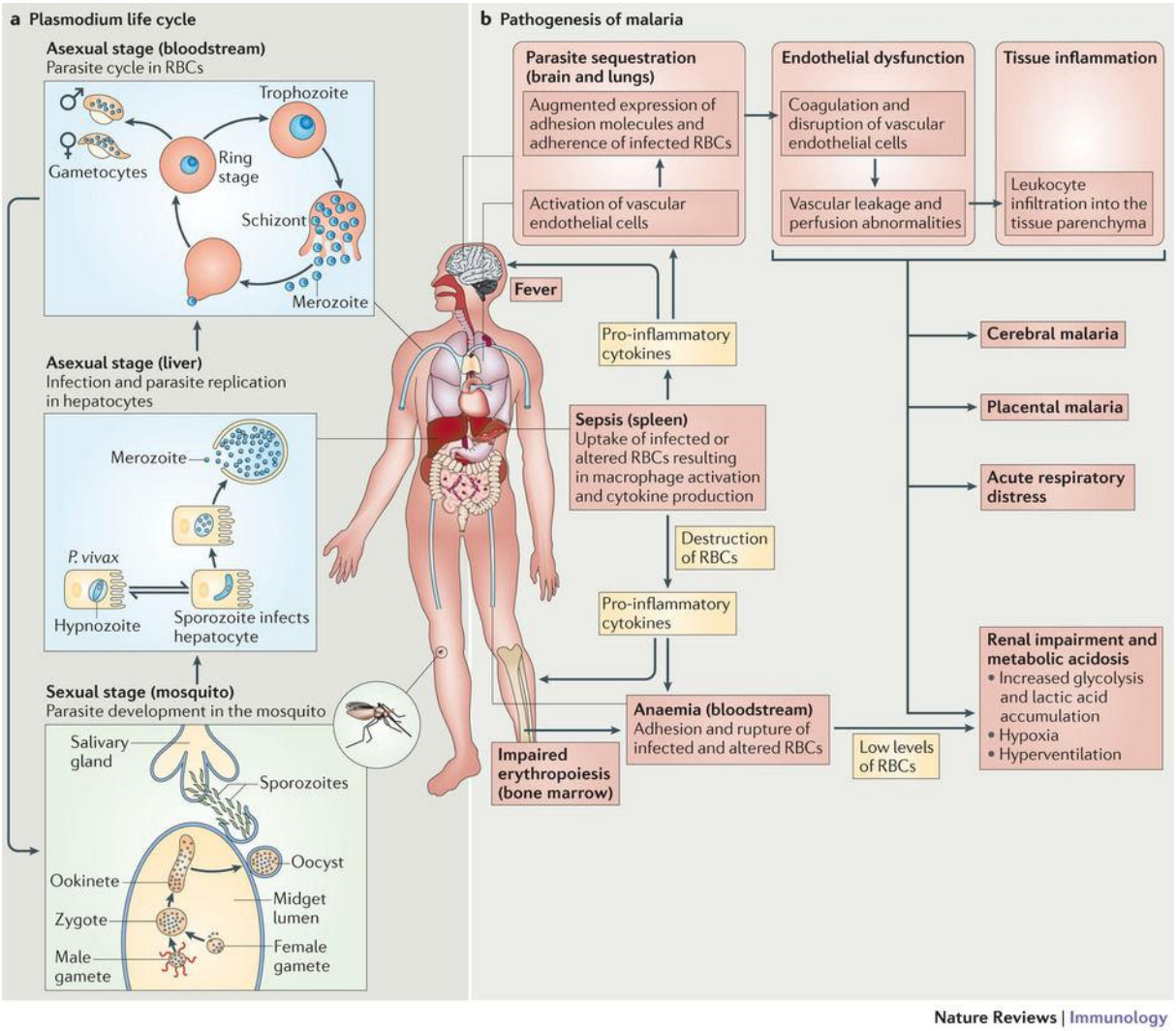

Figure 2: **Pathogenesis of malaria in several organs** modified from Nature Reviews (Gazzinelli, Kalantari et al. 2014)

2.2 The Contribution of vascular occlusion to pathology

Historically severe malaria pathology was attributed to blockage of the microvasculature in afflicted organs because of parasite sequestration and was thought to constitute an event like an ischemic stroke. It was speculated that aggregates of infected and uninfected red blood cells stuck to the endothelial cells walls in the deep vascular beds of the organs would lead to an obstruction of blood flow and eventually rupture of the vessel (Miller, Baruch et al. 2002). However the sequestration of the parasite is a complex mechanism that is influenced by more cell types than just the infected red blood cell and the endothelium (Alencar Filho, Lacerda et al. 2014). Evidence began to emerge demonstrating an important role for immune cells in the development of severe malaria pathology.

2.3 The complex mechanism of parasite sequestration

Parasite sequestration to specific organs is the first requirement and starting event of pathological mechanisms. The parasite changes the shape of the infected erythrocyte quite drastically, especially towards the later stages of its replication cycle, and the iRBCs therefore need to avoid recognition and clearance by splenic macrophages. The family of proteins mostly responsible for this interaction is the infamous erythrocyte membrane protein 1 (PfEMP1) that mediates binding to ICAM1, VCAM1, CD31, CD36 and EPCR, and is thus responsible for binding of the parasitized red blood cells to the endothelium of microcapillaries (de Koning-Ward, Dixon et al. 2016). While this strategy is advantageous for the parasite in that it allows avoidance of the spleen it has the pathological effect of concentrating the parasite in various target organs. This also increases the local concentration of both PAMPs and DAMPs associated with the *Plasmodium* infection and thus potentially creates effects more adverse than the situation in the larger vasculature (Schofield and Grau 2005).

2.4 Immunostimulatory products of parasite growth

The parasite produces a variety of bioactive molecules that can potentially induce an immune response especially when reaching high local concentration due to organ specific sequestration patters. Among these is the glycosylphosphatidylinositol (GPI) of *Plasmodium spp*. Purified GPI induces the expression of various molecules that play a major immunostimulatory role such as TNF, IL1α and IL12, inducible nitric-oxide synthase and importantly the aforementioned endothelial adhesion molecules (Autino, Corbett et al. 2012).

The bioactivity of the parasite pigment hemozoin is less well defined, with some reports showing induction (Coban, Ishii et al. 2002) or inhibition (Skorokhod, Alessio et al. 2004) of dendritic cell maturation. In general, hemozoin seems to have immunosuppressive effects (Schofield and Grau 2005) although some publications claim that by itself it is chemically and immunologically inert (Boura, Frita et al. 2013). These studies suggest that hemozoin can serve as a scaffold or carrier, which binds other immunostimulatory agents such as parasite DNA and transports and presents them to immune cells thus activating for example TLR9 (Coban, Ishii et al. 2005, Kalantari, DeOliveira et al. 2014). Additionally, hemozoin can activate the NOD-like receptor containing pyrin domain 3 (NLRP3) inflammasome complex and lead to the release of IL1β (Autino, Corbett et al. 2012, Olivier, Van Den Ham et al. 2014)

The effects and recognition of both infected red blood cells and free parasites in the blood stream is even less well understood. Splenic macrophages and monocytes respond to those iRBCs that have not sequestered by release of cytokines (Del Portillo, Ferrer et al. 2012) but literature in general is surprisingly sparse. One possible explanation is the reduced immunological control that red blood cells experience in general – they do not express MHC molecules – suggesting that the parasite is hidden from the immune system in the niche it has chosen (Gupta 2005). Additionally, the extracellular episode of the parasite is so short that is might just not suffice for proper immune recognition.

A long-ignored player in the game of malaria pathology is free heme released from the undigested hemoglobin still present in the dying red blood cell as the parasite lyses its former host. In addition, acute malaria infections induce hemolysis of uninfected red blood cells, which

adds to accumulation of free heme in circulation (Jakeman, Saul et al. 1999). Heme is a prosthetic group of many different enzymes such as hemoglobin but is mainly known for its extreme redox potential, requiring the structure to be tightly controlled within a protein scaffold. Lacking target specificity, heme generates free oxygen radicals and thus develops a cytotoxic capacity (Fig. 3).

Systemic release of heme has been hypothesized to activate neutrophils in particular (Chen, Zhang et al. 2014) as they are the most abundant white blood cell in circulation and will therefore be the first cell to be confronted by such systemically released danger molecules.

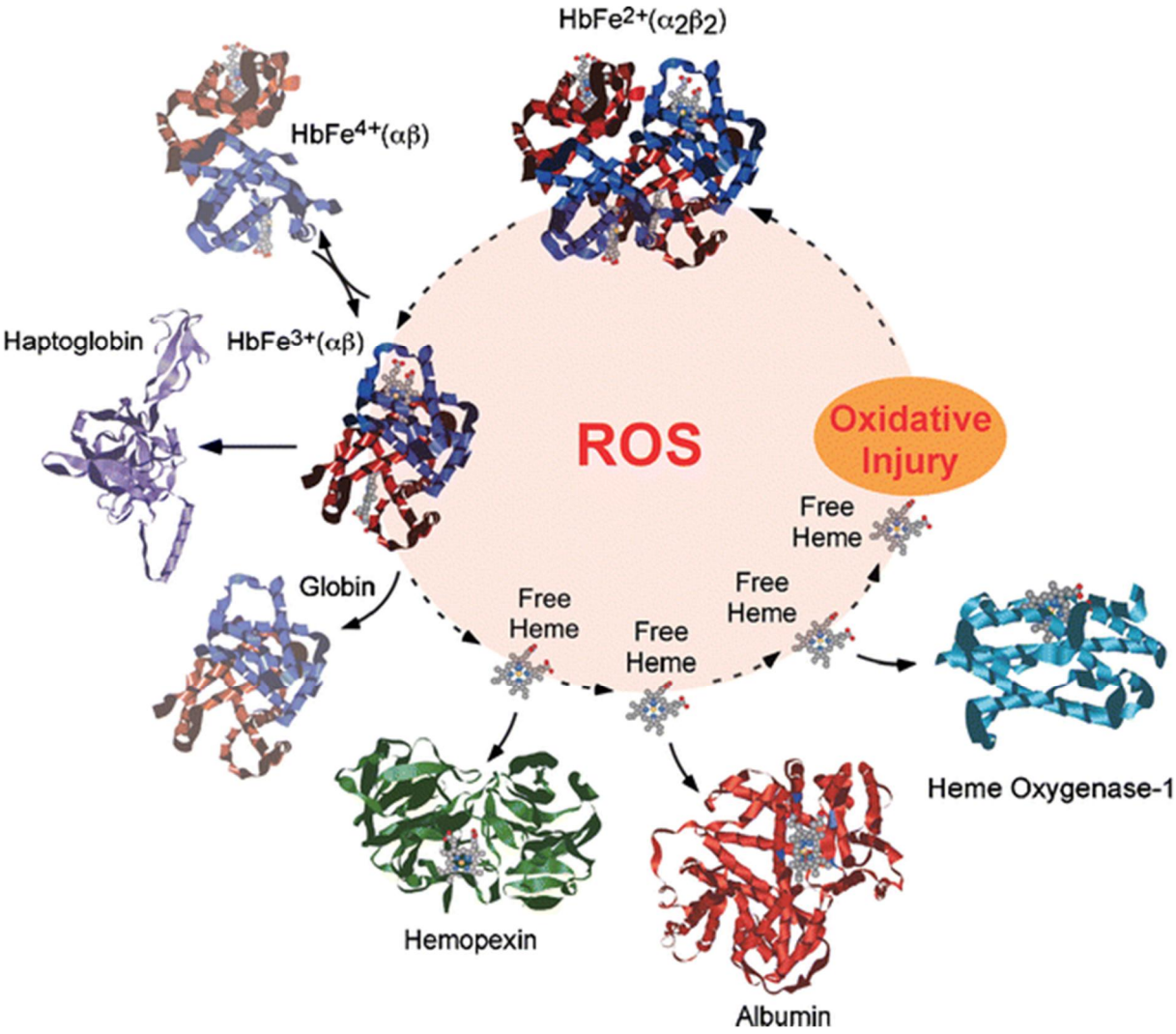

Figure 3: Mechanisms of heme release and detoxification. During *Plasmodium* infection Haptoglobin (Hp) is depleted leading to inadequate clearance of cell free ferric hemoglobin (HbFe3+). This in turn leads to the release of heme into the blood stream. Heme can be scavenged by Hemopexin (Hpx), Albumin or Heme Oxygenase-1 (HO-1). From (Ferreira, Balla et al. 2008).

3. Neutrophils in health and disease

The neutrophil is one of the most abundant immune cell in circulation of mammalian hosts and was long hypothesized to be the primitive foot soldier of the immune system, making up 20- 70 percent of the circulating white blood cells depending on the species. They develop in the bone marrow from precursor cells and are terminally differentiated when they enter the blood stream (Amulic, Cazalet et al. 2012). The recruitment of neutrophils out of the bone marrow relies on the cytokine granulocytes colony stimulating factor (G-CSF). Neutrophils are short lived and therefore have a high turn-over at homeostatic conditions but increased release of G-CSF can induce emergency granulopoiesis. Neutrophils already carry most of the arsenal of highly active molecules that they deploy when a threat is recognized, but to prevent harm these are stored in inactive forms in membrane-bound organelles called granules. Neutrophils express a variety of cytokine and pattern recognition receptors including all TLRs except TLR3, C-type lectin receptors (CLRs), and antibody and complement receptors enabling them to recognize cytokines and many DAMPs emanating from a site of infection. Such signals induce extravasation out of the blood stream and into the underlying tissue where the neutrophils travel along the chemotactic gradient until they meet the focus of infection. Sufficiently activated by the local inflammatory milieu they perform their many antimicrobial and inflammatory functions (Amulic, Cazalet et al. 2012, Kolaczkowska and Kubes 2013, Mayadas, Cullere et al. 2014).

Neutrophils are short-lived cells compared to other effector cells of the immune system. They go into apoptosis after a short aging period which serves to limit the collateral tissue damage caused by their destructive inflammatory functions (Harbort, Soeiro-Pereira et al. 2015). These neutrophil effector mechanisms must be tightly controlled to establish a balance between pro-inflammatory and anti-inflammatory signals, between the right amount of destruction unleashed to kill potential threats and control of this destruction to not damage the self.

3.1 Neutrophil effector functions

Once neutrophils reach their destination they activate various components of their arsenal to combat the infectious agent. Neutrophils produce proinflammatory cytokines to recruit additional immune cells and contribute to microbial clearance through degranulation, production of reactive oxygen species (ROS) and formation of neutrophil extracellular traps (NETs) (Fig.4). These mighty antimicrobials are however indiscriminate in their destructive potential, destroying microbes and host tissue alike (Amulic, Cazalet et al. 2012).

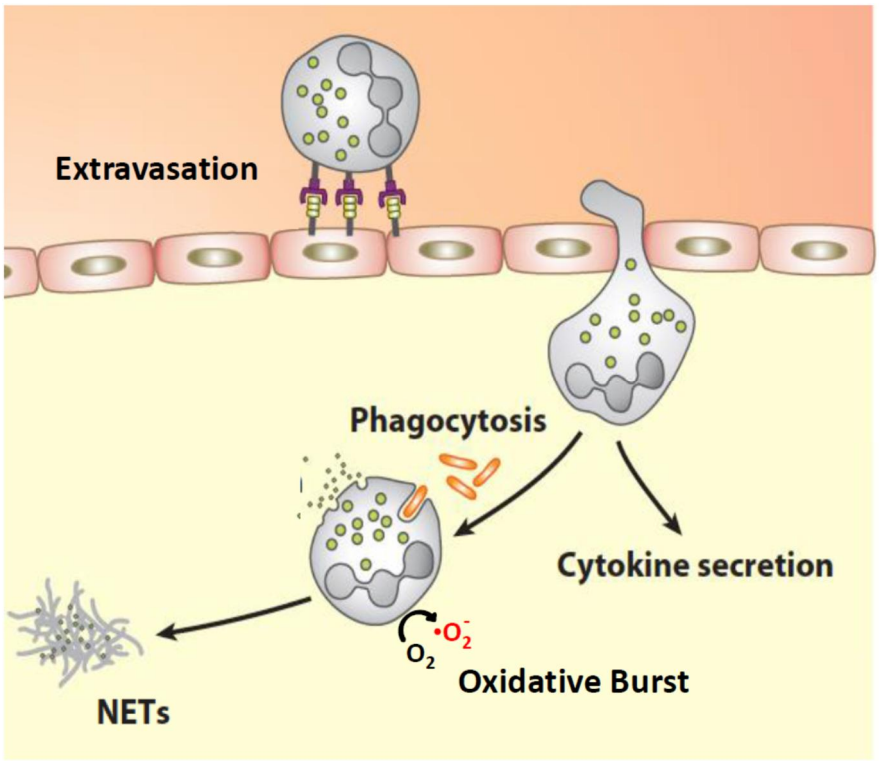

Figure 4: Neutrophils are recruited to sites of inflammation where they carry out their effector functions, figure modified from (Amulic, Cazalet et al. 2012)

3.1.1 Degranulation and phagocytosis

Neutrophils possess a specialized storage organelle – called granule - which serves to store their antimicrobial repertoire in an inactive state until it needs to be released. These are (a) proteases such as neutrophil elastase (NE) and proteinase 3 (Pr3), which can cleave both virulence factors and extracellular matrix proteins, (b) cationic peptides able to form pores in the membrane of

cells and leading to permeabilization, (c) nutrient-binding proteins which sequester and remove essential nutrients and (d) enzymes that mediate the production of ROS. As the neutrophil is activated, these granules can be mobilized in various ways, bringing their weapons to bear. Fusion of the granular membrane with the plasma membrane of the cell results in degranulation, expelling the content of the vesicle into the extracellular space (Amulic, Cazalet et al. 2012). Thus liberated the antimicrobial substances can carry out their destined functions killing microbes but also activating other immune cells and damaging the surrounding tissue (Wang 2018)

A less destructive mechanism is the engulfing of microorganisms that the neutrophil encounters by phagocytosis. The microbe is kept in a phagosome and the granules are fused with this structure, which creates a phagolysosome. In this newly created killing space the content of the granules create extremely harsh conditions which serve to destroy the captured foe (Amulic, Cazalet et al. 2012).

3.1.2 The oxidative burst

Apart from the pregenerated and ready to deploy weaponry that neutrophils store in their granules they can also activate the production of ROS – another potential class of antimicrobials. The generation of these molecules require the activity of the NADPH oxidase, a multimeric enzyme complex that assembles in both the phagosomal and the plasma membranes to reduce molecular oxygen to highly active superoxide (Harbort, Soeiro-Pereira et al. 2015). This radical can be further processed into other species by subsequent enzymatic changes. ROS modify and damage molecules including DNA, proteins and lipids by oxidation. As described for other effector functions of the neutrophil before ROS too are indiscriminate of their target, destroying invading pathogen and bystander host cells alike. In addition to killing microbes in the phagolysosome, ROS have important signaling functions, including in control of NET formation (Harbort, Soeiro-Pereira et al. 2015).

3.1.3 Neutrophil extracellular traps

In a final commitment, the neutrophil can activate a cellular program leading to its own death and the expulsion of a neutrophil extracellular trap (NETs). During this active inflammatory cell death, the neutrophil breaks down the granule membrane in the cytoplasm allowing the enzymes NE and myeloperoxidase (MPO) to reach the nucleus (Papayannopoulos, Metzler et al. 2010). This leads to the decondensation of the chromatin structure and eventually a web-like structure of chromatin decorated with the content of the former granules is released (Brinkmann, Reichard et al. 2004) (Fig. 5). NETosis is an active mechanism, which can be engaged by a variety of stimuli and requires intracellular signaling cascades.

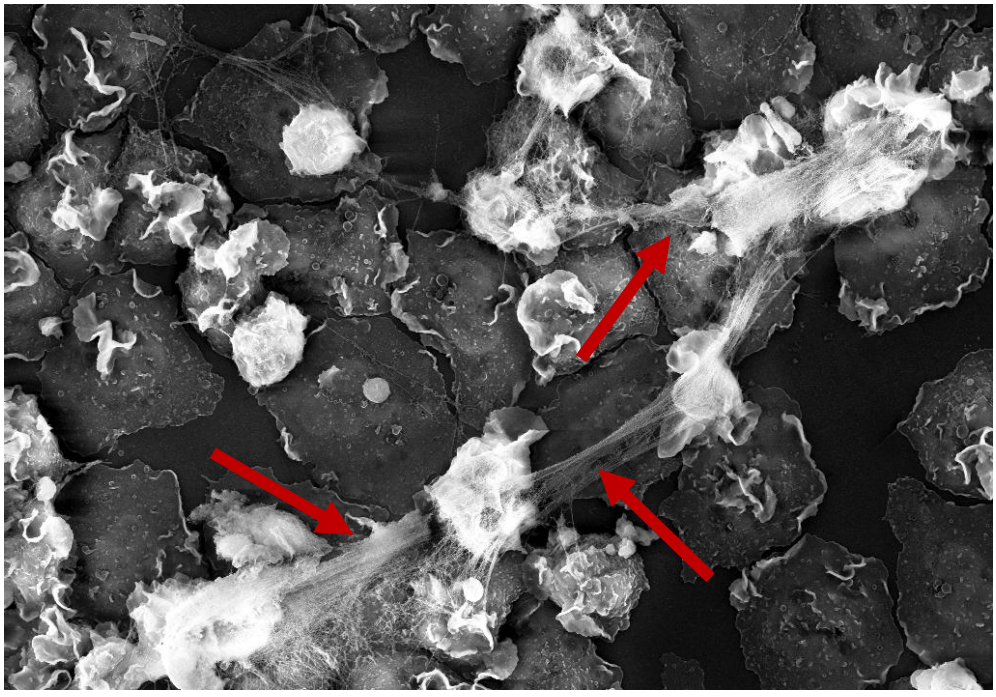

Figure 5: Neutrophil extracellular trap generated *in vitro* and documented by electron microscopy, image courtesy of Volker Brinkmann. Red arrows indicate web-like chromatin structures

3.1.3.1 Diverse stimuli lead to NET induction

A wide range of both biological and chemical stimuli induce NETosis in primary human neutrophils. The first description of NETosis was in response to the mitogenic activator of protein kinase C phorbol myristate acetate (PMA) (Brinkmann, Reichard et al. 2004) which is

still widely used as the positive control. The pathway leading to PMA induced NETs is thus the best characterized. Amulic et al demonstrated that other mitogenic signals such as Concanavalin A (ConA) and phytohaemagglutinin, two plant lectins commonly used to induce proliferation of lymphocytes, also induce NETosis.

Other described stimuli include biological more relevant agents such as *group B streptococcus* (GBS), the hyphae of the fungus *Candida albicans*, free extracellular heme (Chen, Zhang et al. 2014), the calcium ionophore A23187 produced by *Streptomyces chartreusensis* and the potassium ionophore nigericin from *Streptomyces hygroscopicus* (Kenny, Herzig et al. 2017).

3.1.3.2 Mechanism of NET formation

NET formation in response to these diverse stimuli leads to the same web-like structures of extracellular chromatin decorated with the antimicrobials from the granules. The pathway and subcellular requirements differ slightly between different stimuli.

PMA, *C. albicans* and GBS activate PKC and subsequently the Raf/MEK/ERK signaling pathway which eventually leads to the phosphorylation and activation of the NADPH oxidase. The production of ROS by NOX2 is a requirement of PMA induced NETosis (Kenny, Herzig et al. 2017). Interestingly, complete scavenging of ROS can impair *C. albicans* induced NET formation but the lack of NADPH oxidase activity – as assessed by working with cells from patients suffering from chronic granulomatous disease (CGD) – is not required. GBS seems to induce NETosis completely independently of ROS, as the scavenger pyrocatechol fails to inhibit GBS-induced NETs.

Metzler et al showed an important role for MPO during the generation of PMA induced NETs, which has been confirmed for the generation of NETs by both *C. albicans* and GBS (Kenny, Herzig et al. 2017). In the same publication the researches demonstrated an essential role for NE and PR3. These enzymes are stored together with another proteinase called cathepsin G (CG) in the azurophilic granules of the neutrophil. Upon activation of the cell the granules are permeabilized and NE – and potentially Pr3 and CG – travel to the nucleus. NE enters the nucleus and mediates the essential chromatin decondensation by cleavage of histones that eventually leads to the release of NETs (Papayannopoulos, Metzler et al. 2010).

This event coincides with nuclear envelope breakdown, which leads to mixing of cytoplasmic and nuclear components. Eventually the plasma membrane of the cell is also broken down and the NET is released into the extracellular space (Papayannopoulos, Metzler et al. 2010, Kenny, Herzig et al. 2017). Mitogenic signaling is required for NOX2 activation, but also leads to partial cell cycle restart and activation of cyclin dependent kinase 6 (CDK6). CDK6 is required for NET formation but the exact targets of this kinase remain uncharacterized (Amulic, Knackstedt et al. 2017). Importantly, cellular processes that are canonically regulated by CDK6, such as transcription and microtubule polarization, are not involved in the mechanism of NETs.

Interestingly the proteases that initially facilitate the production of NETs, NE and Pr3, remain active and bound to the released structure and carry out important immune modulatory and cytotoxic functions (Kaplan and Radic 2012). It is likely that the production of NETs at least partly serves the purpose of creating high extracellular concentrations of these proteases without allowing their dissemination as would happen if they were released by degranulation.

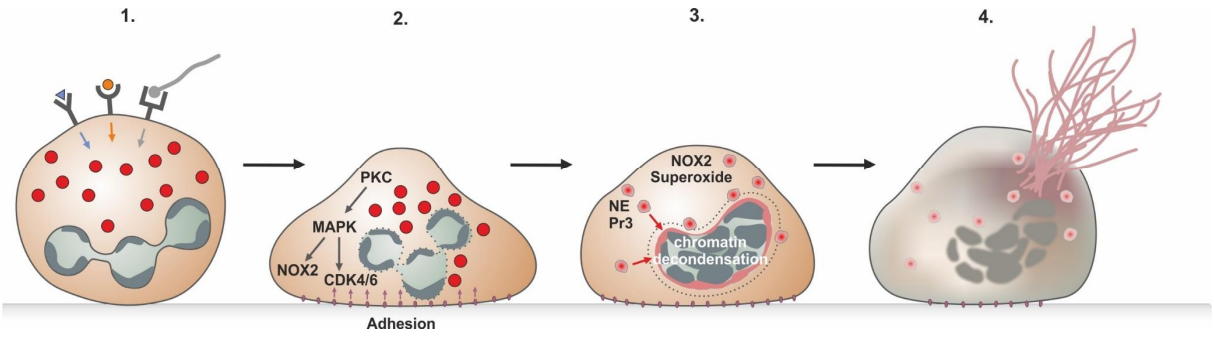

Figure 6: Mechanism of neutrophil extracellular tap formation, figure modified from (Ref Brinkman 2012)

3.1.3.3 The histone citrullination controversy

During transcription, chromatin relaxation can be achieved by post-translational modification of histones. The deimination of positively charged arginine side chains to the neutral and polar citrulline by the peptidylargininedeaminase 4 (PAD4) is such a modification. Several studies have implicated PAD4 activity (Lewis, Liddle et al. 2015) to be crucial for LPS induced NET formation in murine neutrophils and the neutrophil cell line HL-60. Many labs, including ours,

also observe histone citrullination in response to various stimuli, however inhibition of this process by small molecule chemical inhibitors does not influence NET formation. The role of PAD4 in NET formation therefore remains controversial. This dissertation includes experiments to genetically test its involvement in NETosis.

3.2 NETs in health and disease

The release of NETs is a defense mechanism aimed at ensuring local confinement of pathogens. Additionally, NETs are immunostimulatory and control coagulation (Khandpur, Carmona-Rivera et al. 2013, McDonald, Davis et al. 2017). Both NETosis and degranulation are most likely relevant after extravasation into the tissue or on mucosal surfaces where the released substances only have a limited capacity to spread. Activation of such mechanisms in the blood stream might be less associated with pathogen clearance but may have roles in systemic immune activation.

3.2.1 NETs fight infection *in vivo*

Defining the *in vivo* role of NETs during an infection has not been easy mainly due to the lack of a defined NET deficient mouse, although there are several potential candidates including a PAD4 -/-, a NE or NE/Pr3 -/- , a Nox2 -/- and a CDK6 -/- mouse .

While the potential of NETs to kill pathogens, such as *E. coli, in vitro* is well established (Kenny, Herzig et al. 2017) the importance of NETs *in vivo* is less clear and most evidence remains indirect. After undergoing gene therapy a CGD patient was able to clear a persistent *Aspergillus* infection. The patient's neutrophils showed restored NADPH oxidase activity, NET formation and NET mediated killing of *Aspergillus ex vivo* (Bianchi, Hakkim et al. 2009).

More recently the group of Paul Kubes demonstrated a beneficial role of NETs formed in the liver microvasculature of *E. coli* infected mice. These NETs ensnared bacteria in the liver and prevented systemic dissemination of the pathogen. Both depletion of neutrophils by antibody injection and administration of DNase 1 reduced the amount of trapped bacteria (McDonald, Urrutia et al. 2012).

Moreover bacteria that express DNases as virulence factors disseminate more easily in the host, potentially by avoiding entrapment by NETs (Beiter, Wartha et al. 2006, Buchanan, Simpson et al. 2006).

3.2.2 NETs can be detrimental to the host

Unfortunately, excessive or systemic NET formation and impaired degradation of NETs are associated with exacerbated immune responses and can lead to tissue injury (Liu, Su et al. 2016) and unwanted coagulation and thrombus formation (Kaplan and Radic 2012). Interestingly, this effect has been described both in non-infectious autoimmune as well as in infectious settings, some of which are described below.

3.2.3 NETs and vasculitis

Vasculitis is an umbrella-term for a group of chronic diseases caused by the inflammation of blood vessels that can be accompanied by necrotic cell death. Neutrophils play a detrimental role in a subset of these diseases called Small Vessel Vasculitis (SVV) which is characterized by damage occurring in the small vessels and highly associated with the occurrence of autoantibodies directed against proteins that can be found in the neutrophil cytoplasm (Soderberg and Segelmark 2016). Anti-Neutrophil Cytoplasmic Antibodies (ANCA) are by large directed against Pr3 and MPO. Interestingly, exposure of neutrophils to ANCA leads to production of ROS as well as degranulation while TNF primed neutrophils respond to ANCA by undergoing NETosis (Kessenbrock, Krumbholz et al. 2009). Furthermore, neutrophils isolated from patients with SVV spontaneously undergoing NETosis *in vitro*. Further evidence for the involvement of NETs in the pathogenesis of SVV is provided by the observation that NET related histones cause vascular necrosis in severe glomerulonephritis – a major complication associated with SVV (Kumar, Kulkarni et al. 2015).

3.2.4 NETs and systemic lupus erythematosus

NETs and apoptotic neutrophils are normally cleared out of circulation by phagocytes and extracellular enzymes such as DNase 1 (Ren, Tang et al. 2003), as they otherwise provide ample material to induce autoantibodies. Neutrophil cell death is most certainly not the only potential source of autoantigens but it appears to be an important one. Interestingly, the mechanism by which NETs reach the germinal centers and participate in antibody-dependent autoimmunity is not fully understood.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by an overproduction of such autoantibodies directed against dsDNA and chromatin as well as NET-related autoantigens such as MPO, Pr3 and NE (Molnar, Kovacs et al. 2002). These antibodies are thought to be largely responsible for tissue damage observed in patients suffering from flares of disease. This is due to the formation of immune complexes, which, for example, can bind to the glomerular capillaries of the kidney directly inducing necrotic cell death of endothelial cells (Hirose, Itabashi et al. 2017). Nephritis is a major symptom of lupus ranging from mild proteinuria to acute kidney failure (Anders and Vielhauer 2011).

Inhibition of DNase1 activity can occur due to both the accumulation of inhibitors such as g-actin and the protection of NETs from degradation by bound antibodies (Hakkim, Furnrohr et al. 2010). In accordance, earlier findings show that mutations and polymorphism in DNase 1 are associated with SLE (Yasutomo, Horiuchi et al. 2001, Bodano, Gonzalez et al. 2006). The disease can also very clearly be associated with the presence of type I interferons and they seem to play a crucial role in pathogenesis (Hooks, Moutsopoulos et al. 1979) by sustaining a peripheral inflammatory milieu. Interestingly and concurring with the situation describe for SVV, the groups of Lange and Garcia-Romo et al described NETs as a potent inducer of type I interferon release by pDCs, thus underlining the significance of NETs in the pathogenesis of this autoimmune disease.

3.2.5 NETs and sepsis

In sepsis, a bacterial or fungal infection reaches the blood stream and induces hyper inflammation, characterized by massive induction of circulating cytokines. In this setting, platelets get activated by Toll Like Receptor (TLR) 4 mediated recognition of bacterial compounds, bind to neutrophils and induce rapid NET formation in circulation. While this might lead to some clearance of bacteria it mainly induces damage to endothelial cells and propagates an escalating immune response (Caudrillier, Kessenbrock et al. 2012). The pathogenesis of sepsis is largely dependent on extracellular histones (Xu, Zhang et al. 2009) which supports the correlation between sepsis and both SLE and vasculitis. Unlike in the two autoimmune diseases however, the pathogenesis of sepsis does not rely on the generation of antibodies but on other adverse effects described for NETs such as direct destruction of the endothelial lining as well as induction of pDCs. Indeed, septic mice display circulating NET components such as free histones (Xu, Zhang et al. 2009) and MPO and their neutrophils undergo NETosis more readily *in vitro*. Interestingly the same report shows detrimental effects of DNase 1 administration in their model, suggesting an adverse role for liberation of NET components in circulation by extracellular digestion (Meng, Paunel-Gorgulu et al. 2012).

3.2.6 NET components directly induce endothelial damage

A reoccurring theme in all of the above listed diseases is that individual components of NETs seem to be capable of inducing endothelial damage. Indeed, two independent studies described direct effects of NETs on both the endothelium and the epithelium. NETs can be induced by activated endothelial cells (Gupta, Joshi et al. 2010) but then they in turn appear to induce cell death programs in the endothelium directly leading to endothelial leakage and barrier dysfunction (Saffarzadeh, Juenemann et al. 2012). This effect seems to be mainly mediated by histones as treatment of the NETs with anti-histone antibodies prior to challenging the endothelial cells abrogated the induction of cell death.

However, histones are not the only molecule associated with NETs that has been described to induce adverse effects in endothelial cells. Grechowa et al show that human neutrophil elastase can directly induce endothelial cell death by induction of C/EBP-homologous protein (CHOP). Additionally NE and Pr3 can directly induce apoptosis in endothelial cells by direct cleavage of the transcription factor NF-kappaB (Preston, Zarella et al. 2002, Pendergraft, Rudolph et al. 2004). Taken together these reports suggest that systemic release of NETs promotes endothelial cell death, leakage and barrier dysfunction.

3.2.7 NETs and thrombosis

When blood vessels are injured it is of utmost importance that the organism seals these breaks quickly, so that damage to the tissue and excessive bleeding can be prevented. Thrombi or blood clots are formed by platelet aggregation and activation and can lead to red blood cell accumulation in a fibrous matrix. This process is immensely advantageous as it prevents the loss of crucial blood volume but must, on the other hand, be extremely tightly controlled. Blood needs to flow in both large and small vessels in order to guarantee a constant supply of oxygen to the tissues. Blockage of blood vessels by dysregulated thrombus formation can lead to vessel rupture, blood leakage and eventually loss of oxygen supply causing the underlying tissue to die (Deb, Sharma et al. 2010).

NETs generated in circulation can trap platelets and red blood cells and thereby serve as a scaffold for thrombus formation *in vitro* and *in vivo*. Accordingly, experimental generation of thrombi in a primate model revealed extracellular DNA as a backbone of the clump (Fuchs, Brill et al. 2010) Additionally, NE present on NETs in high concentrations can cleave and inactivate tissue factor pathway inhibitor (TFPI) leading to increased procoagulant activity (Massberg, Grahl et al. 2010). Moreover tissue factor (TF) itself is deposited onto the NETs thus further increasing their procoagulant capacity (von Bruhl, Stark et al. 2012). This mechanism of NET induced coagulation and thrombus formation shows, as was observed before, signs of self-propagation. Procoagulant activity induces platelet activation and those activated platelets can then trigger NETosis again (Clark, Ma et al. 2007).

NETs are therefore implicated in the pathogenesis of deep vein thrombosis (Brill, Fuchs et al. 2012) and ischemic stroke (Laridan, Denorme et al. 2017) because they can lead to a dysregulation of the coagulation cascade and hence induce vascular obstruction and eventually rupture.

3.3. Neutrophils and malaria

Neutrophils constantly patrol the blood stream, the compartment where *Plasmodium* infection takes place and where parasites can often reach high densities of more than 250,000 iRBCs per µl. It is therefore very likely that neutrophils are involved in either resolution or progression of disease. Interestingly, people from malaria endemic regions such as people of African descent and some ethnic groups from the Middle East display low basal neutrophil counts (benign neutropenia). Malaria has exerted an enormous evolutionary selective pressure on populations living in such endemic areas and has selected for gene variants that promote tolerance. Decreased neutrophil counts could therefore indicate a detrimental role for neutrophils in malaria infections. Moreover neutrophilia is very commonly observed in patients suffering from both *P. falciparum* and *P. vivax* malaria (Maina, Walsh et al. 2010, Olliaro, Djimde et al. 2011, Kotepui, Piwkham et al. 2015) and is associated with disease severity.

Indeed, two reports suggest that neutrophils might play a detrimental role in the development of severe malaria (Chen, Zhang et al. 2000, Rocha, Marques et al. 2015) although the older one uses an outdated antibody for the depletion of neutrophils that has in the meantime been shown not to be specific for neutrophils. Another publication implicates NETs in pathogenesis of acute respiratory distress in a *P. berghei* model (Sercundes, Ortolan et al. 2016). The authors successfully treat disease employing the same strategies commonly used in patients with cystic fibrosis (NE inhibitors and active DNase, both applied by inhalation).

In patients a recent transcriptomic analysis that compared blood from severe and uncomplicated *P. falciparum* malaria patients identified a G-CSF-induced neutrophil granulopoiesis signature as a specific feature of cerebral malaria. Interestingly the highest log-fold upregulation between severe and uncomplicated malaria was apparent in genes encoding

neutrophil granule proteins such as NE and metalloproteinase-8 (MMP-8). In accordance with this observation children with retinopathy-positive cerebral malaria display an accumulation of externalized neutrophil proteins such as NE, Pr3 and MPO (Feintuch, Saidi et al. 2016).

In addition to the accumulation of neutrophil granule components, complicated malaria is associated with increased cell-free nucleosomes in circulation, which are partly host derived. NETs are an important platform for the externalization of both chromatin and neutrophil proteases. Initial reports have indicated the presence of NETs in both human patients (Baker, Imade et al. 2008) and mouse infections (Sercundes, Ortolan et al. 2016).

4. Aim of the study

Many of the neutrophil antimicrobials are indiscriminate in their killing capacity and can induce collateral damage by destruction of host cells. While this trade-off can be beneficial when occurring in spatially confined compartments it becomes detrimental when occurring systemically. Neutrophil components and specifically NETs are pathogenic in autoimmune diseases such as SLE and SVV and infectious diseases such as sepsis. These diseases seem rather different at first glance but are tied together by the common theme of systemic NET release. A *Plasmodium* infection represents another condition that can induce such systemic release of neutrophil components. While there already have been initial reports of neutrophil involvement in the pathogenesis of malaria a conclusive analysis of the effects of systemic NET release during *Plasmodium* infection is still missing.

The aim of this study was to investigate the function of NETs in the context of human and rodent malaria, to define their mode of action and to determine whether they represent a potential new therapeutic target.

We therefore set out to test whether NETs are present in the circulation of human patients and if so what other parameters correlate with the amount of extracellular NET components.

We addressed the mechanisms of NET formation induced by potential malaria associated stimuli. The insights into the requirements of malaria PAMP or DAMP induced NET formation would then allow us to transition to a mouse malaria model infect the proper NET deficient genotype and analyze neutrophil pathogenic mechanisms *in vivo*.

We thus aimed to determine mechanistically what systemic and local changes are induced by malaria associated NET formation and to thereby identify potential targets of intervention for novel adjunctive therapies.

RESULTS

5. Malaria leads to NET induction via heme and TNF

5.1 NETs are present in the circulation of malaria patients

Malaria is associated with accumulation of circulating NET components such as NE, Pr3, MPO and nucleosomes. Furthermore, levels of these proteins are correlated with the severity of a *P. falciparum* infection (Feintuch, Saidi et al. 2016). However, NETs themselves have so far not been clearly identified in the circulation of infected individuals (Baker, Imade et al. 2008) and it remains unknown if the above mentioned granule proteins are released via NETs or by degranulation. To analyze NET production in malaria patients we conducted a field study at the Albert Schweitzer Hospital in Lambaréné in Gabon. The province of Moyen Ogoove is endemic for malaria, there is year round transmission which peaks during wet seasons. The region is mainly afflicted by *P. falciparum*, but *P. malariae* and *P. ovale* can also be observed (Scherbaum, Kosters et al. 2014).

We recruited patients of both sexes at the ages between 1.5 – 84 years, who were confirmed to be infected with *P. falciparum* by the diagnostic laboratory by Giemsa thick smear analysis. As a control, we also recruited healthy individuals in a similar age range that were confirmed to be *P. falciparum* negative also by thick blood smear analysis. We determined NETs by sandwich ELISA as complexes of the granule protein NE and DNA in the plasma of these patients. The components are normally separated in intact neutrophils but they co-localize on NETs. We observed that a significant proportion of patients had NET complexes in their plasma (Fig. 7 A).

While the parasite lives in the red blood cells it uses hemoglobin as its source of nutrition. Leftover hemoglobin is released into the blood stream. Additional hemoglobin is released by the lysis of uninfected bystander cells. Extracellular hemoglobin releases its prosthetic group heme which can therefore serve as a marker for hemolysis occurring during a malaria infection. A previous study proposed extracellular heme as an inducer of NETs in sickle cell disease (Chen,

Zhang et al. 2014), which although not an infectious is also a hemolytic disease. Therefore we determined the concentration of heme in the patient samples (Fig 7 C) and, as expected, observed a significant increase when compared to healthy individuals. Interestingly, the amount of heme can be positively correlated with the presence of NETs in the circulation (Fig 7 B). When this analysis is restricted to the patients who display NET levels above those of the healthy control only (responders) (Fig. 7 D) this correlation becomes even more pronounced, suggesting a direct link between hemolysis and intravascular NET formation.

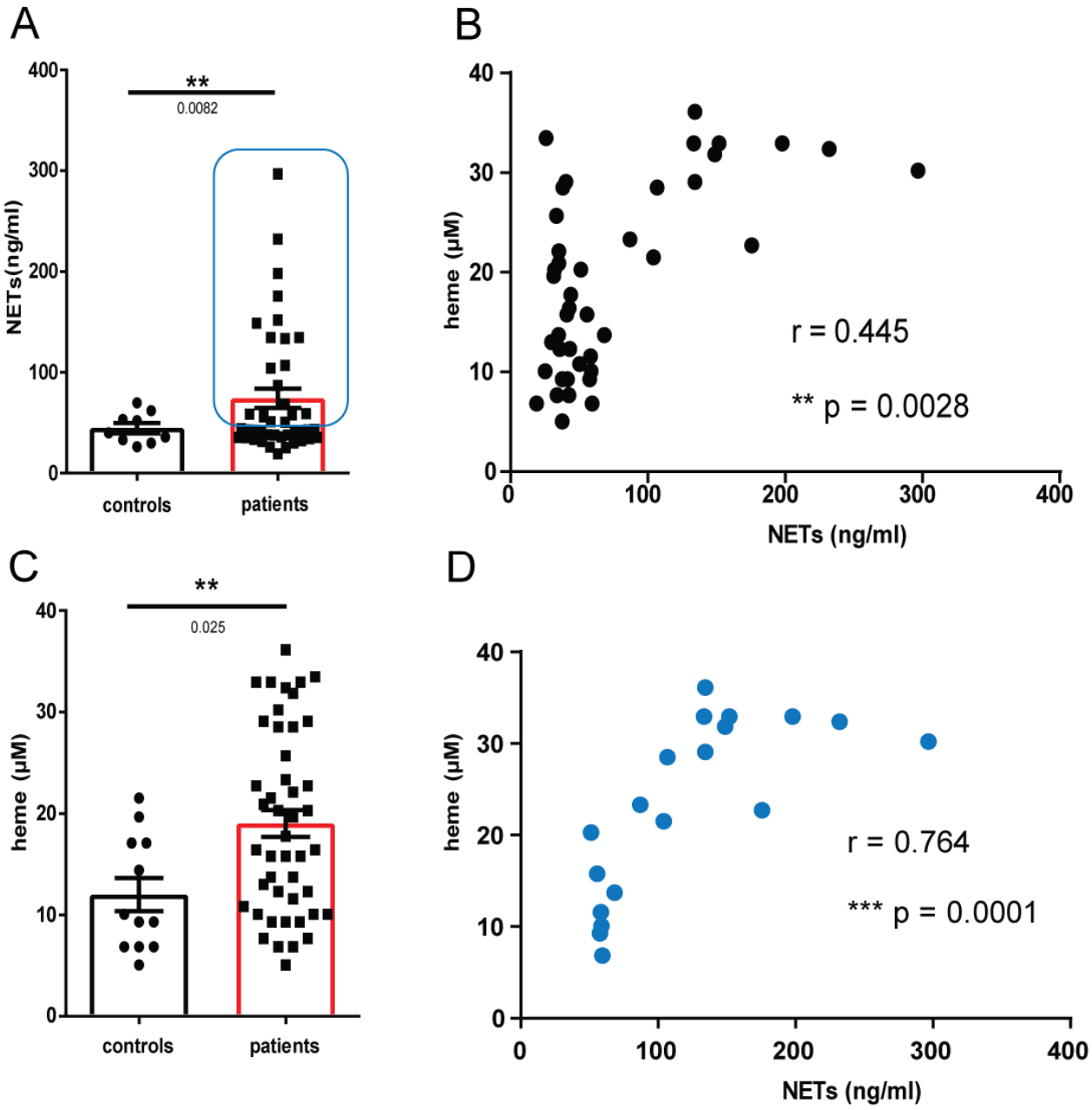

Figure 7: *P. falciparum* infection induces accumulation of extracellular NETs and free heme
Plasma was isolated from patients suffering from malaria and healthy individuals and subsequently analyzed by sandwich ELISA for NE/DNA complexes (A). The concentration of extracellular heme was determined from the same samples (C) and values were correlated by Spearman correlation (B and D). Data is presented as the mean ± standard error of the mean (SEM). Asterisks indicate significance: *P<.05, **P<.01, ***P<.001 by Welch's t-test.

5.2 Extracellular heme and TNF can drive neutrophils into NETosis

We then determined how neutrophils respond to the PAMPs and DAMPs present during a *Plasmodium* infection. Understanding the trigger that might lead to the activation of neutrophils and the subsequent release of cellular contents into the circulation is crucial to develop intervention strategies.

We isolated neutrophils from peripheral blood of healthy donors by density separation, resulting in highly pure, inactivated neutrophils (Harbort, Soeiro-Pereira et al. 2015). These were then stimulated *ex vivo* with PMA (positive control) and the following malaria-associated immunostimulatory components:

The **infected red blood cell** itself, isolated from *in vitro* cultures of *P. falciparum*. It represents the largest immunostimulatory entity during the infection, comprising all potential PAMPs such as GPI anchors, parasite surface antigens and the misshapen erythrocyte.

The **free merozoites** represents the only form of the parasite that is entirely visible to the immune system, between lysis of the old and infection of a new red blood cell. Although this period is short it represents a potential for immune recognition.

The **digestive vacuole** is the organelle the parasite uses to detoxify the heme released by digestion of hemoglobin. As merozoites burst from the dying erythrocyte this vacuole containing the hemozoin crystal is released into the blood stream where it can be phagocytosed by immune cells.

Free heme as we have observed before in circulation of malaria patients is the degradation product of extracellular hemoglobin. It has a strong redox potential, can produce radicals and is thus cytotoxic. It is also a danger signal to the immune system signifying uncontrolled hemolysis.

After 4h, cells were fixed and analyzed for NET formation by immunofluorescence analysis (IFA) as described before (Brinkmann, Goosmann et al. 2012). We observed no NET formation with either infected red blood cells, free merozoites, isolated digestive vacuoles or even free heme in unprimed neutrophils whereas PMA (positive control) induced NET formation as published (Fig. 8 A, top panel).

To reproduce the conditions existing during malaria *in* vitro we co-stimulated isolated neutrophils with the cytokine most commonly associated with a *Plasmodium* infection. Tumor necrosis factor (TNF) is present in the circulation of malaria patients at very high concentrations and has a priming effect on neutrophils (Chen, Zhang et al. 2014). We therefore primed neutrophils for 15 min with 2 ng/ml TNF followed by stimulation with the aforementioned PAMPs and DAMPs. We observed no NET induction in response to iRBCs, free merozoites and digestive vacuoles but free heme induced NET formation comparable to PMA in TNF primed neutrophils (Fig. 8 A, lower panel and B) yet again suggesting that neutrophils respond to the inflammatory and hemolytic conditions present during malaria by undergoing NETosis.

To more carefully asses the *in vivo* activation status of neutrophils we recruited adult malaria patients of both sexes between the ages of 18 – 84, from whom we obtained larger volumes of blood, enabling us to isolate peripheral neutrophils in the same fashion as described above. These neutrophils were plated *ex vivo* without any further stimulation and NET formation was assessed after a 4h incubation period. We observed that neutrophils from malaria patients undergo NETosis to a significantly higher rate than those from healthy donors *in vitro* (Fig. 8 C) suggesting that the NETotic program was already initiated in the circulation of those patients and thus carried out *in vitro* without the need of any further activating signals.

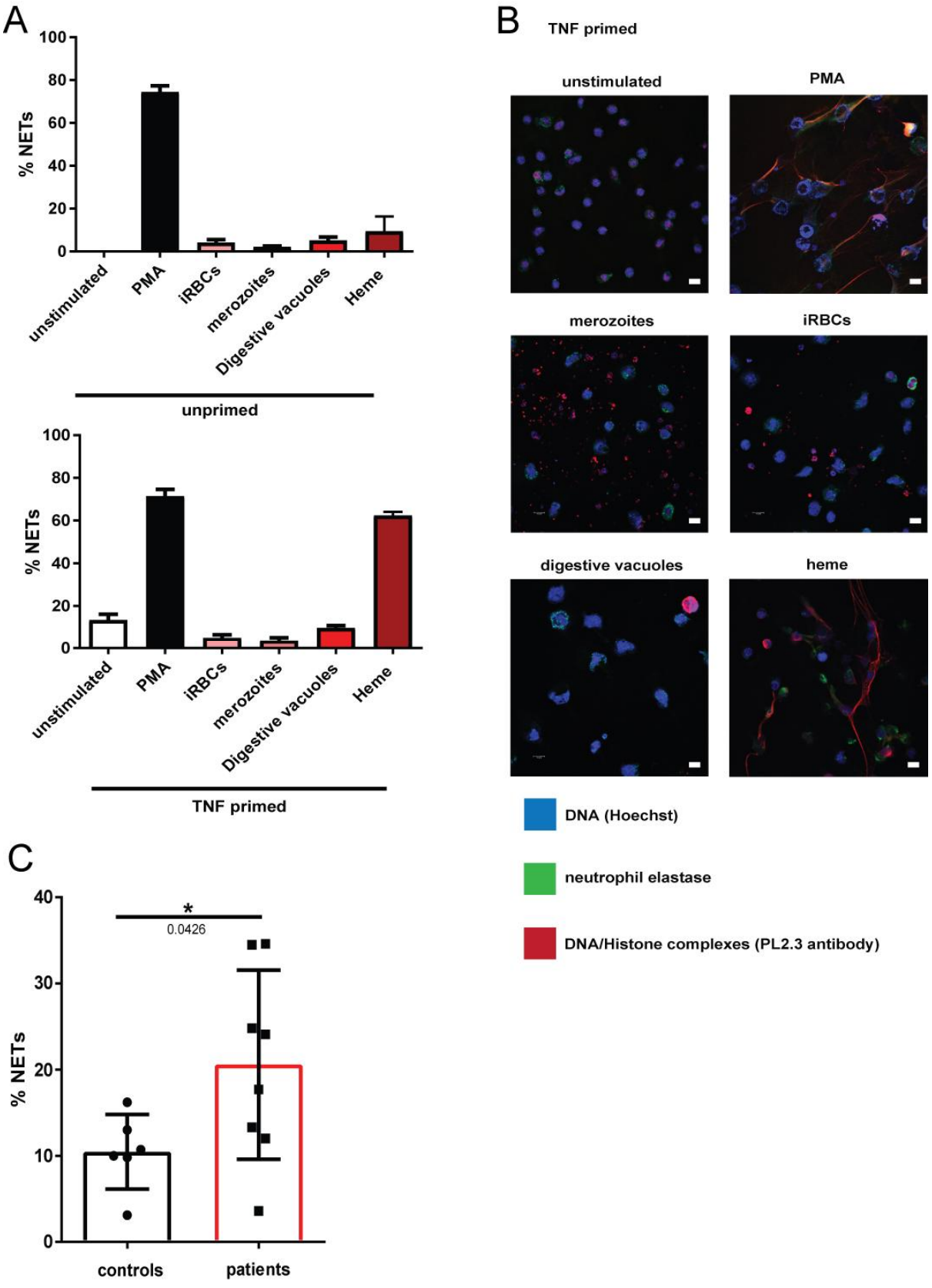

Figure 8: TNF/heme stimulation induces NET formation in primary neutrophils. (A) Quantification of NETosis in response to malaria PAMPs and DAMPs in unprimed and TNF primed (2 ng/ml) neutrophils. Neutrophils were fixed and analyzed by immunofluorescent staining. (B) Representative images of the staining. (C) Quantification of NETosis by neutrophils isolated from healthy individuals and malaria patients. Scale bar = 20 µm

5.3 Heme/TNF induced NETs require NOX2-independent oxidants and serine protease activity but do not require protein translation nor citrullination

We and others (Chen, Zhang et al. 2014) have demonstrated the ability of heme to induce NETs in combination with TNF but the underlying mechanism has not yet been thoroughly explored. Heme can be sensed through TLR4 in macrophages (Dutra and Bozza 2014), suggesting an active sensing of this DAMP and hence the engagement of distinct pathways during heme induced activation of cells. The characterization of signaling and effector molecules involved in TNF/heme induced NETosis is an important step towards specific inhibition of this mechanism. If NETs are indeed pathogenic in the setting of a *Plasmodium* infection these targets might represent a strategy for intervention with disease progression.

We set out to determine by which mode of action heme induces NET formation as several different NETosis pathways have been described (Kenny, Herzig et al. 2017). First we aimed to determine whether heme induced NETosis depends on the generation of ROS by the NADPH oxidase. We isolated primary neutrophils from healthy donors and patients suffering from chronic granulomatous disease (CGD), who possess a dysfunctional NADPH oxidase and are therefore not able to produce ROS. We observed that PMA induced NETosis is abrogated in CGD patients but heme was still able to induce NETs (Fig. 9 A) indicting that heme induced NETosis proceeds independently of the NADPH oxidase. We confirmed the abrogation of ROS production in CGD neutrophils by luminescence measurements (Fig. 9 B).

To further characterize heme induced NET formation we subsequently performed inhibitor experiments with neutrophils from healthy donors. The cells were treated with the published NET inhibiting concentrations of each inhibitor (Kenny, Herzig et al. 2017) for 30 minutes before stimulation.

Both PMA and heme are activators of the important signaling mediator protein kinase C (PKC) (Graca-Souza, Arruda et al. 2002) and inhibition of PKCs function inhibits PMA induced NET

formation. We observed that heme induced NET formation also depends on the activity of PKC as the inhibitor treated cells were unable to produce NETs in response to heme (Fig. 9 C). Interestingly, we observed that complete scavenging of ROS (Fig 9 D) by pyrocatechol did abrogate heme induced NET formation (Fig. 9 C) suggesting that this type of NETosis is dependent on a different source of ROS. This could potentially be mitochondrial ROS or ROS derived from other enzymatic sources such as xanthine oxidase.

We find that heme induced NET formation in primary human neutrophils is independent of both protein translation (cycloheximide) and the conversion of arginine to citrulline by PAD4 (Fig. 9 B). The activity of cycloheximide was confirmed by IL-8 release assay in response to LPS (Fig. 9 E). Amulic et al. demonstrate that NETosis relies upon enzymes associated with cell cycle progression in actively dividing cells. Neutrophils however are terminally differentiated cells and incapable of cell division but seem to have repurposed part of the cell cycle machinery (Amulic, Knackstedt et al. 2017). In accordance with these prior findings about other NET inducing stimuli, heme/TNF induced NETosis can be abrogated by inhibition of the cyclin-dependent kinase 6 (Cdk6). Furthermore, we find that heme induced NET formation is dependent on protease activity by the neutrophil proteases NE and Pr3 (Fig. 9 C).

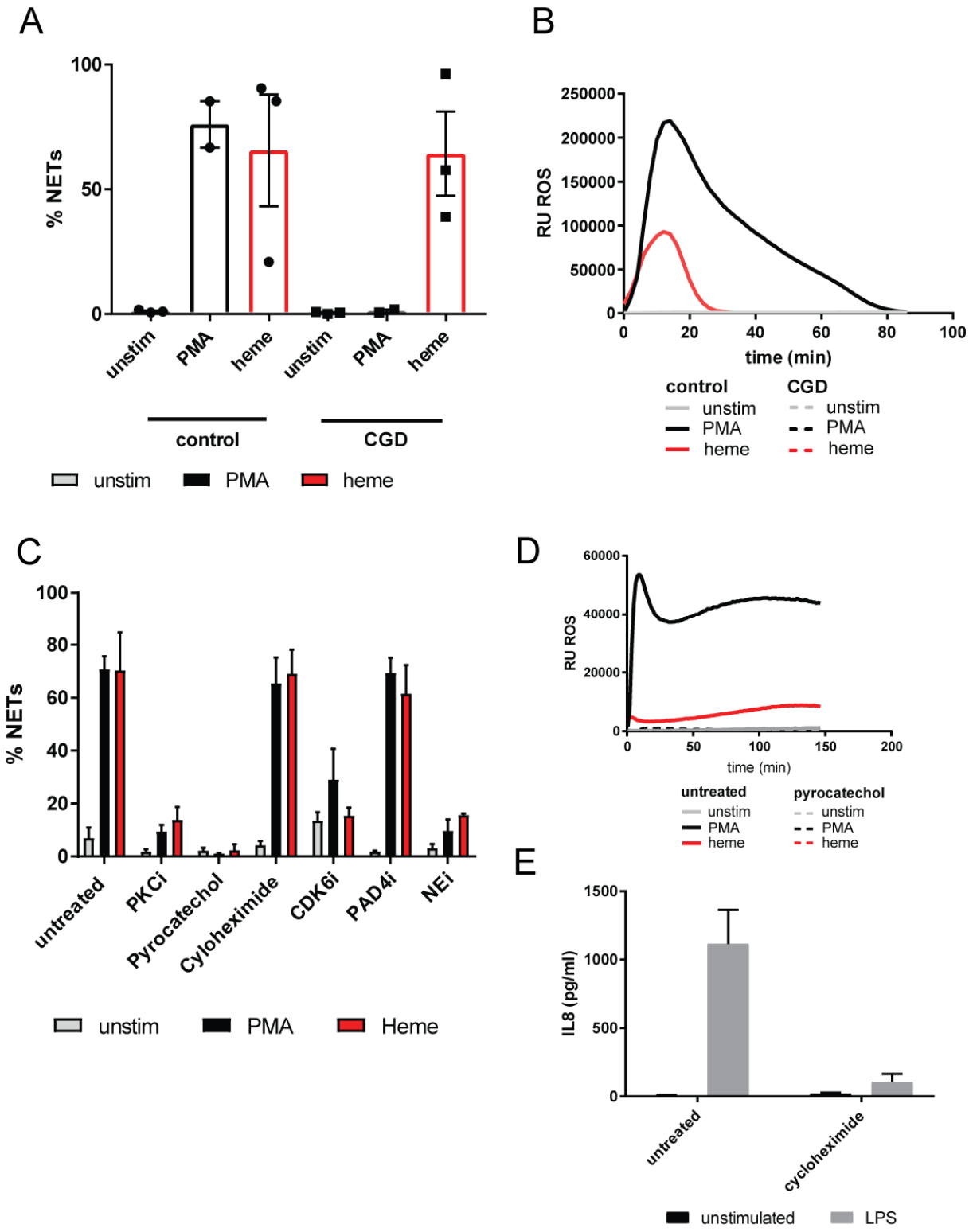

Figure 9: Heme/TNF induced NETs require NOX2-independent oxidants and serine protease activity. (A) Quantification of NETosis in neutrophils from healthy donors and CGD patients in response to PMA and heme. Quantification was performed as described before by IFA. (B) Oxidative burst in PMA- and heme-stimulated neutrophils. Neutrophils were incubated with luminol and horseradish peroxidase to measure ROS production by luminescence. Luminescence was measured kinetically in a luminometer and expressed as relative light units. (C) Quantification of NETosis in response to PMA and heme in inhibitor treated neutrophils. (D) ROS assay of pyrocatechol treated neutrophils. (E) IL-8 release from *de novo* synthesis after 18h LPS stimulation of untreated and cycloheximide treated primary neutrophils.

5.4 Murine Heme/TNF induced NETs require serine protease but not protein arginine deaminase nor DNase activity

NETs are involved or implicated in three distinct pathological mechanisms:

First the **macrostructure of NETs** can act as a scaffold inducing platelet activation, coagulation and eventually thrombus formation and is therefore implicated in vascular occlusion in ischemic strokes as well as atherosclerosis (Qi, Yang et al. 2017). Vascular occlusion has long been hypothesized as one major factor in the development of organ pathology in malaria, especially because iRBCs bind to both other RBCs (infected or not) and the endothelium of the microvasculature. In a healthy individual the serum protein DNase 1 counteracts such accumulation of large filamentous chromatin based structures by unspecific cleavage of the DNA backbone. This enzyme is also responsible for the clearance of NETs in circulation (Hakkim, Furnrohr et al. 2010) thus preventing these structures from facilitating coagulation.
Secondly the NET components can be **directly cytotoxic** (Xu, Zhang et al. 2009) and can cause damage to endothelial cells (Gupta, Joshi et al. 2010).
Thirdly NETs and their individual components are recognized as DAMPs by both immune and endothelial cells and lead to a **proinflammatory response** which might itself aggravate disease.
To address which of these mechanisms might be important we require an *in vivo* model in which we can compare situations where (a) NET formation is blocked and (b) the NETs that are produced cannot be digested and keep their intact superstructure without dissemination of individual components.
In accordance with our human data (Fig. 9), we selected NE -/- as well as NE/Pr3 -/- mice as putative NET deficient genotypes. These two proteases are structurally very similar sequence homologous (Campanelli, Melchior et al. 1990) and possess similar substrate specificity, meaning that they may each be able to compensate for the loss of the other (Warnatsch, Ioannou et al. 2015). The third neutrophil serine protease CG possesses a structurally identical catalytic center but a slightly different substrate binding groove. CG therefore preferentially hydrolyses peptide bonds after aromatic amino-acid residues whereas NE and Pr3 favor

cleavage after valine residues (Pham 2006) We assessed both the NE -/- and the NE/Pr3-/- genotypes for their ability to produce NETs but did not include a CG -/- mouse as the protease seems to play a less dominant role in NETosis.

We also decided to test a PAD4 -/- mouse despite the inhibitor not showing an effect on human heme induced NETs. There is ongoing controversy in the field as to whether NET formation requires PAD4 activity or not as described in the Introduction (Konig and Andrade 2016). We therefore aspired to back our human inhibitor data with the appropriate observations in the mouse.

Lastly we decided to assess the capability of DNase 1 deficient mice to make NETs, as they would later in the infection model allow us to distinguish between effects mediated by the NET macrostructure and effects dependent on the solubilization of protein components initially associated with NETs. No role is described for DNase 1 in the formation of NETs, but neutrophils from DNase deficient mice were never tested with regards to their capacity to make NETs *in vitro*. We therefore decided to include DNase deficient neutrophils in our analysis.

As shown in Figure 10, only neutrophils isolated from the peritoneum of mice deficient in both NE and Pr3 were entirely unable to produce NETs in response to heme/TNF costimulation. While PAD4 deficiency showed no effect, mice deficient for NE showed an intermediate phenotype with a reduction of NET formation of about 50% in response to heme/TNF (Fig. 10 B). As expected the DNase 1 deficient neutrophils produced NETs normally. Interestingly microscopic analysis revealed that neutrophils from NE/Pr3 -/- mice still become permeable in response to heme but fail to fully decondense their chromatin (Fig. 10 A).

This finding allowed us to proceed with our *in vivo* analysis of NETs in *Plasmodium* infection as we have fulfilled the requirements outlined above. The NE/Pr3 deficient mice are incapable of producing NETs in response to TNF/heme costimulation and will therefore allow us to assess the role of NETs in general. The DNase 1 deficient mice on the other hand are still able to produce NETs but fail to solubilize NET components by extracellular digestion of the DNA backbone. Infection of these two mouse strains will allow us to determine whether NETs are

involved in the pathogenesis of malaria and whether this is due to their macrostructure or to the systemic dissemination of individual components.

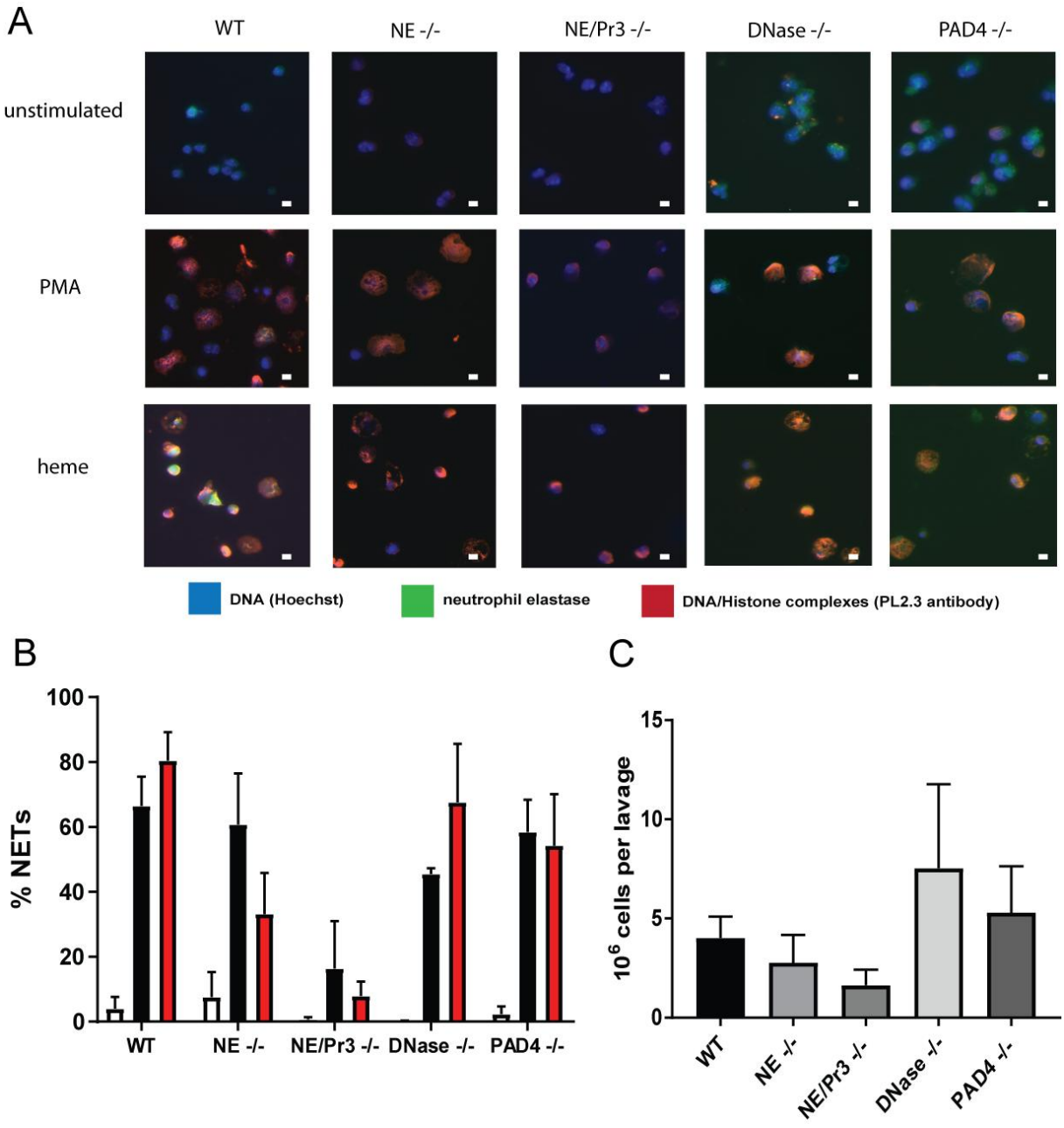

Figure 10: murine heme/TNF induced NETs require serine proteases but not protein arginine deaminase nor DNase 1 activity. (A) Representative images of immunofluorescence staining of murine neutrophils isolated from peritoneal lavage fluid after repeated casein injection. Cells were stimulated with PMA or heme after TNF priming *in vitro* and subsequently fixed in 2% PFA. (B) Quantification of NET formation of different genotypes in response to PMA and heme. The quantification was carried out using the method described by Brinkman et al. (C) Neutrophil cell counts in the peritoneal lavage fluid after repeated injection of casein. Neutrophils must extraversate out of the blood into the peritoneum to be detected here. Scale bar = 10 µm

To verify that our knockouts specifically affect NETs, we also quantified neutrophil extravasation rates into the peritoneal cavity. Neutrophils infiltrate into the afflicted organs of

mice and humans suffering from severe cases of malaria (Rocha, Marques et al. 2015, Bostrom, Schmiegelow et al. 2017). Neutrophil extravasation has been proposed to be at least partly dependent on the activity of proteases such as NE and Pr3. We therefore assessed the neutrophil efflux in response to an inflammatory stimulus. We injected a 9 % casein solution into the peritoneum of mice of all genotypes. We then lavaged the peritoneum with sterile PBS and determined the number of cells that had migrated there (Fig. 10 C). No significant differences were seen although the DNase deficient mice seemed to show a trend towards more neutrophil mobilization.

6 NET components are pathogenic in malaria

WE show that, just like human neutrophils, murine neutrophils commit to NETosis in response to heme/TNF stimulation and that this process depends on the activity of the granular proteases NE and Pr3 while DNase 1 deficiency does not abrogate this process. These observations equipped us with the right tools to address the potential mechanism of NET induced pathology.

We decided to use the *P. chabaudi* mouse model of malaria due to two important reasons:

a) The infection resembles the one that occur in humans as it is synchronized and cyclic. Same as in the human host parasites burst from the infected red blood cell all at once. Furthermore, *P. chabaudi* cytoadheres to the endothelium in a well-defined pattern, unlike other rodent malaria models. The infection is chronic, hemolytic and eventually leads to organ damage – in this case in the liver. Unlike the infection with *P. berghei,* there is no mortality described for *P. chabaudi*.
b) A role for neutrophils in the pathology of this infection has already been established (Rocha, Marques et al. 2015). In brief, mice were depleted of neutrophils by repeated antibody injection over the course of the *P. chabaudi* infection. This treatment abrogated organ damage.

Organ damage is induced during a *P. chabaudi* infection in the tissues that the parasitized red blood cells sequester to, namely the liver and the lung. The read out for cytoadhesion-mediated organ pathology in this model are the hepatocyte specific enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are released into circulation only upon necrotic cell death in the liver. Pathology in the lung can be assessed by pathological scoring of lung sections, which can identify necrotic events and immune cell infiltration.

6.1 NET components in the blood of *P. chabaudi* infected mice

We infected mice of the three described genotypes (WT, NE/Pr3 -/- and DNase -/-) with $1x10^4$ iRBCs injected *i.v.* directly into the bloodstream, and mice were sacrificed on days 7, 9, 11 and 13 post infection. On these days, blood samples were collected and the livers were fixed in paraformaldehyde for microscopic analysis. The blood was separated into plasma and the cellular fraction. Plasma samples were further analyzed by ELISA whereas the cell fraction was analyzed by fluorescence activated cell sorting (FACS).

Other groups have described that neutrophils do not participate in the clearance of parasites and that the depletion of neutrophils does not affect the growth of the parasite (Rocha, Marques et al. 2015, Sercundes, Ortolan et al. 2016). In accordance with this we found that neither the absence of the proteases NE and Pr3 nor of the endonuclease DNase 1 had any significant effect on the growth of the parasite (Fig. 11 A) as determined by counting of the percentage of infected iRBCs in thin blood smears of the mice (parasitemia).

Interestingly we could observe an accumulation of NETs in circulation of WT mice which was not observed in the NE/Pr3 deficient mice, consistent with our *in vitro* findings. Surprisingly, DNase I deficient mice also showed absence of circulating NETs (Fig. 11 C). NETs were measured as NE/DNA complexes in the plasma of mice by sandwich enzyme linked immunosorbent assay (ELISA). We initially expected the DNase -/- mice to exhibit increased NET concentrations in circulation due to an impaired clearance, however NETs bind to endothelial cells (McDonald, Urrutia et al. 2012) and are sequestered away from circulation if they are not broken into smaller fragments. DNase 1 is therefore required for solubilizing NET components and releasing them systemically.

We went on to determine the concentrations of individual NET-associated molecules and observed an accumulation of both extracellular nucleosomes and NE in the circulation of infected WT mice. Strikingly, neither component was present in either of the knockout genotypes (Fig. 11 D and E).

To gain a better understanding of how neutrophils contribute to pathology, we determined neutrophil numbers in the blood stream by FACS using an anti-Ly6G/C antibody in combination with an anti-CD115 antibody as describe elsewhere (Dunay, Fuchs et al. 2010). This combination of antibodies allows a clear distinction of neutrophils from other circulating immune cells such as inflammatory monocytes. Neutrophils can be observed as Ly6G/C high and CD115 negative. This analysis revealed an increase in the neutrophil population from day 7 of the infection in WT mice. Neutrophil numbers continuously rise over the course of the infection, but drop back to basal levels as parasite burden decreases. Neither of the deficient genotypes displayed an accumulation of neutrophils comparable to the WT at any time during the infection (Fig. 11 B).

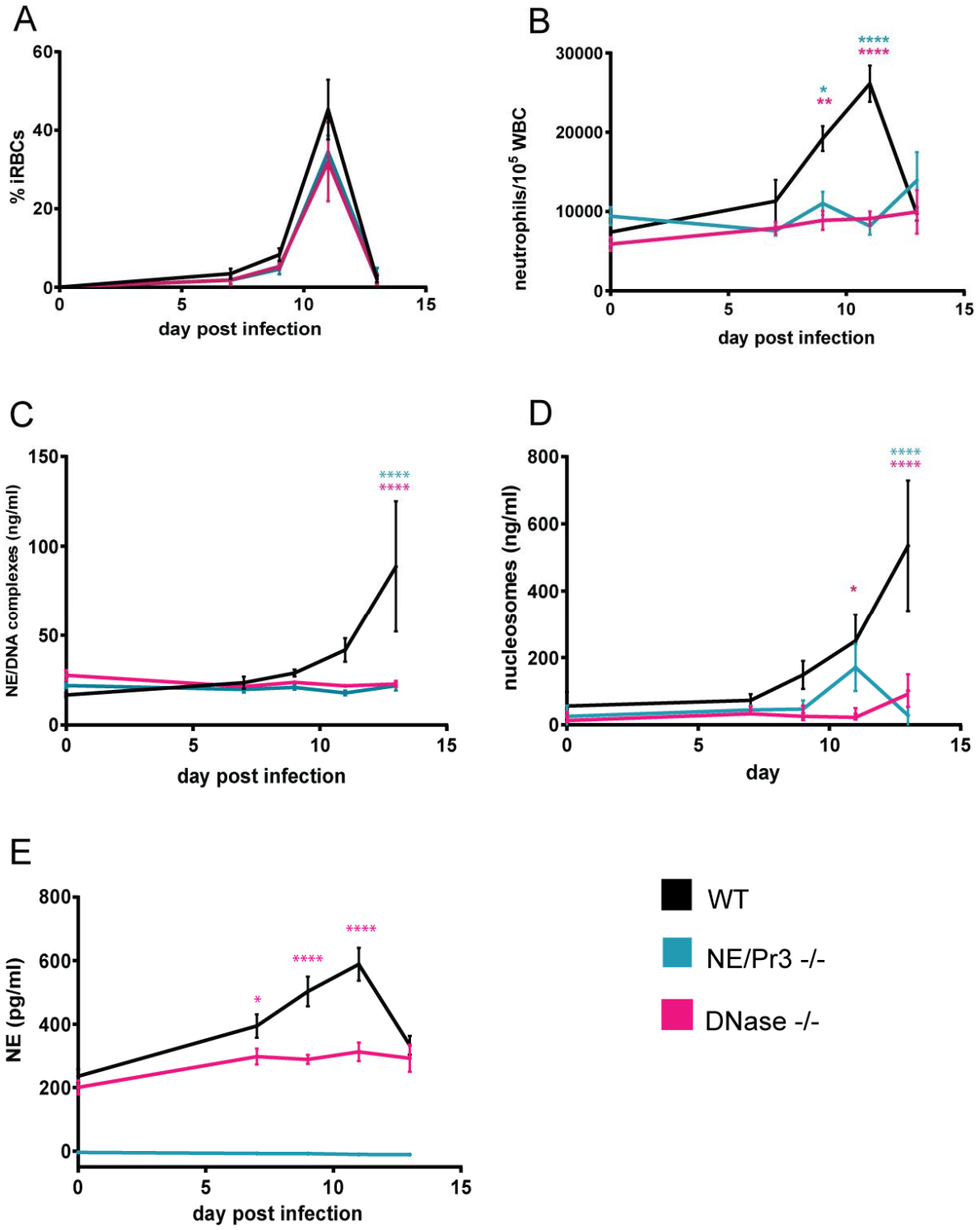

Figure 11: Increased NET components and neutrophil to leukocyte ratio in *P. chabaudi* infected mice. (A) Parasitemia of infected mice as determined by counting of thin blood smears. Images were taken at a 63X magnification and at least 500 red blood cells were counted per samples. (B) Ratio of neutrophils to leukocytes over the course of the infection determined by FACS analysis of whole blood samples. Neutrophils were defined as CD45 +, CD3 -, Ly6G/C high, CD115 -. The population was confirmed by SSC/FSC location. (C) NET quantification in plasma of mice by sandwich ELISA detecting NE/DNA complexes. Quantification of extracellular nucleosomes (D) and neutrophil elastase (E) by sandwich ELISA.
The group size of all data presented is n = 6. Data is presented as the mean ± standard error of the mean (SEM). Asterisks indicate significance: *P<.05, **P<.01, ***P<.001 and ****P<0.0001 by two-way analysis of variance (ANOVA) comparison of 3 groups. Color of the asterisks indicate which genotype they refer to.

6.2 NET components liberated by serum DNase 1 drive pathology

We observed prominent macroscopic changes in the livers of the animals over the course of the infection. We noted that necrotic patches start to manifest on the organs as early as day 9 and the organs display severe darkened discoloration at the later time points of the infection (Fig. 12 A). Interestingly neither white necrotic patches nor the change in color was apparent in the livers of deficient mice.

WT mice showed a drastic increase in extracellular AST in the plasma starting from day 9 of the infection, which peaked on day 11 together with the parasitemia (Fig. 12 B). This indicates liver damage and hepatocyte death. Remarkably both knockout genotypes exhibit a complete abrogation of liver damage, as measured by circulating AST and when livers are assessed macroscopically (Fig. 12 A and B). To verify this phenotype, we assessed liver damage of all three genotypes by pathological scoring of hematoxylin and eosin (H&E) stained liver sections of the infected mice (Fig. 12 C and D). This analysis was carried out in a blinded fashion by the iPATH Charité University Hospital pathology division and revealed large necrotic patches in the livers of infected WT mice which were almost completely absent in both deficient genotypes (Fig. 12 C). We were also able to observe an infiltration of immune cells into the liver as well as the accumulation of malaria pigment over the course of the infection (arrows figure 12 D) both of which were absent in the knockout mice.

The absence of pathology and discoloration in the livers of NE/Pr3 -/- mice indicates that NETs are a crucial factor in the development of organ pathology during a malaria infection. Furthermore, the absence of pathology in DNase 1 -/- mice indicates that this systemic effect is due to extracellular digestion of the DNA backbone of NETs and thus release of individual NET components.

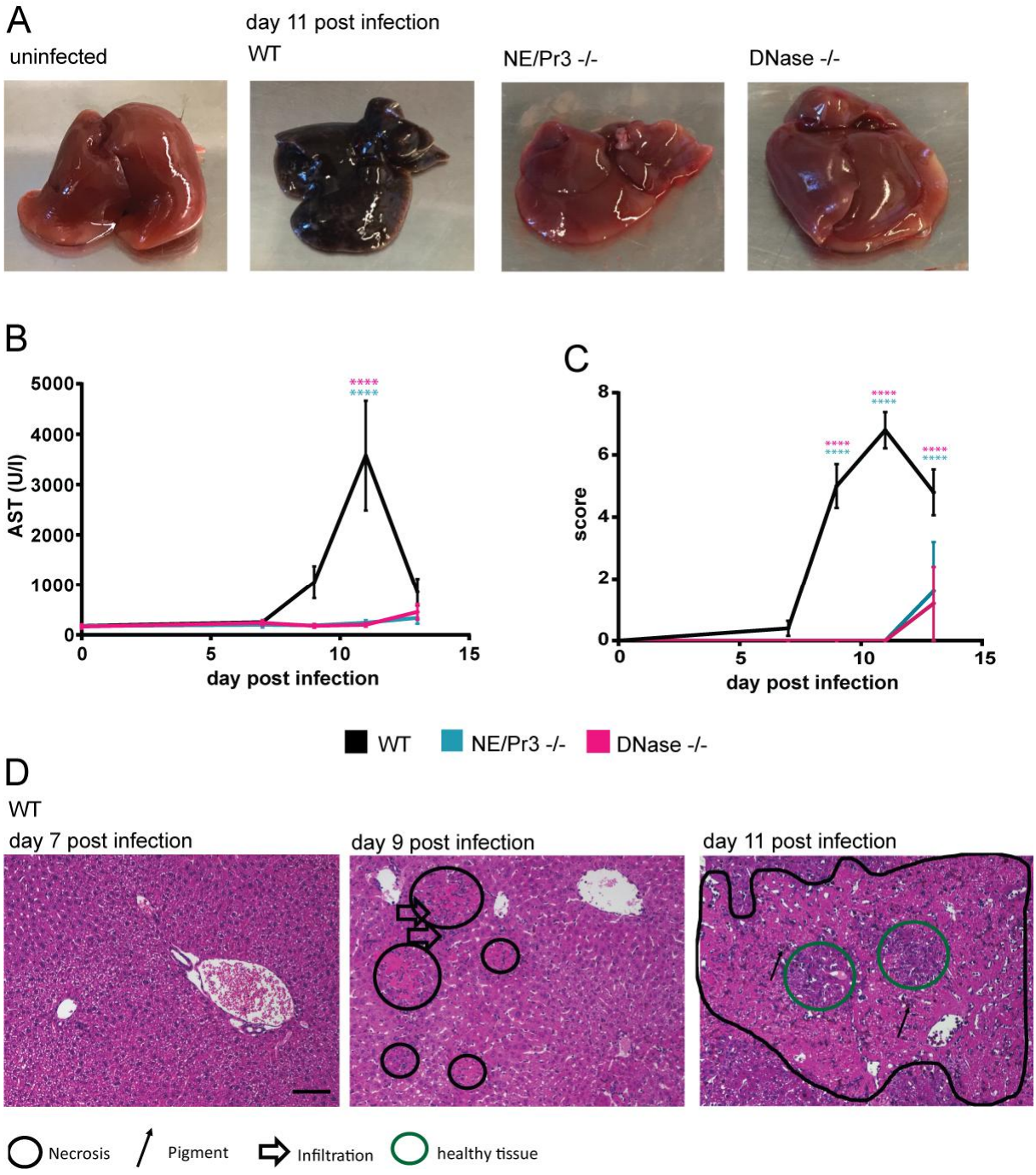

Figure 12: NET components liberated by serum DNase 1 are pathogenic in a P. chabaudi mouse model of malaria. (A) Representative images of dissected livers of uninfected mice and 11 days post infection of all genotypes. (B) Concentration of aspartate aminotransferase in plasma of mice over the course of the experiment. Measurements were conducted by a routine veterinary laboratory (SYNLAB vet). (C) Pathological score of livers over the course of the infection, determined blind by the pathology unit of the Charité University Hospital. (D) Representative images of H&E stained liver sections used for pathological scoring.
The group size of all data presented is n = 6. Data is presented as the mean ± standard error of the mean (SEM). Asterisks indicate significance: *P<.05, **P<.01, ***P<.001 and ****P<0.0001 by two-way analysis of variance (ANOVA) comparison of 3 groups. Color of the asterisks indicate which genotype they refer to.
Scale Bar = 100 µm

6.3 NET components drive pathology by controlling parasite sequestration and neutrophil infiltration of livers

The absence of discoloration and pigment in the NET release or degradation deficient genotypes led us to determine the sequestration frequency of infected red blood cells in the liver. The black discoloration of organs occurs during human disease as well (Haldar, Murphy et al. 2007) and indicates accumulation of hemozoin in the afflicted organs.

As described before, the parasite binds to the endothelium of liver micro capillaries in infected WT mice (Fig.13 A). Such accumulation was absent in both NE/Pr3 and DNase 1 -/- mice (Fig. 13 A). Representative H&E stained images are shown in Fig.13 C. The first picture shows a large blood vessel in the liver and green arrows point to infected RBCs in circulation. These represent parasitemia and are not counted as sequestered parasites. The two lower images show parasites in the microvasculature of the tissue (black arrow) which are counted as sequestered parasites (Fig.13 C).

Parasite sequestration both leads to and is enhanced by endothelial activation. Parasitized red blood cells bind to adhesion factors such as VCAM, ICAM, CD36 and EPCR (Cunningham, Lin et al. 2017, Kessler, Dankwa et al. 2017) that are all upregulated upon activation of endothelial cells. Sequestration of some iRBCs therefore increases the sequestration of other iRBCs in a forward cycle. The same adhesion molecules also mediate the recruitment of immune cells into the microvasculature of afflicted organs, which then in turn enhance the inflammatory environment by producing and releasing their own repertoire of immune mediators.

We wanted to determine whether the sequestration, or lack thereof, in the deficient genotypes was mirrored in the number of neutrophil infiltrates present in the liver. The quantification in the livers was performed by immunofluorescent staining of paraffin sections using a neutrophil-specific anti-calgranulin A antibody generated at the Max-Planck Institute for Infection Biology in Berlin. Entire liver sections were then scanned using a ZEISS Axio Scan Z1

and the signal intensity was quantified. Representative images are shown in figure 13 D (Calgranulin A shown in red).

Quantification showed an influx of neutrophils into the liver of infected WT mice starting on day 9 of the infection, which decreased as the infection was cleared towards day 13. Neither the NE/Pr3 nor the DNase deficient mice showed comparable infiltration of cells into the liver (Fig. 13 B).

The lack of sequestration and immune cell infiltration in the deficient genotypes potentially explains the lack of liver damage in these animals, as well as the absence of macroscopic changes. The black discoloration visible in the livers of infected WT mice is most likely due to the pigment hemozoin present in both parasites and professional phagocytes binding to the microvasculature of the organ and since this does not happen in the knockout genotypes no discoloration occurs. We conclude that liberated NET components are involved in the activation of endothelial cells and thereby upregulate surface adhesion receptors leading to both sequestration of infected red blood cells as well as accumulation by immune cells.

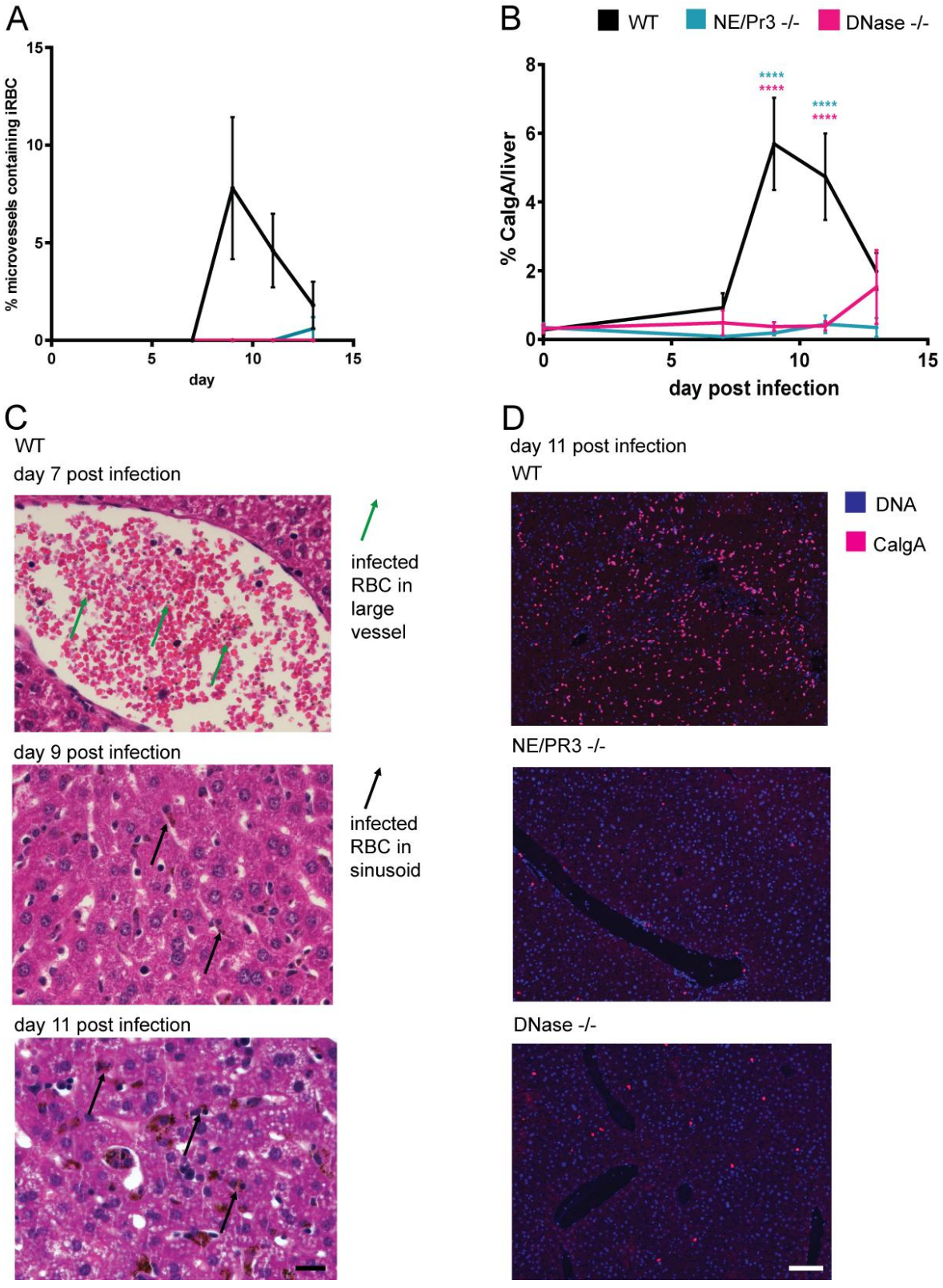

Figure 13: NET components drive pathology by controlling parasite sequestration and neutrophil infiltration of livers. (A) Quantification of parasite sequestration in the liver performed by microscopic analysis of H&E stained liver sections. (B) Quantification of neutrophil infiltrates into the livers of all three genotypes over the entire liver section performed using an Axioscan Z1 (C) Representative images of H&E stains at 400X magnification, black arrows indicate sequestered parasites. (D) Representative images of immunofluorescent stains of liver sections from all three genotypes at peak liver pathology, using the neutrophil marker Calgranulin A. Scale bars: (C) = 20 µm and (D) = 100 µm

6.4 NET components induce an inflammatory environment favoring neutrophil recruitment and extravasation

So far we were able to show that NET components are involved in inducing neutrophil expansion in the blood, infiltration of neutrophils into the liver and in liver pathology. We hypothesized that liberated NET components regulate *de novo* production of neutrophils, also known as emergency granulopoiesis.

Neutrophils are produced in the bone marrow from hematopoietic precursors and this process is regulated by cytokines, most notably G-CSF and the IL-1 family. (Summers, Rankin et al. 2010). We thus set out to define the inflammatory environment in the different genotypes. To do so we performed a multiplex cytokine analysis using a Bio-Plex Multiplex Immunoassay system. This bead based assay allows detection of multiple immune mediators in the same plasma sample. Analysis of the data revealed a proinflammatory environment with increased levels of IL-1α, IL-6, IL-17, G-CSF, IFN-γ and TNF in the infected WT mice (Fig. 14 A). Once again, the deficient mice showed decreased levels of all the above-mentioned cytokines in circulation although differences were not always clearcut. Strikingly, G-CSF and IL-1α, which regulate emergency granulopoiesis, were greatly reduced in knockout mice (Fig. 14 A), explaining the observed neutrophilia in infected WT mice that is absent in the deficient genotypes. Reduced circulating levels of G-CSF were confirmed by regular ELISA in the same samples (Fig. 14 B). Furthermore, IL-17, another proinflammatory cytokine that activates granulopoiesis via G-CSF induction, was also significantly reduced in knockout mice. Interestingly IL-17 and TNF can act synergistically on endothelial cells to induce release of G-CSF but also to promote ICAM display and neutrophil extravasation (Bosteen, Tritsaris et al. 2014)

We conclude that the absence of NETs (NE/Pr3 -/-) or NET processing (DNase 1 -/-) prevents expression of inflammatory cytokines driving emergency granulopoiesis. The immunomodulatory effect of NET components was not limited to granulopoiesis, as other cytokines with pleiotropic effects on the inflammatory cycle, such as TNF, IFNγ and IL-10, were also affected.

We next wanted to test if this effect is specific for neutrophils or if other immune cells are also affected.

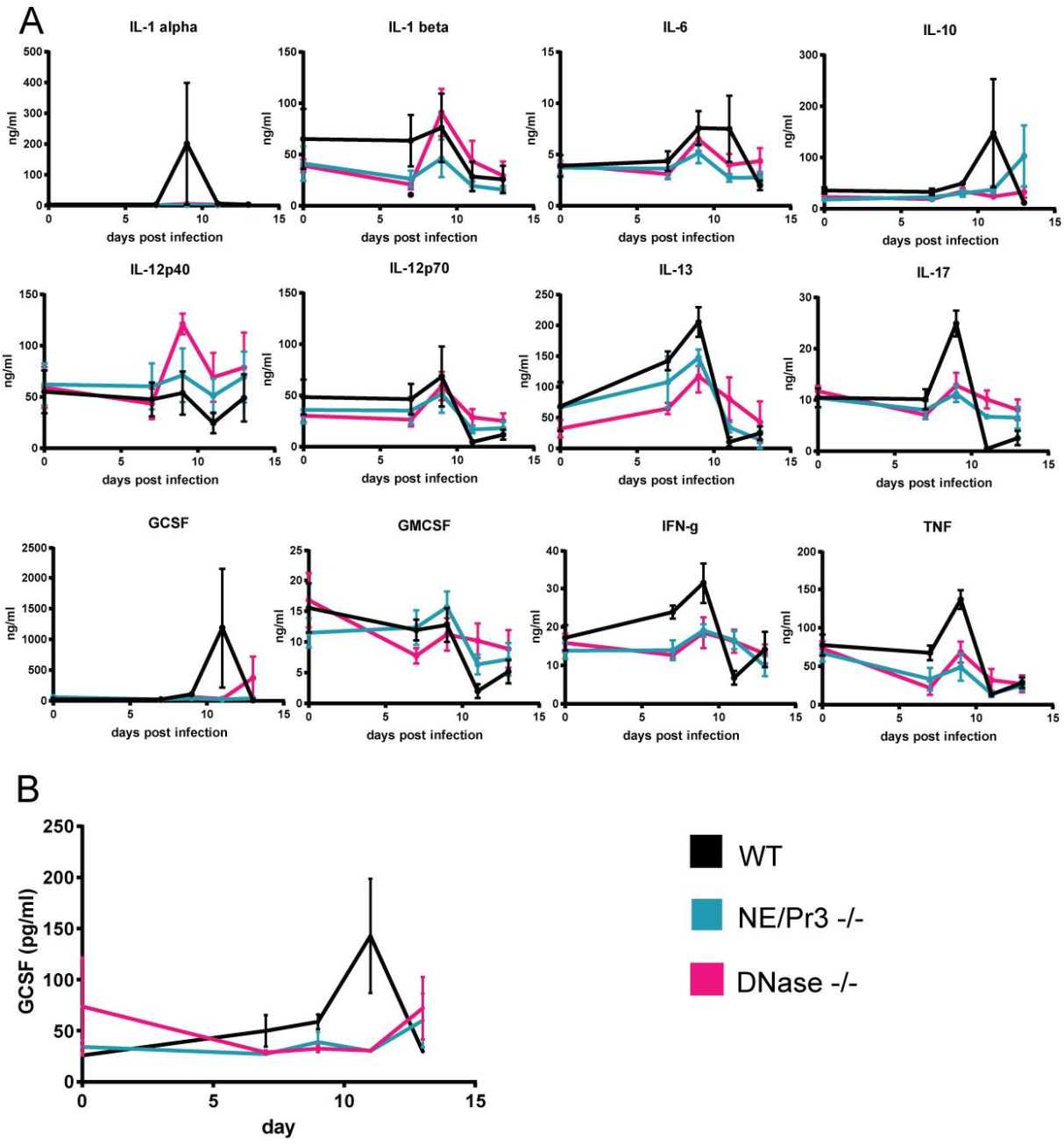

Figure 14: NET components have a proinflammatory effect in a P. chabaudi mouse model of malaria. Bioplex analysis of the plasma generated by infection of WT, NE/Pr3 -/- and DNase 1 -/- mice with the rodent parasite *P. chabaudi*. The group size of all data presented is n = 6. Data is presented as the mean ± standard error of the mean (SEM).

6.5 Reintroduction of NET components restores pathology in NET-deficient mice

To confirm that the observed lack of pathology is due to absence of circulating NETs, rather than some other function of neutrophils or DNase 1. We also wanted to gain more insight into the mechanism by which NETs cause emergency granulopoiesis and endothelial activation.

We therefore decided to infect NET-deficient NE/Pr3 -/- mice in the same fashion as before and then complement them with exogenously-provided NETs via injection, to prove that NETs themselves are pathogenic in a malaria setting.

The NETs to be injected were generated *in vitro* from WT peritoneal neutrophils. These cells were stimulated for 18 hours with PMA. The PMA was washed away and the remaining NETs were scraped of the tissue culture dish. In their untreated form, NETs form viscous clumps that are impossible to accurately pipet or inject. Samples were therefore sonicated before the DNA concentration was determined by picogreen assay. We injected each mouse with an amount of NET DNA that corresponds to the total amount measured in previous experiments (400 ng/mouse).

To identify which specific component of the NET structure is responsible for the observed effect we also decided to inject the following:

DNase treated NETs: We treated the NETs to completion with DNase 1 which was afterwards removed by immunoprecipitation. If the DNA part of the NETs is responsible for the immunostimulatory effects, the reinjection of these samples should exhibit no effect.

Nucleosomes: Nucleosomes are complexes of DNA and histones and represent the major structural component of chromatin and NETs. We purified mouse nucleosomes from chromatin from bone marrow derived macrophages (BMM) by subcellular fractionation. If the neutrophil specific proteins on the NETs are responsible for the effects that were observed before, the injection of these samples should exhibit no effect on the pathology.

Sivelestat treated NETs: The neutrophil proteases NE and Pr3 are not only important for the formation of NETs but also remain associated and continue to be active while bound to NETs. Furthermore they have also been identified as mediators of NET immune stimulatory functions.

Sivelestat irreversibly inhibits the activity of these two proteins. We treated *in vitro* produced NETs with the inhibitor and subsequently washed it away, before injection into mice. If the proteases are responsible for the observed effects, injection of these samples should be unable to restore pathology.

As displayed in Figure 15 A the injection of different types of NET preparations did not affect parasite growth in the animals. Interestingly injection of sonicated or DNase treated NETs fully restored pathology in NE/Pr3 -/- mice, as demonstrated by detection of circulating liver enzymes (AST) comparable to the levels in WT mice, while once again the infected NE/Pr3 -/- treated with only buffer showed very little to no pathology (Fig. 15 B). On the other hand, injection of protease inhibitor-treated NETs or purified nucleosomes failed to restore pathology in NE/Pr3 knockouts. These findings confirm that NET components are the detrimental factor driving the liver pathology and identify neutrophil serine proteases as the key immunomodulatory molecules.

We then measureed G-CSF in the circulation of these animals and observed a result comparable to the behavior of the liver enzymes (Fig. 15 C). No circulating G-CSF is found in uninfected mice of either genotype when they are injected with sonicated NETs, indicating that NET-associated proteases are necessary but not sufficient for induction of this cytokine. This situation is perhaps functionally analogous to the 'two-hit'-mechanism required for TNF/heme activation of NETosis. As shown in Fig.14 a broad range of cytokines is present in the blood stream of infected mice before G-CSF can be detected. Interestingly IFNγ can enhance the production of G-CSF by adherent monocytes upon LPS stimulation in humans (Ref 1993). G-CSF production is also regulated by TNF, IL-1α and IL-17, which are present early on during *P. chabaudi* infection (Fig 13).

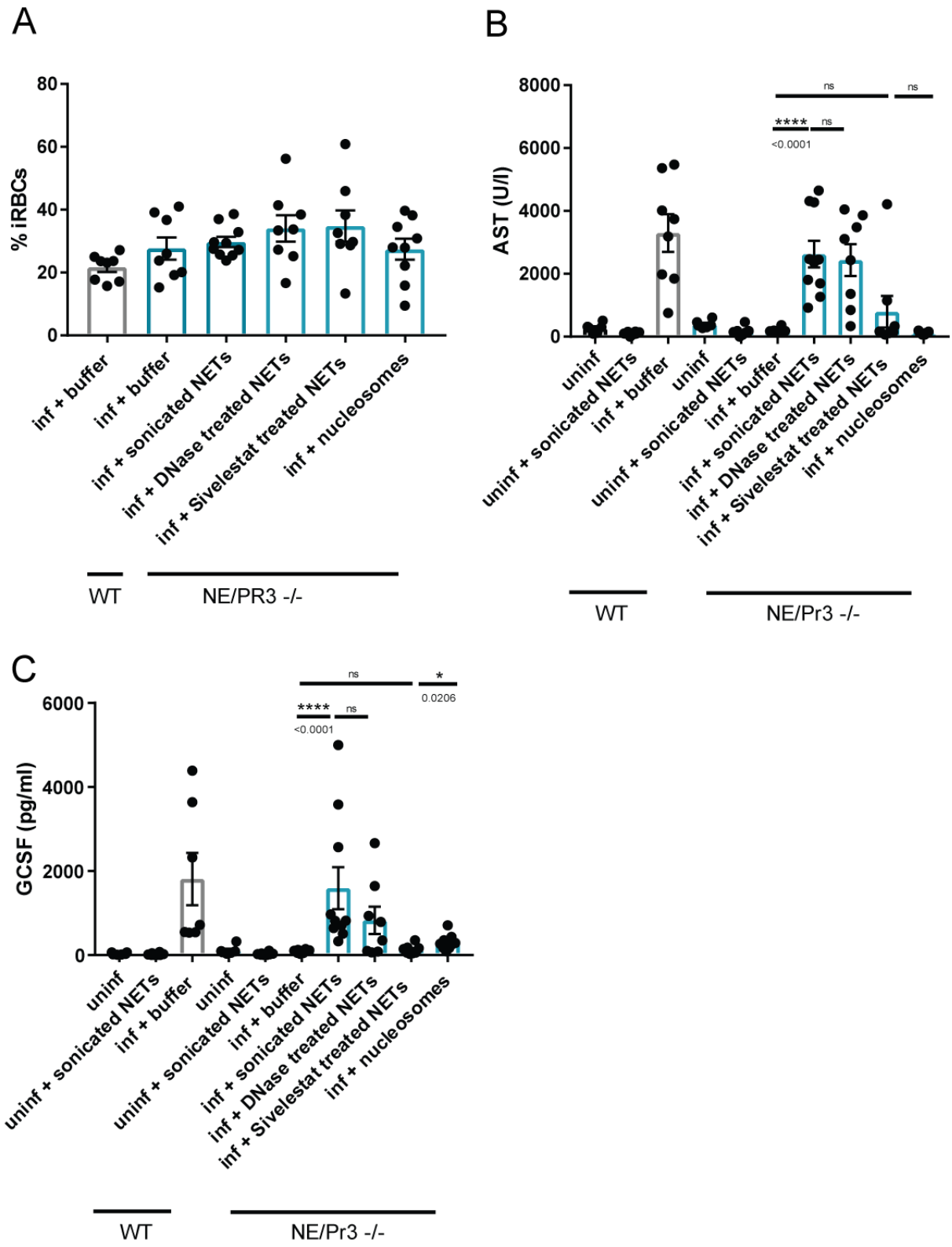

Figure 15: NET components are sufficient to restore pathology in NET-deficient P. chabaudi infected mice. (A) Parasitemia of infected mice as determined by counting of thin blood smears. Images were taken at a 63X magnification and at least 500 red blood cells were counted per samples. (B) Concentration of aspartate aminotransferase in plasma of mice over the course of the experiment. (C) Concentration of G-CSF was determined by sandwich ELISA of plasma samples. The group size of all data presented is n = 5 -10. Data is presented as the mean ± standard error of the mean (SEM). Asterisks indicate significance: *P<.05, **P<.01, ***P<.001 and ****P<0.0001 by Mann-Whitney test.

6.6 NET components induce adhesion molecules in *Plasmodium chabaudi* malaria

Our work shows a lack of pathology in mice deficient in either the production or the extracellular digestion of NETs. This phenomenon coincides with a lack of G-CSF release, reduced neutrophil infiltration into affected organs and reduced sequestration of infected red blood cells in the microvasculature of the liver.

The pathology of *P. falciparum* – the parasite responsible for most malaria related deaths still occurring in the world – relies partly on the binding of iRBCs to the endothelium of afflicted organs by ICAMs, VCAMs, CD36 and most recently described endothelial protein C receptor (EPCR) (Tuikue Ndam, Moussiliou et al. 2017)

The *P. chabaudi* mouse model reflects human disease closely as pathology is dependent on the expression of ICAM-1. It is required for cytoadhesion and hence sequestration of iRBCs in the microvasculature. ICAM-1 deficient mice show less pathology during a *P. chabaudi* infection (Cunningham, Lin et al. 2017).

Because we observed decreased sequestration in both deficient strains we set out to determine the expression of ICAM-1 in the livers of the infected cells. Liver sections were stained by antibody staining, scanned using a ZEISS Axio Scan system (representative images in Fig. 16 B) and percent ICAM-1 signal was quantified blinded by a trained technician. We observed an increase in ICAM-1 expression in the livers of infected WT mice over the course of the infection (Fig. 16 A) that coincides with the peak in parasite burden, sequestration and liver damage. Interestingly, both the NE/Pr3 and the DNase deficient mice did not show an increase in ICAM-1 expression in the liver during infection.

ICAM-1 can be shed from the surface of cells by an uncharacterized mechanism and to unknown consequence. The presence of soluble ICAM-1 in circulation can however serves as an indicator for vascular activation and an overall inflammatory setting (Witkowska and Borawska 2004). We measured sICAM-1 in the plasma of infected WT mice by sandwich ELISA and observed a significant increase when compared to uninfected mice. Interestingly the injection of NETs by themselves did not induce sICAM-1 in uninfected WT mice (Fig. 16 C).

NE/Pr3 deficient mice failed to induce sICAM-1 in response to *P. chabaudi* infection. Strikingly, and in accordance with our observations regarding liver damage and G-CSF production, injection of NETs into infected NE/Pr3 mice restored sICAM-1 levels. In contrast to our previous findings however, this is also true for Sivelestat treated NETs (Fig. 16 C) and might indicate that a less specific neutrophil protease inhibitor might be an even more promising treatment candidate. In DNase deficient mice we observed no ICAM-1 induction on the liver vasculature throughout the infection but do observe an increase in sICAM-1 comparable to WT mice (Fig. 16 C).

EPCR is a transmembrane receptor classically known for its capacity to bind protein C and activated protein C and thus being an important factor in anticoagulation. Membrane-bound EPCR also exhibits cytoprotective effects by inhibiting apoptosis (Mohan Rao, Esmon et al. 2014). Interestingly, infection, production of cytokines and ROS can lead to the cleavage of membrane-bound EPCR by metalloproteases and the release of a soluble form of the receptor (Xu, Qu et al. 2000). In stark contrast to its former role, sEPCR binds APC and inhibits its function thus propagating a coagulation and thrombus formation (Mohan Rao, Esmon et al. 2014).

The role of EPCR in the pathogenesis of severe malaria is just now beginning to emerge and there is no coherent understanding of the underlying mechanisms. Parasite binding to membrane-bound EPCR through specific subtypes of PfEMP1 is clearly associated with disease severity (Turner, Lavstsen et al. 2013) but EPCR also seems to be lost at sites of parasite sequestration (Moxon, Wassmer et al. 2013). Soluble EPCR is positively correlated with disease severity in children suffering from cerebral malaria (Moussiliou, Alao et al. 2015).

To further our understanding of NET mediated malaria pathology, we measured sEPCR concentrations in the plasma of all three genotypes. WT mice showed a drastic increase in plasma sEPCR levels at the peak of pathology that was completely absent in both NE/Pr3 and DNase -/- mice. Astonishingly, reinjection of fragmented NETs completely restored plasma sEPCR levels in NE/Pr3 deficient mice (Fig. 16 D).

Taken together these data suggests that NET components, and specifically NET-bound serine proteases, are crucial in generating a proinflammatory environment during a malaria infection, leading to cytokine production, immune cell recruitment and endothelial activation. These changes promote parasite sequestration and immune cell infiltration and eventually lead to organ damage.

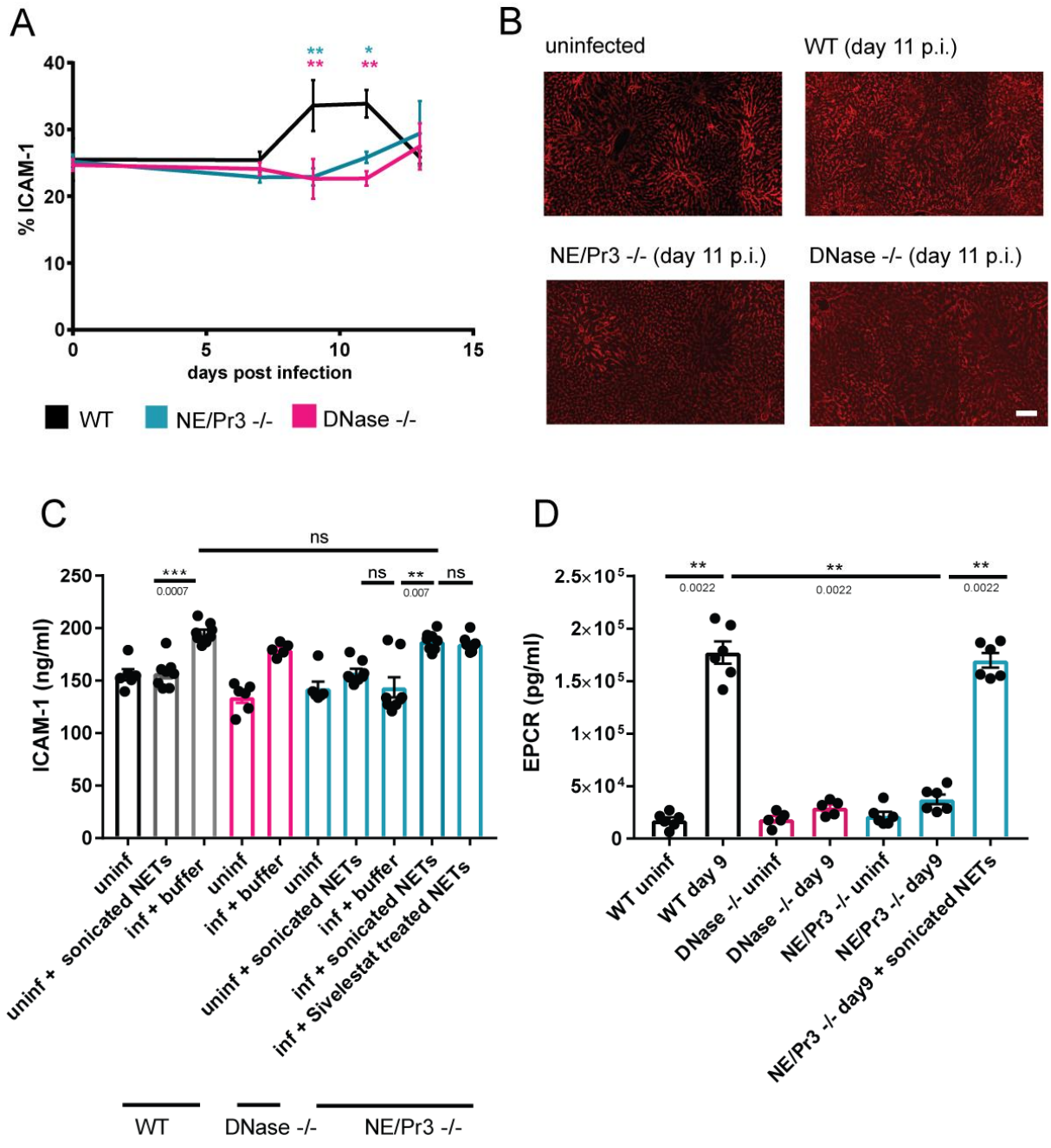

Figure 16: NET components induce endothelial activation in *P. chabaudi* malaria. ICAM-1 induction was determined in livers of infected animals by immunofluorescent staining and subsequent microscopy. The scanning system used is a ZEISS Axio scan and quantification was carried out blindly by a trained technician (A & B). ICAM-1 concentrations in plasma of infected animals was determined by sandwich ELISA (C). EPCR concentration in plasma was determined by sandwich ELISA (D). The group size of all data presented is n = 5 - 10. Data is presented as the mean ± standard error of the mean (SEM), each dot represents one biological replicate. Asterisks indicate significance: *P<.05, **P<.01, ***P<.001 and ****P<0.0001 by two-way analysis of variance (ANOVA) comparison of 3 groups (A) or Mann-Whitney test (C & D). Scale bar = 100 μm

7 G-CSF blocking antibodies decrease malaria pathology

We continued our work by trying to translate the findings described above into a veritable therapeutic approach against organ damage in malaria. We have seen an increase in circulating G-CSF that coincides with increased levels of circulating neutrophils as well as ultimately organ damage. Additionally, G-CSF is found in human patients suffering from complicated malaria and is quickly reduced upon treatment with anti-malarials (Ref 2000 Stoiser).

We set out to treat mice with anti-G-CSF blocking antibodies similar to previous studies (Ref Morris 2015). We used a single dose of 100 µg of antibody. Anti-G-CSF treatment did not affect the parasitemia (Fig 17 A) but significantly decreased the amount of circulating liver enzymes (AST), compared to animals injected with an isotype control (Fig. 17 B). These data suggest that therapies targeting G-CSF induction may represent a treatment strategy in malaria

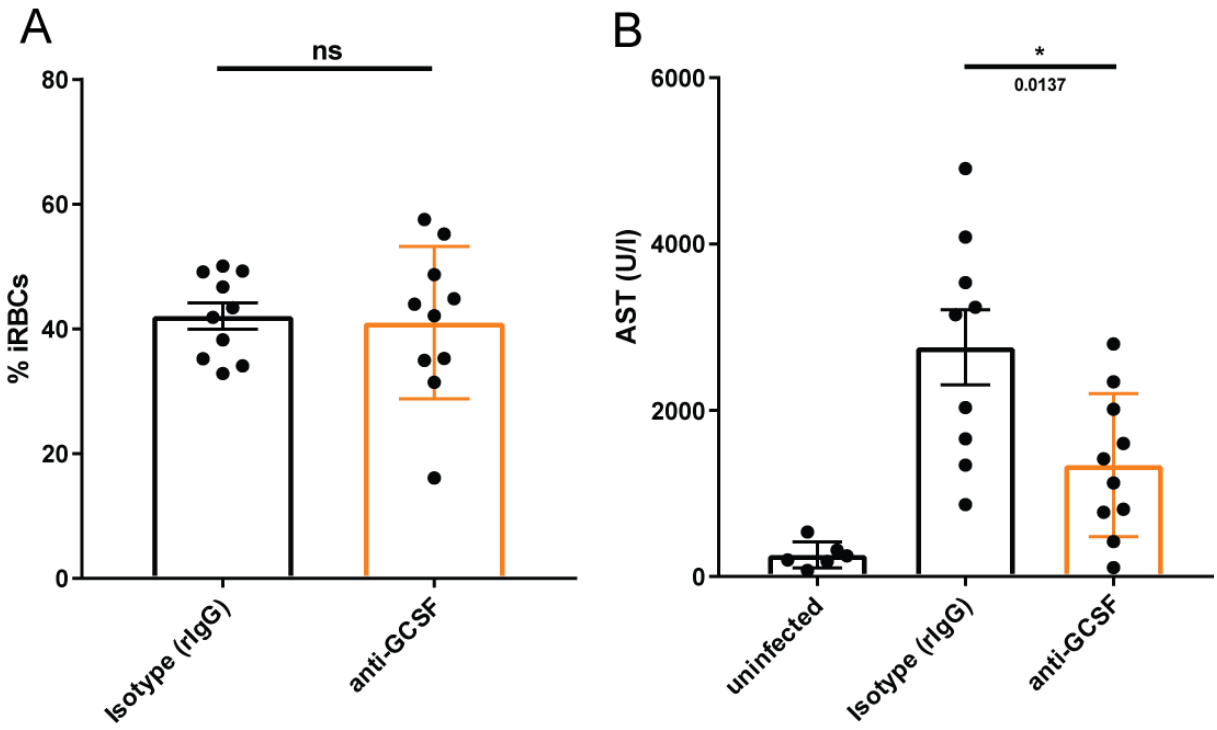

Figure 17: G-CSF blocking is protective in *P. chabaudi* malaria. (A) Parasitemia of infected mice as determined by counting of thin blood smears. Images were taken at a 63X magnification and at least 500 red blood cells were counted per samples. (B) Concentration of aspartate aminotransferase in plasma of mice over the course of the experiment. The group size of all data presented is n = 6 - 10. Data is presented as the mean ± standard error of the mean (SEM), each dot represents one biological replicate. Asterisks indicate significance: *P<.05, **P<.01, ***P<.001 and ****P<0.0001 by student's t-test

Considering the positive effect, we observed by treating mice with anti-G-CSF blocking antibodies we evaluated whether this might be a possible approach in human patients. We therefore quantified the amount of G-CSF present in the plasma of the patients that we recruited to our initial study. We observed a significant increase of G-CSF in *Plasmodium* infected individuals as compared to healthy donors (Fig.18)

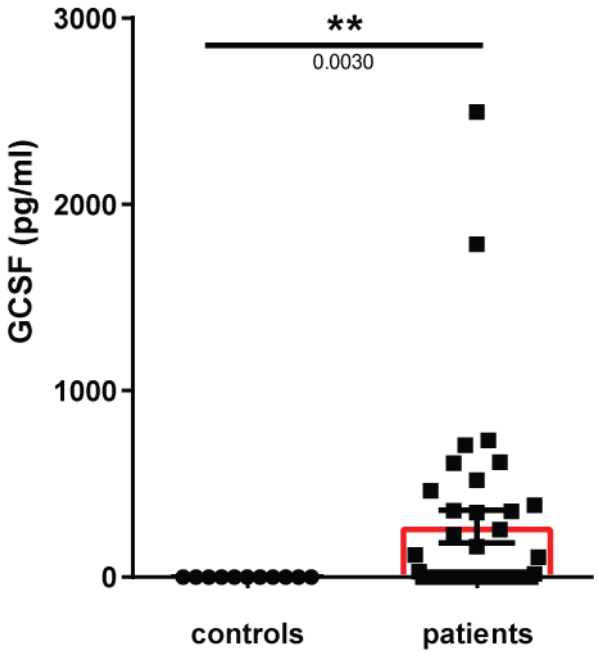

Figure 18: G-CSF is induced in patients suffering from malaria. Concentration of G-CSF was determined by sandwich ELISA of plasma samples. Data is presented as the mean ± standard error of the mean (SEM), each dot represents one biological replicate. Asterisks indicate significance: *P<.05, **P<.01, ***P<.001 and ****P<0.0001 by Welch's t-test

DISCUSSION

Malaria is an extremely diverse disease with symptoms that range from mild fatigue, fever and rigor to single or multiple organ failure, respiratory distress, jaundice and eventually lethal neurological complications in cerebral malaria (CM) (Coban, Lee et al. 2018). In spite of extensive research on malaria pathology in hope of novel therapeutic approaches, the underlying mechanisms remain elusive.

Historically the pathogenesis of malaria was attributed to vascular occlusion mediated by parasite adherence to the endothelial walls of the microvasculature and to other infected and non-infected red blood cells, leading to the formation of thrombus-like complexes that block and rupture vessels. These events are comparable to micro strokes. The immune system plays an important role in the pathological processes underlying organ damage in a *Plasmodium* infection. In the late 1980s and early 1990s, TNF- and IFN-γ blocking antibodies were shown to prevent CM in the *P. berghei* mouse model (Grau, Heremans et al. 1989, Grau, Piguet et al. 1989). Subsequently a role for almost every immune cell including γδ-Tcells, NKT-cells, CD8- T cells, monocytes, macrophages and neutrophils and a wide range of cytokines and chemokines - CXCL10, IL-17, IL-12p40, IL-10, IFNγ, TNF and GCSF - were described in malaria pathogenesis (Artavanis-Tsakonas, Tongren et al. 2003), highlighting the complexity of disease mechanisms.

Prior to this project, there was fragmented information about the role neutrophils play in a malaria infection. By Ly6 G/C mediated depletion, neutrophils were attributed a role in the pathophysiology of experimental cerebral malaria (Chen, Zhang et al. 2000) although this effect is hard to interpret because the antibody also depletes monocytes, macrophages and to some degree CD8+ T-cells. In a more specific approach Rocha et al. showed that depletion of neutrophils by anti-Ly-6G antibody injection abrogates liver damage and reduces neutrophil infiltration into the liver in *P. chabaudi* infected mice (Rocha, Marques et al. 2015). They also report an inflammatory and type 1 interferon responsive phenotype for human neutrophils isolated from *P. vivax* infected individuals. This observation coincides with several other findings, namely by Mohammed et al. and Feintuch et al., who observed a direct correlation

between circulating neutrophil granular proteins such as NE, Pr3, MPO, lysozyme and lipocalin in circulation of patients and severity of disease (Mohammed, Elghazali et al. 2003, Rocha, Marques et al. 2015, Feintuch, Saidi et al. 2016).

NETs have been described in patients infected with *P. falciparum* albeit the authors show blood smears from peripheral blood of these patients stained with either Giemsa or DAPI containing cloud-like DNA structures but did not assess colocalization with neutrophil proteins (Baker, Imade et al. 2008). A second paper addressing the possibility of NET formation published by Sercundes et al. shows cloud like structures of MPO in tissue sections of lungs of *P. berghei ANKA* infected DBA/2 mice but does not address the systemic release of NETs (Sercundes, Ortolan et al. 2016). Here, we provide definitive evidence that *bona fide* NETs are released during *P. falciparum* infections and show that they are induced via a heme-/TNF and ROS dependent mechanisms.

Furthermore, we demonstrate, in a *P. chabaudi* mouse model, that NETs are proinflammatory during a malaria infection. NETosis releases molecules that induce proinflammatory cytokines, emergency granulopoiesis, endothelial activation, immune cell recruitment and tissue infiltration as well as parasite sequestration. These results suggest that NET formation might be a potential treatment target in malaria.

8 NET formation in response to Plasmodium DAMPs and PAMPs

The first part of this thesis addresses whether NETs are released in the circulation of patients suffering from malaria. We further investigate by which mechanism *Plasmodium* infection might lead to such an induction of NETosis. Here we interpret our findings and discuss their relevance to the field as well as implications for future research conducted in the field of malaria pathology.

8.1 Correlation between hemolysis and circulating NETs

We conducted a field study in a malaria endemic region in Gabon and measured NETs in the circulation of malaria patients. We showed a positive correlation between hemolysis (by extracellular heme quantification) and NET concentration (Fig. 7).

The presence of NETs was previously suggested in malaria patients (Baker, Imade et al. 2008), but this study only showed cloud-like DNA-containing structures on Giemsa stained blood smears of infected individuals. To properly identify a NET and characterize its neutrophilic origin it is necessary to observe colocalization of DNA with neutrophil proteins such as NE, Pr3 or MPO. The sandwich ELISA approach that we used to identify the presence of NETs in circulation of infected individuals is based on this colocalization of NE and DNA. Additionally, using *in vitro* generated NETs to produce a standard curve allows us to quantitatively asses the amount of NETs present in individual patients. Future studies might use this tool to study the correlation between NETs and disease severity or onset, thus possibly supplying an interesting diagnostic tool to predict whether severe disease might develop.

Because malaria is a hemolytic disease, the presence of extracellular heme in circulation of malaria patients is not all together surprising, but has important implications for the disease progression. Heme consists of a tetrapyrrole ring with a central iron atom (Fe) coordinated to the pyrrole rings, which readily and reversibly changes its oxidation state. It is a ubiquitous prosthetic group used to catalyze oxidative reactions, electron transfer processes and the delivery of oxygen to cells (Dutra and Bozza 2014). While these abilities are essential to many biological processes, they can also be pathological. Free heme oxidizes lipids and proteins (Vincent 1989) and damage DNA (Aft and Mueller 1984). Furthermore, the hydrophobic porphyrin ring intercalates in lipid layers destabilizing biological membranes by increasing their permeability leading to lysis of the affected cells (Schmitt, Frezzatti et al. 1993). On top of that, heme also leads to the production of free radicals through the Fenton reaction, which catalyzes hydroxyl radicals from the reaction of Fe with H_2O_2.

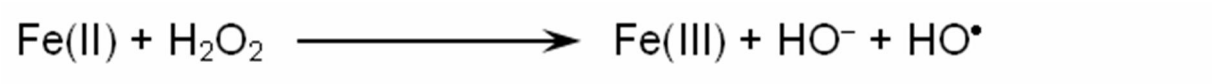

Fig.19: Chemical equation of the Fenton reaction.

Despite these hydrophobic properties, cells sense heme by specific receptors. In macrophages recognition of free heme by TLR4 leads to activation of MyD88 but not TRIF dependent intracellular signaling cascades. MyD88, in turn, activates the mitogen-dependent proteins kinases (MAPK) and further downstream NF-κB thus inducing inflammatory cytokines like TNF, IL-6 and KC (Takeuchi and Akira 2010). Heme also induces leukotriene B4 (LTB4), a chemoattractant for neutrophils, by macrophages *in vivo* (Monteiro, Pinheiro et al. 2011). Interestingly, heme induces neutrophil migration *in vitro* in the absence of macrophages which is inhibited by Pertussis toxin indicating the involvement of a G-protein coupled receptor (GPCR) in heme sensing by neutrophils (Porto, Alves et al. 2007). Although heme induced NET formation itself is clearly established, the specific receptor required for this activation is not known. Both TLR4 as well as a range of GPCRs are potential candidates.

Heme is present in large quantities within the hemoglobin contained in red blood cells making it a critical factor to be released in all hemolytic diseases (sickle cell disease, hemorrhagic fevers, malaria, β-thalassemia, etc.). The danger of free heme is reflected in a plethora of control mechanisms aimed at dealing with it. Hemoglobin is bound by Haptoglobin in circulation preventing free heme from being released from the protein (Melamed-Frank, Lache et al. 2001). Additionally, the heme scavenger hemopexin binds free heme, inhibits its oxidative property and transports it to intracellular compartments through the macrophage receptor CD91 (Hvidberg, Maniecki et al. 2005). Intracellularly heme is catabolized by heme oxygenase 1 (HO-1) into billiverdin, CO and Fe, which is then recycled by ferritin. In a final fail-safe mechanism, heme binds and is cleared by serum albumin. Nevertheless, in severe hemolysis these endogenous scavenging mechanisms are overwhelmed and heme accumulates in biological fluids.

Interestingly, the survival of mice infected with *P. berghei* depends on their ability to express HO-1 indicating deleterious effects of heme in *Plasmodium* infections (Ferreira, Balla et al.

2008). Similarly, heme induces NETs and drives the pathology in patients and mice suffering from sickle cell disease (Chen, Zhang et al. 2014). The positive correlation we observed between the amount of NETs and free heme in circulation supports the notion that a heme related mechanism of disease progression exists in malaria patients. Larger studies should address the importance of heme in malaria patients since it might represent a promising therapeutic target. Decrease of heme concentration and activity might have at least four beneficial effects on afflicted patients: (1) Heme lyse RBCs (Chiu and Lubin 1989) and could lead to bystander hemolysis and severe anemia in malaria patients. This seems to be a self-propagating mechanism since lysis of RBCs will release hemoglobin and eventually heme into circulation. (2) Heme activates endothelial cells to express adhesion molecules, like ICAM-1 and VCAM-1 (Belcher, Chen et al. 2014), which mediate both parasite sequestration and neutrophil adhesion. (3) Heme activates macrophages to produce inflammatory stimuli, especially TNF by engaging TLR4 (Figueiredo, Fernandez et al. 2007) thus propagating inflammation and activation of other immune cells such as neutrophils (see next section). Once again, this is a positive feedback loop since TNF, in turn, primes macrophages for necroptosis, an inflammatory programmed form of necrotic cell death. (4) Lastly, heme activates PKC in neutrophils leading to ROS generation, inducing the expression of adhesion molecules, the production of IL-8 and the release of NETs (Chen, Zhang et al. 2014). The induction of NETosis by heme has previously been described, although the underlying mechanism, the required signaling pathways and effector molecules remain to be determined.

8.2 Mechanism of malaria associated NET formation

Strategies targeting extracellular heme are a possible approach to block malaria pathogenesis but would only combat one potential stimulus. Malaria is a complex and multifactorial disease and although we did not observe NET formation in response to iRBCs, merozoites, the parasite digestive vacuole or even heme itself in resting unprimed neutrophils, we showed that NETs are produced in response to malaria associated free heme after TNF priming in a ROS and

protease activity dependent manner (Fig. 8 & 9). The inflammatory environment in the blood stream of infected individuals may however favor NET release in response to the other stimuli. While the term 'neutrophil priming' is liberally used throughout the literature and associated with overall increased performance including enhanced phagocytosis, degranulation and NET formation, and, contradictory, to the latter enhanced longevity of the cells, the actual underlying mechanisms are not understood (Condliffe, Kitchen et al. 1998). Interestingly, TNF priming of neutrophils induces increased levels of H_2O_2 in the cells, which in cooperation with free heme might lead to free radical formation (see above). The induction of H_2O_2 is also observed in the presence of GCSF, IL-1α and IL-6 (Elbim, Bailly et al. 1994), all of which are induced in malaria patients and infected mice.

I*n vitro* experiments with neutrophils isolated from CGD patients showed that no NADPH oxidase activity is required for heme generated NETs. Other stimuli, like silica and the calcium ionophore A23187 induce NETosis (Brinkmann, Goosmann et al. 2012, Douda, Khan et al. 2015) independently of NOX2 relying, at least partially, on mitochondrial ROS (Douda, Khan et al. 2015). In these cases ROS are produced by enzymes of the electron transport chain or other cellular oxidant enzymes such as xanthine oxidase (Kalogeris, Bao et al. 2014). Our data show that heme, unlike PMA, relies on alternative sources of ROS and is mechanistically more similar to NET inducers like microcrystals.

In accordance with previous findings, heme induced NETs require PKC, CDK6 and the neutrophil specific protease NE. This finding underlines regulated sensing and response as opposed to secondary necrotic cell death that could also lead to the release of chromatin from neutrophils. PKC and CDK6 are important regulators of cell activation and proliferation, respectively (Altman and Kong 2014, Sherr, Beach et al. 2016) and therapeutic or experimental inhibition of their activity may lead to detrimental effects apart from NETosis inhibition. The inhibition of the neutrophil specific protease NE and Pr3, on the other hand, is a promising strategy to inhibit NETosis in human and murine (Fig. 10) neutrophils. As previously suggested (Kessenbrock, Dau et al. 2011) we observed that suppression of NE activity alone is not sufficient to abrogate NETosis completely (Fig. 10), arguing for the necessity of a dual neutrophil serine protease (NSP) inhibitor for therapeutic approaches in hemolytic diseases. In

agreement with our results, the neutrophil elastase 'specific' inhibitor Sivelestat can reduce the symptoms in a rat model of sepsis (Suda, Takeuchi et al. 2010, Li, Jia et al. 2016) but not abrogate them. Interestingly, Sivelestat inhibits Pr3 but to a far lower degree than it inhibits NE, probably leaving some residual activity. NE and possibly NET formation is a target for several other diseases such as chronic obstructive pulmonary disease (von Nussbaum and Li 2015) and *Pseudomonas aeruginosa* lung infection (Benarafa, Priebe et al. 2007) indicating that discoveries concerning the pathogenic mode of action of NETs and NSPs could have a general therapeutic impact.

Although PAD4 has been described as an essential enzyme to release NETs, we showed that neither inhibition nor genetic deficiency in the PAD4 gene have any effect on either PMA or heme/TNF mediated NET formation. Indeed, there is a controversy in the field as to whether PAD4 activity is required for NET formation (Konig and Andrade 2016) or whether it is a mechanism by which protein function on the released structure can be altered. Our data points towards a non-essential role of protein deimination, however PAD4 requirement was initially described for LPS induced NETs (Li, Li et al. 2010) and might just be specific to certain stimuli. There appear to be several paths leading to NETosis.

9 NET components drive cytoadherence and inflammatory pathogenesis

9.1 *P. chabaudi* malaria

The second part of this thesis utilizes the *Plasmodium chabaudi* mouse model to address the mechanism by which neutrophils provoke organ damage of a blood borne hemolytic infection. As mentioned before, we chose the *P. chabaudi* mouse model for two main reasons: First, The human pathogenic *Plasmodia* are synchronous, meaning that development in the infected RBCs happens all at the same time throughout the entire host. This has important implications since it leads to synchronous iRBCs sequestration in the microvasculature of afflicted organs leading to high local parasite load. Furthermore, synchronous rupture of iRBCs leads to a sudden and drastic surge in local PAMPs and DAMPs and subsequently cytokine concentration (Autino, Corbett et al. 2012, Gazzinelli, Kalantari et al. 2014). *P. chabaudi* has a synchronous replication cycle, *w*hile other mouse parasites strains such as *P. berghei ANKA* and *P. yoelii* do not.

Second, human severe disease and tissue damage are associated with sequestration of infected red blood cells into the deep bed vasculature of afflicted organs (Autino, Corbett et al. 2012). This sequestration depends on parasite receptors such as the infamous PfEMP1 and host adhesion molecules such as CD36, ICAM-1 and EPCR (Chakravorty and Craig 2005, Turner, Lavstsen et al. 2013). While *P. berghei ANKA* is the model of choice to investigate cerebral malaria, the most prominent form of severe malaria, *P. berghei* does not sequester and shows no binding to endothelial cells via surface adhesion molecules. We aimed to study neutrophils and their externalized components in the development of cytoadherence mediated tissue damage. In the *P. chabaudi* mouse model the involvement of neutrophils (Rocha, Marques et al. 2015) as well as ICAM-1 (Cunningham, Lin et al. 2017) are established, making it our model of choice.

The *in vitro* experiment shown in figure 10 allowed us to choose the appropriate mouse genotypes to address the two following questions. First, NE/Pr3 deficient mice are unable to produce NETs and allow us to test whether they are relevant in malaria. Second, DNase 1

deficient mice release NETs but do not degrade the macrostructure and therefore allow us to answer the question whether pathogenic effects of NETs are mediated by the large intact structure or by the release and dissemination of individual components.

9.2 The mechanism of NET component mediated tissue damage

In this study we show that NETs are formed in the circulation of *P. chabaudi* infected mice and lead to (1) the induction of emergency granulopoiesis via the generation of GCSF, (2) the upregulation of the endothelial adhesion markers ICAM-1 and EPCR thereby facilitating (3) parasite cytoadhesion and (4) neutrophil influx in the liver which ultimately lead to (5) liver damage. Prevention of NET formation in animals were NE and Pr3 are deleted abrogates the entire process. Furthermore, we demonstrate that these mechanisms of pathogenesis rely on the systemic dissemination of individual NET components as they are also abrogated in mice still capable of forming NETs but unable to digest NET structures in circulation (Fig. 11 – 18). Our findings underline that there are different mechanisms by which systemically produced NETs lead to adverse effects to the host. In direct contrast to what we observe in *P. chabaudi* infected mice, others showed that the application of recombinant DNase 1 can be advantageous in diseases linked to NET formation. The autoantibody-driven pathology of systemic lupus erythematosus for example is propagated by a decrease in DNase 1 activity in the plasma (Hakkim, Furnrohr et al. 2010) suggesting systemic DNase 1 application as a potential treatment. Moreover, the failure to degrade NETs in small vessels induce thrombosis and tissues damage in several mouse models of vascular occlusion (Brill, Fuchs et al. 2012, Jimenez-Alcazar, Napirei et al. 2015). Lastly local DNase 1 application prevents tissue damage in the lung of both LPS treated animals (Liu, Su et al. 2016) and in an acute lung injury model of malaria (Sercundes, Ortolan et al. 2016). Contrary to these studies and in accordance with our findings Dnase 1 is detrimental in a cecal ligation and puncture model of sepsis. Administration of the recombinant enzyme to septic animals decreases survival and increases inflammatory cytokines and tissue damage and neutrophil infiltration in livers and lungs (Meng, Paunel-Gorgulu et al. 2012).

It seems that comparable to human disease, the pathogenic event in the *P. chabaudi* model is the sequestration of the parasite into the microvasculature of afflicted organs. This mechanism does not play a role in sterile pathogenic conditions such as SLE and deep vein thrombosis, which might explain the drastically different roles DNase 1 seems to play under different conditions. Additionally, local application of DNase 1 by inhalation restricts the effect of the enzyme to NET structures occurring in the actual tissue of the organ thus showing a beneficial effect (Liu, Su et al. 2016, Sercundes, Ortolan et al. 2016). Such a local application is not possible in internal organs such as the liver, kidneys, spleen and brain which are afflicted during a systemic *Plasmodium* infection in the human host. Unfortunately, neither of the groups measured the impact such a local administration of DNase 1 had on the systemic levels of either NETs or neutrophil components such as NE, Pr3, MPO and lipocalin that are associated with severe disease in humans. The lack of this information makes speculations about differences and/or similarities between the studies almost impossible.

Neutrophil proteases regulate or induce inflammation and inflammatory tissue damage in multiple ways in addition to being imperative for NET formation (Fig. 10 and Fig. 11). Unfortunately, we can only speculate on the effects of these proteases in malaria since our experiments cannot establish a direct causal relationship between individual protease functions and disease progression. Indeed, the lack of tissue damage and cytokine release in uninfected mice injected with sonicated NETs (Fig. 15) argues for a multifactorial pathogenic mechanism, where infectious agents (PAMPs) and host response (DAMPs) contribute. Notably, Sivelestat treatment of NETs prior to injection into infected mice abrogated the adverse effects arguing for a role of neutrophil protease activity.

9.2.1 Neutrophil proteases and cytokines

In addition to regulating NETosis the neutrophil proteases are implicated in the intracellular regulation of IL-1β, as an alternative to the inflammasome in a caspase 1 independent manner (Netea, van de Veerdonk et al. 2015). We can exclude a role of such intracellular regulatory mechanisms based on the NET injection experiments describe in figure 15.

Extracellular neutrophil proteases can process cytokines to alter their activity (Clancy, Henry et al. 2017). This mechanism can either in- or decrease cytokine activity. For example, neutrophil proteases activate IL-8 through N-terminal cleavage (Shpacovitch, Feld et al. 2008) in humans. Although mice do not have IL-8, the aspect of cytokine activity modulation during a malaria infection might be an interesting topic to address in the *P. chabaudi* mouse model in the future. Interestingly, older studies suggest that neutrophil proteases generate a more active form of TNF (Kriegler, Perez et al. 1988). We observed that TNF escalates the inflammatory response by inducing NETosis together with heme. In addition, TNF activates the endothelium to upregulate cell adhesion markers such as ICAM-1 (Burke-Gaffney and Hellewell 1996, Chan, Shum et al. 2008). Although we have not addressed the effect of TNF in our model, ICAM-1 is vital for both parasite and immune cell sequestration. NET associated NE can also lead to the activation of IL-1α (Clancy, Henry et al. 2017) and this might, in an autocrine manner, lead to more production of the cytokine itself leading once again to a positive feed-forward loop. Absence of autocrine cytokine stimulation might explain the absence of many proinflammatory cytokines in the NET production and dissemination deficient genotypes (Fig. 14).
Interestingly so far, the role of NETs or their components in GCSF production and emergency granulopoiesis has not been described. We do demonstrate such a mechanism but only in the context of infection once again emphasizing the interplay between PAMPs and DAMPs.

9.2.2 NET components and adhesion molecules

NE upregulates the expression of ICAM-1 on endothelial cells directly (Ishihara, Yamaguchi et al. 2006) or, as described above, indirectly by regulating cytokine activity and expression. This is in accordance with our findings that ICAM-1 upregulation is decreased on the endothelium of infected animals deficient in NET production and processing (Fig. 16).
We also observe increased levels of soluble ICAM-1 (sICAM-1) and interestingly EPCR. It is possible that the upregulation of cell bound ICAM-1 binds to neutrophils (discussed below) and that these cells as well as extracellular neutrophil proteases cleave both ICAM and EPCR on the endothelial cell surface. At first glance, this appears to have a favorable effect since we

propose that it decreases binding of iRBCs, neutrophils and other immune cells. Moreover sICAM-1 decreases the adhesion capacity of neutrophils when binding to circulating cells (Spark, Scott et al. 1999), although this effect is debated and some scientists argue that sICAM-1 might be a shedding product and a marker for the upregulation of cell-bound ICAM-1 (Witkowska and Borawska 2004).

Whilst it is well established that cell bound EPCR is cytoprotective and negatively regulates the coagulation cascade and formation of thrombi by activating protein C (Mohan Rao, Esmon et al. 2014), the role of soluble EPCR (sEPCR) is less well understood. sEPCR binds APC blocking its activity propagating coagulation. Interestingly, a recent study shows that APC inhibits NET formation (Healy, Puy et al. 2017). The release of sEPCR in infected animals might lead to more NET formation in circulation aggravating the disease.

Lastly, several studies report direct induction of endothelial cell death by NET structures (Gupta, Joshi et al. 2010, Saffarzadeh, Juenemann et al. 2012, Meegan, Yang et al. 2017) which leads to vascular leakage and expose the underlying tissue to molecules present in circulation, suggesting another link between NETs and malaria pathogenic principles.

9.2.3 NET components and immune cell recruitment

Once NE and Pr3 are released from cells, either by degranulation or bound to NETs, they can degrade extracellular matrix proteins such as elastin, fibronectin and collagen (Mainardi, Hasty et al. 1980, McDonald and Kelley 1980, McGowan and Murray 1987) promoting tissue permeability and facilitating immune cell infiltration. This mechanism becomes especially relevant in the context of endothelial cell apoptosis. We observed neutrophil recruitment to the liver of infected mice which is absent in both the N/Pr3 and DNase deficient genotypes, although we cannot assess whether these cells left the vasculature and entered the underlying tissue (Fig. 13). It is also not possible to gage whether neutrophil recruitment to the liver is cause or effect of the damage to hepatocytes and whether other cell types might be involved.

In the *P. berghei* model of cerebral malaria several other cell types are involved in pathogenesis. Monocytes and/or macrophages accumulate in the brain of these animals (Srivastava, Field et

al. 2010). Neutrophil products attract monocytes to sites of infection either by modulating the cytokine environment (see above) or by releasing granular proteins through degranulation and NETosis (Soehnlein, Lindbom et al. 2009) and these activated monocytes might contribute to endothelial dysfunction by the release of ROS (Woller, Brandt et al. 2008). The accumulation of monocytes in afflicted organs also occurs in human patients (Dorovini-Zis, Schmidt et al. 2011) and might represent a translatable phenomenon to address in our model.

T-cells have a controversial role in the pathology of human diseases. Both CD4+ and CD8+ T-cells are required to develop experimental cerebral malaria (ECM). Potentially CD4+ cells prime CD8+ cells to carry out detrimental functions (Belnoue, Kayibanda et al. 2002). In addition, and in agreement with our findings that mere injection of NETs into uninfected mice does not cause pathology, adoptive transfer of CD8+ cells from infected to naive mice does not cause pathology (Nitcheu, Bonduelle et al. 2003). The controversy, however, stems from the fact that to this date no CD8+ Tcell involvement in human disease has been demonstrated, not even in post mortem histological studies. T-cells accumulate in the livers of infected mice and are essential for pathology as revealed by lack of organ damage in Rag -/- mice (Brugat, Cunningham et al. 2014). It will be interesting to test if T-cell recruitment is also dependent on neutrophil proteases.

9.3 Intervention strategies

In severe malaria anti-parasite therapy often fails to save patients (Varo, Crowley et al. 2018) highlighting the need for adjunctive therapies. Indeed, a wide range of immunomodulatory therapies were tested (Varo, Crowley et al. 2018). So far, none were sufficiently protective and the need for novel approaches remains. Our study suggests several potential targets of intervention.

Inhibition of NE/Pr3 to improve disease outcome in several infectious and non-infectious diseases is a well-established idea (Kaneko, Kudoh et al. 1997) and has already been shown to be partially effective in a malaria model (Sercundes, Ortolan et al. 2016). Our data confirms that

a pan-neutrophil serine protease inhibitor might be beneficial. The development of such a drug is an avenue worth exploring.

To prevent NET formation in malaria patients, it might be relevant to apply hemopexin, which was shown to work in other hemolytic conditions (Chen, Zhang et al. 2014). Furthermore, our data suggest that this therapeutic approach might have an especially beneficial effect when combined with anti-TNF therapy (Varo, Crowley et al. 2018).

As mentioned above to prevent NETosis an anti-sEPCR therapy is a possibility that might reduce the coagulatory potential and inhibit systemic NETosis by the release of the NET inhibitor APC (Healy, Puy et al. 2017). While antibodies against sEPCR exist (as used in the ELISA) and could be used to inhibit its activity, antibody therapy is hardly a viable approach to treat malaria (see below). It would therefore be of interest to develop small molecule inhibitors that can selectively act on either cell bound or soluble EPCR. Another approach might be to inhibit the shedding of EPCR from the endothelial surface by Withaferin (Ku, Han et al. 2014). The effects of such a treatment might be ambiguous, and might decrease NETosis but increase parasite and neutrophil adhesion.

We observe beneficial effects of anti-GCSF antibody therapy in infected mice, which might represent a powerful way to suppress emergency granulopoiesis that allows a sustained inflammatory environment including NETosis, cytokine release and endothelial activation. Unfortunately, there is currently no anti-GCSF antibody therapy available in patients and the development of such therapeutic agents is a long and difficult road. There are two major problems with antibody therapies that apply specially to developing countries: Firstly, while some forms of cancer are treated by antibody therapies in developed countries (Willems, Gauger et al. 2005), these therapies are expensive and unusable to control a disease that is associated with poverty. Secondly, antibodies must be applied intravenously and while this can and is done in malaria patients suffering from severe disease in urban hospitals, it is not practical in remote rural regions.

10 Translation of our findings to human disease

The observation that people of African origin and a few groups from the Middle East are neutropenic in homeostasis is in agreement with a detrimental role of neutrophils in malaria infections (Thobakgale and Ndung'u 2014). This condition is known as ethnic or benign neutropenia. Moreover, this condition is directly linked to single nucleotide polymorphisms in CDK6 and GCSF (Reiner, Lettre et al. 2011), which are directly involved in the NET and hematopoiesis driven mechanism of pathology we describe here. It is plausible that gene variants that promote tolerance to malaria are selected in endemic regions because this disease has exerted evolutionary selective pressure on human populations.

Similarly to what we observe in *P. chabaudi* infected mice, neutrophilia is common in patients suffering from *P. falciparum (*Maina, Walsh et al. 2010) and *P. vivax* (Rocha, Marques et al. 2015) infection and these increased neutrophil counts can be linked to symptoms of severe malaria (Squire, Asmah et al. 2016). As shown by us and others malaria is accompanied by elevated GCSF levels (Stoiser, Looareesuwan et al. 2000), suggesting a potential to translate the therapeutic success shown in Figure 17 to human disease.

Interestingly the role of endothelial adhesion molecules such as ICAM-1 (Chakravorty and Craig 2005, Cunningham, Lin et al. 2017) and EPCR (Turner, Lavstsen et al. 2013, Kessler, Dankwa et al. 2017) are conserved between human and mouse making our findings translatable to human disease. This opens a future line of investigation as discussed below.

11 Conclusions and Outlook

During this study, we demonstrated that NETs are formed in the circulation of humans and mice infected with *Plasmodium spp*. We identified TNF and extracellular heme as stimuli for this intravascular NET formation and observed a positive correlation between the amount of extracellular heme and extracellular NETs in human patients.

In the *P. chabaudi* model of malaria, we demonstrated that NETs are released over the course of the infection and are digested by Dnase 1 leading to the systemic dissemination of active NET components. These propagate a proinflammatory environment by inducing cytokine production and emergency granulopoiesis, activating the endothelium to express adhesion markers and thereby mediating the sequestration of infected red blood cells and neutrophils to the microvasculature of the liver. In conclusion, we showed that activated neutrophils perpetuate an inflammatory feed forward loop that leads to malaria associated organ damage (Fig. 19).

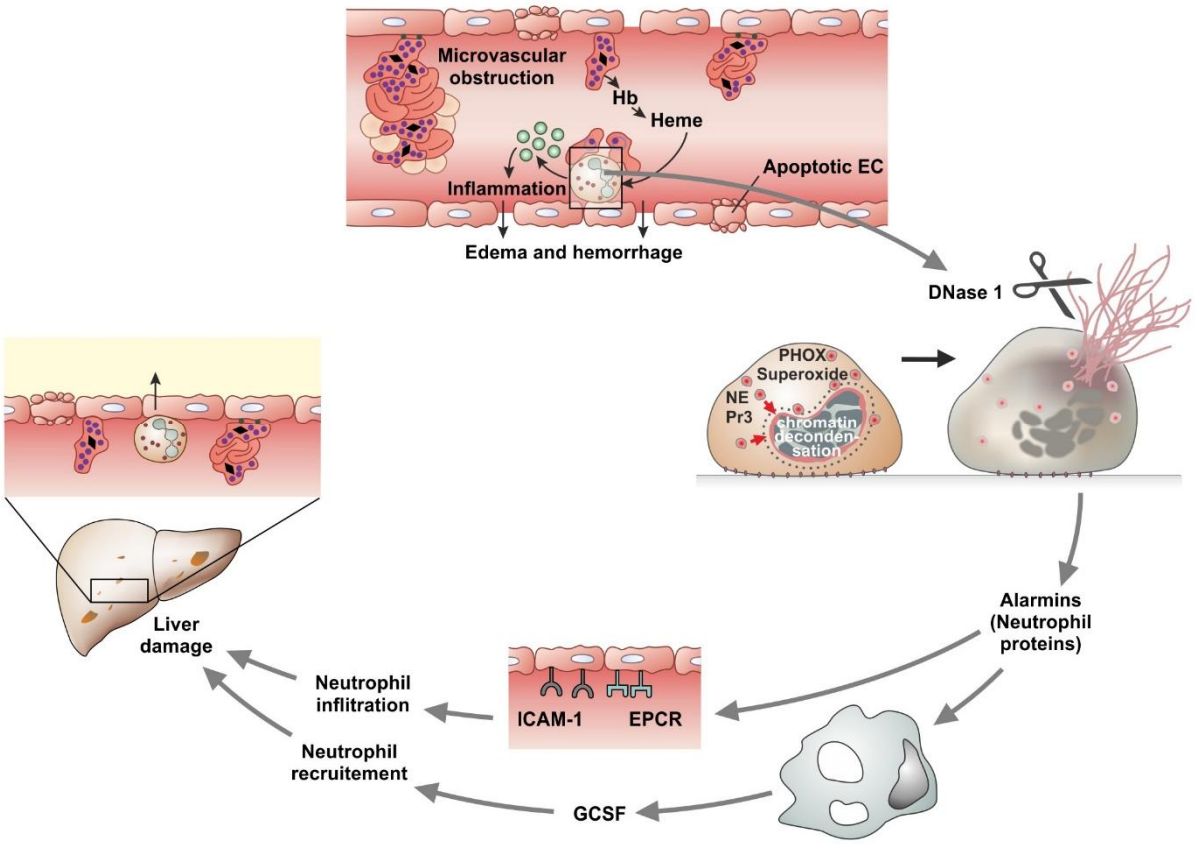

Fig.19: Graphic representation of the mechanism of neutrophil induced malaria pathogenesis. NETs are induced in circulation, digested by DNase 1and subsequently lead to the production of G-CSF and the upregulation of endothelial surface marker ICAM-1 and EPCR. This combination leads to neutrophilia and neutrophil and parasite sequestration into the liver

Future research should focus on the mechanism by which neutrophil components lead to endothelial activation since it might have therapeutic implications that extend beyond malaria and might be useful in the treatment of other hemolytic disease such as sepsis and sickle cell disease. It is important to identify what part of the NETs contributes to what degree to endothelial activation. It is also crucial to identify how these factors are sensed by endothelial cells, which intracellular signaling pathways are activated and exactly what array of surface molecules are upregulated in response to NET components. We observed that the inhibition of protease activity decreases the capacity of NETs to reconstitute pathology but the mechanism remains elusive. It would be an interesting initial experiment to inject proteases by themselves into infected NET deficient mice and monitor disease progression.

Finally, these findings should be taken to other fields of research where NETs and their interaction with endothelial cells might play a detrimental role such as sepsis, SLE and ischemic stroke. If we could prevent the uncontrolled activation of the endothelium by dying neutrophils this would hand us a mighty therapeutic tool.

MATERIALS & METHODS

12 Materials

12.1 Buffers and solutions

Casein solution	10X PBS, 9% (w/v) casein
ELISA general sample diluent	1% BSA in PBS-T
ELISA wash buffer	0.05 % Tween in PBS
FACS buffer	PBS, 2.5 % FCS, 0.1 %NaN_3
Human NET assay media	RPMI 1640, 10mM HEPES buffer, 0.05 % (v/v) Human serum albumin
Microscopy blocking buffer	PBS, 5 % (v/v) normal donkey serum. 15 % (v/v) Cold water fish gelatin, 1 % (w/v) bovine serum albumin, 0.05 % (v/v) Tween 20
Mouse NET assay media	RPMI 1640, 1% plasma from DNase k.o. mice,
Neutrophil preparation wash buffer	PBS, 0.05 % (v/v) human serum albumin
P. falciparum complete growth medium	RPMI (500 ml: 10.4 g RPMI, 5.95 g HEPES (25mM), 0.05 g hypoxanthine) Add: 25 ml Albumax II (10% in ddH_2O) 15 ml Sodium Bicarbonate (7% in ddH_2O) 0.1 ml Gentamicin (50mg/ml)
Peritoneal lavage solution	PBS, 0.02 % (v/v) EDTA
Posphate buffered saline (PBS)	137 mM NaCl, 2.7 mM KCl, 4.3 mM NA_2PO_4, 1.47 ,; KH_2PO_4
Bone marrow derived macrophage medium	DMEM + 10 % (v/v) FCS, 20 % (v/v) L929 cell conditioned supernatant, 1 % (v/v) L-glutamine, antibiotics to liking
Buffer A	10 mM HEPES, 10 mM KCl, 3 mM NaCl. 3 mM $MgCl_2$, 1 mM EDTA, 1mM EGTA, 2 mM dithiothreitol 2 mM phenylmethylsulfonyl fluoride 100 µg/ml soybean trypsin inhibitor 1 mM benzamidine 2 mM levamisole 1 mM NA_3VO_4 10 mM NAF 20 mM glycerophospaht 60 µl/5 x 10^6 cells inhibitor mixture (Sigma P-8340)
Buffer NE	20 mM HEPES, 25 % glycerol, 0.8 , KCL, 1mM MgCl2, 1% Nonidet P-40, 0.5 mM EDTA, 2mM dithiothreitol + inhibitors as in buffer A

12.2 Cell culture reagents

Reagent	Product Number	Manufacturer
Heparin natrium	H-NA-25000	Ratiopharm
Histopaque	11191	Sigma-Aldrich
Percoll	17-0891-01	GE Healthcare
RPMI	12633020	Gibco
Fetal calf serum	12003C	Sigma-Aldrich
	G7513	Sigma-Aldrich
Penicillin/Streptomycin	P4333	Sigma-Aldrich
HEPES	H0887	Sigma-Aldrich
PMA	P 8139	Sigma-Aldrich
Albunorm 20 %	200271	
Murine G-CSF	2505	PEPROTECH
Murine TNF	315-01A	PEPROTECH
Human TNF	300-01A	PEPROTECH
Heme (Hemin)	H651-9	Frontier Scientific

Inhibitor	Target	Chemical name	Concentration	Manufacturer
Go6976	PKC	5,6,7,13-Tetrahydro-13-methyl-5-oxo-12*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-12-propanenitrile	10 µM	Tocris
Pyrocatechol	ROS scavenger	1,2-Dihydroxybenzene	30 µM	Sigma-Aldrich
Cycloheximide	translation	3-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide	1 µg/ml	Sigma-Aldrich
LY2835219	CDK4/6		1 µM	Selleck
TDFA	PAD4	N-Acetyl-L-threonyl-L-α-aspartyl-N5-(2-fluoro-1-iminoethyl)-L-ornithinamide trifluoroacetate	200 µM	Tocris
GW311616A	NE	(3S,3aS,6aR)-3-Isopropyl-1-(methanesulfonyl)-4-[4-(1-piperidinyl)-2(E)-butenoyl]perhydropyrrolo[3,2b]pyrrol-2(1H)-one hydrochloride	20 µM	Sigma-Aldrich

12.3 ELISA and bioplex kits

Reagent	Product Name	Manufacturer
Elastase, Human, ELISA kit	HK319-02	Hycult Biotech
Cell Death Detection ELISA Plus	11920685001	Roche Diagnostics
Human Interleukin 8 Quantikine ELISA	S8000C	R&D Systems
Mouse Neutrophil Elastase Duo Set ELISA	DY4517-05	R&D Systems
Mouse G-CSF Quantikine ELISA Kit	MCS00	R&D Systems
Human G-CSF Quantikine ELISa Kit	SC S50	R&D Systems
Bio-Plex Pro Mouse Cytokine 23-plex Assay	M50-MSDS	BIORAD
Mouse ICAM-1 /CD54 Quantikine ELISA Kit	MIC100	R&D Systems
Mouse EPCR ELISA Kit	LS-F3979	LifeSpan BioSciences, Inc.

12.4 Antibodies & Dyes

Target	Product Name	Manufacturer	Assay
Biotin-anti-NE	Ab79962	abcam	NET ELISA
Anti-neutrophil elastase	481001	Calbiochem	IFA of cells
ICAM goat polyclonal antibody	AF796	Novus Biologicals	IFA of tissue
VCAM goat polyclonal antibody	AF643	Novus Biologicals	IFA of tissue
Calgranulin A [8A6a] rat monoclonal AB		MPIIB (Robert Hurwitz)	IFA of tissue
Hoechst33342	639	ImmunoChemistry	IFA of cells/tissue

13 Methods

13.1 Donor consent

Donors gave consent to blood drawing in accordance with the Helsinki Declaration. All patients or their parents in the case of children gave informed consent to the blood donation. The blood samples were collected with approval from the ethical committees responsible for each institution.

13.2 Human cell isolation and sample preparation

13.2.1 Human neutrophil isolation

Human neutrophils were isolated by two subsequent density gradient separations as has been established in the field (Fuchs, Abed et al. 2007). Blood was collected into EDTA KE containing tubes (monovette system) and subsequently layered onto an equal volume of Histopaque 1119 and centrifuged at 800 x g for 20 minutes with acceleration and brakes set at 7 and 3, respectively. The PBMC layer was then discarded and the neutrophil-rich layer collected, washed with neutrophil preparation wash buffer and pelleted by centrifugation at 300 x g for 10 minutes, acceleration and brakes set at 9. The resulting pellet was resuspended in 2 ml wash buffer and layered onto a discontinuous PBS-buffered Percoll gradient (85%, 80%, 75%, 70%, 65% in 2ml layers). The preparation was again centrifuged at 800 x g for 20 minutes with acceleration and brakes set to 7 and 3. The lower neutrophil band was collected, washed and resuspended in wash buffer. Cell concentration was determined using either a CASY cell counter or a Neubauer chamber. Neutrophils were further processed as soon as possible after isolation.

13.2.2 Human plasma preparation

Plasma from human malaria patients and healthy control donors was obtained from venous blood collected into EDTA KE containing monovette system tubes. 1 ml of blood was transferred to low-bind 1.5 ml Eppendorf and centrifuged at 10,000 x g at 4°C for 10 minutes. Plasma samples were snap frozen in liquid nitrogen until further use. Samples were always thawed on ice.

13.2.3 *P. falciparum* in vitro culture

The red blood cells necessary for P. falciparum culture were generated from the pelleted RBCs in the first step of the standard neutrophil isolation procedure. They were washed and resuspended to 50 % hematocrit in RPMI and stored at 4°C for up to two weeks.

Frozen parasites were removed from the freezer and thawed at room temperature, the 550 µl aliquot was transferred to a 50 ml Falcon tube. Very slowly and under constant agitation 5-7 drops of 12 % NaCl were added to the parasites and they were left standing at room temperature for five minutes. Afterwards 4.5 ml of 1.65 % NaCl were added at constant agitation and cells were incubated for five minutes at room temperature. Cells were pelleted by centrifugation at 3000 rpm for four minutes and washed with 4.5 ml 0.9 % NaCl/ 0.2 % Dextrose and then pelleted again. Then cells were resuspended in 5 ml culture medium and 300 µl red blood cells at 50 % hematocrit were added. Cluture flask was gased (to reach 5 % O_2) and placed at 37°C. *P. falciparum* was grown at 5% hematocrit at 5 % O_2 and cultures were generally maintained at a parasitemia below 20%.

13.2.4 *P. falciparum* infected red blood cell isolation

The mature-stage *P. falciparum* culture was washed and taken up in 2 ml of RPMI at up to 50% hematocrit, which were then layered onto 5 ml of a 60% Percoll solution. The mixture was centrifuged at 2000 x g at room temperature for 20 minutes with acceleration and brake set to 7 and 0, respectively. The 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 at 1500 rpm at room temperature for 3 minutes.

13.2.5 Free merozoite preparation

After enrichment the trophozoite infected red blood cells were pelleted once more and take up in 60 µl 0.03 % saponin solution and kept on ice for 5 min. Subsequently the sample was centrifuged at 16,000 x g at 4°C for 15 minutes, washed three times with PBS and taken up in 100 µl RPMI. Concentration of merozoites was determined by use of a Neubauer chamber.

13.3 Animal experiments

13.3.1 Ethics approval

Animal experiments are in compliance with the German animal protection law and have been officially approved by the Landesamt für Gesundheit und Soziales, Berlin.

13.3.2 Mouse neutrophil isolation

Murine neutrophils were isolated as described previously (Swamydas, Luo et al. 2015). Mice were injected once in the evening and once in the morning of the following day with 1 ml of 9% sterile casein solution. Three hours after the second injection mice were euthanized and the peritoneum was lavaged with 5 ml peritoneal lavage solution. The cells were subsequently pelleted by centrifugation at 200 x g for 10 minutes at room temperature and washed twice with PBS. Finally the pellet from each mouse was resuspended in 1 ml PBS and layered onto a 90% Percoll solution at room temperature in a 10-ml Beckman ultracentrifuge tube. The mixture was then spun for 20 minutes at 60,650 x g at 4°C. Neutrophils can subsequently be collected from the second opaque layer of the gradient, washed and used as soon as possible for experimental procedures.

13.3.3 *P. chabaudi* infection, plasma and organ preparation of WT, NE/Pr3 and DNase deficient mice

Mice of all genotypes were infected by injection of $1x10^4$ viable parasites *i.v.*. To ensure viability of the parasites a frozen stabilite was thawed and injected *i.p.* into a transfer mouse. Parasitemia of that mouse was monitored from day 3 after stabilite injection onward. Once the parasitemia had reached between 1 – 10% the amount of iRBCs/µl blood was calculated as:

$A = \%\ \text{parasitemia} \times 7 \times 10^6$

This allowed us to calculate the amount of blood needed to be diluted in 200 µl of PBS to achieve a suspension containing $1x10^4$ iRBCs/200 µl. The volume injected into each mouse was adjust 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 thin blood smear. On the prior established days post infection mice were bleed by cardiac

puncture under deep isoflurane anesthesia, leading to painless death by blood loss. Afterwards organs were harvested and fixed for 20h at room temperature in 2% PFA. The blood was kept from coagulating by addition of 50µM final concentration of EDTA and blood was kept on ice immediately after preparation. Plasma was generated by centrifugation at 10,000 x g at 4°C for 10 minutes. Plasma was aliquoted, frozen in liquid nitrogen and stored at -80°C until further use. Plasma was always thawed on ice.

13.3.4 Determination of liver enzyme concentration in mouse plasma

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

13.3.5 FACS analysis of mouse whole blood

After samples were spun down to collect plasma the cell pellet was resuspended to a volume equivalent to the collection volume. 100 µl of the sample were transferred to a FACS tube and stained with an equivalent 100 µl of FACS antibody diluted 1:100 in FACS buffer and incubate for 30 minutes at room temperature protected from light. Afterwards 3 ml of 1X FixLyse solution (Invitrogen 00-5333-54) was added to the samples and they were incubated for 60 minutes at room temperature protected from light. Samples were spun down at 300 x g at room temperature for 10 min and washed at least twice. Data was collected on a MACS Quant Analyzer after appropriate compensations between channels and analyzed using FlowJo software.

13.4 NET formation assay

13.4.1 Culture and stimulation of neutrophils

To be able to perform immunofluorescence staining, 24 well plates were prepared by adding glass cover slips to the bottom of each well. For the NETosis assays, $1x10^5$ neutrophils were added directly into 500 µl of NET assay medium for each well of a 24 well plate. In case of inhibitor experiments the medium already contained the appropriate inhibitor and the cells

were incubated for 30 minutes at 37°C. Afterwards TNF was added and cells were incubated for another 15 minutes. Afterwards 500 µl medium containing inhibitor, TNF and the appropriate stimulus (at 2X) was added to each well. TNF was used at 2 ng/ml, PMA at 50 nM and heme at 20 µM is not indicated differently. All reagents were freshly prepared on the day of the experiment.

Human neutrophils were fixed after three hours of stimulation.

Murine neutrophils were fixed after 18 – 20 hours of stimulation.

13.4.2 Quantification of NET formation

The quantification of NETosis was carried out as described here (Brinkmann, Goosmann et al. 2012). Briefly, cells were fixed for 30 minutes at room temperature in 2 % paraformaldehyde (PFA) post NET induction, 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 (Losman, Fasy et al. 1992). The secondary antibodies goat anti-mouse Alexa Fluor 568 and goat anti-rabbit Alexa Fluor 488 as well as Hoechst 33342 (Sigma-Aldrich) were used. 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.

13.5 Sample preparation for reinjection experiments

NETs (sonicated and DNase treated): Murine NETs were prepared as described above, washed to remove residual PMA, scraped from the plate and sonicated. Subsequently the DNA content was quantified by PicoGreen assay as described below. Where applicable sample was then treated with 2 U DNase 1 overnight at 37°C. Complete digestion of DNA was confirmed both by agarose gel and PicoGreen measurement.

NETs (Sivelestat treated): These samples were prepared as described above but Sivelestat was added two hours before dedicated harvest time and subsequently washed away before NETs were scraped from the plate.

Chromatin from bone marrow derived macrophages: bone marrow derived macrophages were thawed from frozen stock by carefully placing the vials in a 37°C water bath. Once thawed the cells were transferred to a 50 ml Falcon tube and 10 ml culture medium was added dropwise. Cells were then transferred to a tissue culture plate, the medium was changed after 4 hours to remove residual DMSO and non-adherent dead cells.

When cells were confluent and ready to be harvested, medium was removed and 5 ml cold (4°C) PBS was added. The petri dishes were then placed at 4°C for 15-30 minutes before they were washed off the plate with a pipette. Cells were then washed and counted, per 5 x 10^6 cells 300 µl of hypotonic buffer A 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. 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 and samples were stored at -80°C.

13.6 Oxidative burst chemiluminescence assay

ROS production was measured by luminol chemiluminescence. Horse reddish peroxidase catalyzes the oxidation of luminol by hydrogen peroxide generated by the respiratory burst, which generates a quantifiable signal when measured in a luminometer. Neutrophils were seeded in a 96-well plate at 1x10^5 cells per well in 100 µl of NET assay media supplemented with 50 µM luminol and 1.2 units/ml HRP (both from Sigma Aldrich). Cells were incubated 30 min at 37°C and afterwards stimulated by addition of 100 µl of 2X solutions of the indicated stimuli. Luminescence was measured over the indicated period of time in a VICTOR Light luminescence counter (Perkin Elmer).

13.7 Quantification of total heme – formic acid assay

To make heme standard curve, the heme stock used for cell culture experiments was diluted in ddH_2O. The top standard was 16 µM, followed by a 1:2 serial dilution. Plasma samples were diluted 1:50 in ddH_2O. In a 96 well flat bottom plate, 50 µl of each standard and sample were added per well, followed by addition of 50 µl formic acid (Sigma Aldrich). Both heme stock, standard and subsequently the plate were protected from light. The absorbance was read at 405 nm and the background was subtracted at 490 nm.

13.8 Quantification of DNA – picogreen assay

The Quant-iT ™ PicoGreen ® dsDNA quantification kit was used to determine the concentration of dsDNA in solutions containing *in vitro* generated NETs according to manufacturer's instructions. In brief samples were diluted in supplied TE buffer and 100 µl of standards and samples were plated in a white 96 well flat bottom plate. Samples were normally diluted 1:20, 1:200 and 1:2000 because too much material can saturate the signal. The Quant-iT ™ PicoGreen ® reagent was diluted 1:200 and 100 µl were added to each well (resulting in a 1:400 dilution of the reagent). Plates were incubate for 2-5 minutes protected from light and fluorescence was detected using a Fluoroscan plate reader.

13.9 Enzyme linked immunosorbent assays (ELISAs)

13.9.1 General ELISA protocol

ELISAs are used to quantify protein concentrations based on an antibody binding specificity. In a sandwich ELISA one antibody is used to capture the protein of interest and immobilize it on the well. A secondary antibody recognizing a different epitope of the same (or a different) protein is used to detect the protein of interest. The secondary antibody is normally labelled with biotin, allowing for detection with streptavidin-HRP, which converts a quantitative colorimetric substrate.

If ELISA kits were used the assay was performed as to manufacturers specifications.

For R&D ELISAs the assay was performed as follows. Plates were coated with capture antibody over night at room temperature, washed 4 times with general ELISA wash buffer, and blocked

with 1% BSA for at least two hours at room temperature. 100 µl of diluted sample was added, the dilution factor for each assay has to be determined individually, as they can vary greatly depending on the analyte. Samples were incubated for two hours at room temperature and plates were washed 4 times. Detection antibody was added and plates were incubated for two hours at room temperature. Plates were then washed, incubated with streptavidin-HRP, washed again and incubated with substrate. Substrate development was stopped by acid stop and measured in a Spectramax M5 microplate reader (Molecular Devices). Data was analyzed either using SoftMax® Pro software or using excel and www.myassay.com to generate a standard curve.

13.9.2 Human NET ELISA

To detect NETs in the plasma of human malaria patients and healthy control donors the following assay was used.

Human neutrophils were induced to make NETs *in vitro* (see below) by PMA stimulation overnight and scraped from the plate the following morning. The sample was sonicated and the DNA content measured by picogreen assay (see below) and the samples were adjusted to 1 µg/ml as highest standard for the standard curve. The curve was generated by 1:2 serial dilution of that top standard.

Samples and standard were diluted in general sample diluent and 50 µl was added to the precoated anti-NE plate of the Human anti-NE Hycult ELISA kit, followed by two hours incubation at room temperature with agitation at 350 rpm. The plate was washed 4 times and 50 µl of the anti-DNA-POD diluted 1:100 in its supplied dilution buffer was added for two hours at room temperature with agitation at 350 rpm. Plates were washed again five times and subsequently developed with ABTS substrate supplied with the Cell Death Detection ELISA kit. Plates were stopped and measured in a microplate reader.

13.9.3 Mouse NET ELISA

To determine NETs in the plasma of mice we used the following assay:

Neutrophils generated by peritoneal lavage (see above) were induced to make NETs *in vitro* (see below) by PMA stimulation overnight and NETs were scraped from the plate the following morning. The samples were sonicated and the DNA content was measured using a picogreen assay. The samples were then adjusted to 1 µg/ml as highest standard for the standard curve. The curve was generated by 1:2 serial dilution of the top standard.

The biotinylated anti-NE antibody was coated onto the streptavidin coated plates supplied with the Roche Cell Death Detection ELISA kit at 4°C overnight. The antibody was previously diluted 1:100 and 50 µl were added per well. Coating was followed by washing three times with PBS-T and blocking in 1 % BSA at room temperature for two hours. 50 µl of diluted standards and samples were added afterwards and plates were incubated two hours at room temperature with agitation at 350 rpm.
Plates were washed at least three times with PBS-T and 50µl of anti-DNA-POD antibody diluted 1:100 in its supplied dilution buffer was added to the plate for two hours at room temperature and with agitation at 350 rpm. Plates were washed again and subsequently developed with ABTS substrate supplied with the Cell Death Detection ELISA kit. Plates were stopped after sufficient color change could be observed and measured in a microplate reader.

13.9.4 Cytokine measurement by Bio-plex assay

To simultaneously detect multiple cytokines in plasma samples that are restricted in volume multiplex assays were performed in accordance with manufacturer's instructions (BioRad). Multiplex assays rely on a capture antibody coupled to differentially sized beads that can be sorted according to cytokine they recognize. Cytokines are detected by biotinylated secondary antibodies that bind streptavidin coupled to PE.
In brief the filter plate was washed, polystyrene beads added and the plate was washed again. The 50 ml of plasma was added per well, the samples were diluted 1:4. After 30 minutes incubation on a plate shakers the plate was washed, detection antibody added and the plate incubated for 30 minutes while shaking. The plate was washed again and streptavidin-PE was

added, followed by a 15 minutes incubation. The plate was then washed again, beads resuspended in assay buffer and measured on a Bio-Plex 200 system (BioRad).

13.10 Immuno-/Histochemical assessment of mouse organs

The scoring of liver pathology as well as the counting of parasites sequestered to the microvasculature of the livers was performed by trained pathologists at the iPATH-Berlin Core Unit for immunopathology of experimental model organisms. The laboratory also performed the sectioning of paraffin blocks and the staining necessary for the individual quantification.

13.11 Immunofluorescence of Tissue Sections

The 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 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 with deionized water three times, PBS pH7.4 one time, 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 RT. We used secondary antibodies raised in donkey and pre-absorbed against serum proteins from multiple host species (Jackson Immuno Research). Dilution and blocking buffer was PBS supplemented with 1% BSA/2% donkey NS/5% cold water fish gelatin/0.05% Tween 20/0.05%Triton X100.

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SELBSTSTÄNDIGKEITSERKLÄRUNG

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