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THE ROLE OF NEUTROPHILS IN IMMUNE RESPONSES IN MALARIA INFECTION

Presented by

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Abstract

Malaria remains a major health challenge worldwide with an estimated incidence of more than 200 million cases per year, and is one of the leading causes of morbidity and mortality in tropical and subtropical regions. *Plasmodium falciparum* malaria causes severe morbidity and mortality, especially in young children and pregnant women. Clinical malaria is caused by blood-stage parasites. The mature stage of *P. falciparum* parasites produces the clonally variant adhesion protein called *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) which is expressed on the surface of infected erythrocytes (IE) and mediates attachment to endothelial surface receptors. If malaria is caused during pregnancy, *P. falciparum* parasites produce PfEMP1 VAR2CSA protein on the surface of IEs and mediate the binding to placental receptor, chondroitin sulfate A. Repeatedly malaria-exposed individuals develop immunity and become protected from severe clinical malaria. Naturally acquired antibodies against VAR2CSA are produced during successive pregnancies, and VAR2CSA is also an important vaccine candidate. Parasite sequestration and inflammation are assumed to be the two main mechanisms of malaria pathogenesis and complications. Phagocytic cells play an important role in innate immunity to malaria. Neutrophils are the most abundant innate immune cells, and play a crucial role in pathogen clearance. However, neutrophil responses to human malaria are understudied. This dissertation aims to investigate the role of neutrophils in the immune response in malaria, including in phagocytosis of malaria-infected cells, and formation of neutrophil extracellular traps, or NETs in human tissues and *in vitro*.

In chapter four, we evaluate functional antibody responses to pregnancy-specific IEs and VAR2CSA DBL antigens in association with protection against placental malaria. We used plasma samples from PNG pregnant women collected upon enrollment into a randomized controlled trial intermittent preventive treatment in pregnancy. Women were followed up until delivery and were grouped into placental malaria (PM) and infected but no placental malaria (no PM) and noninfected at delivery, based on light microscopy of Giemsa stained placental sections and peripheral blood smear together with PCR of peripheral blood. To measure ADNP activity in test plasma, neutrophils were isolated from whole blood collected from healthy donors by negative selection and were incubated with placental binding IEs or beads coated with VAR2CSA DBL antigens, and phagocytosis was

measured by flow cytometry. Functional antibodies induce neutrophil phagocytosis of IEs expressing placental binding parasites and antibody-dependent neutrophil phagocytosis (ADNP) for IE and DBL2 coated beads, expressed as the average of 3 neutrophil donors, differed between pregnant women with malaria infection but no placental malaria and women with placental malaria. However, we observed no evidence of a difference between no PM and PM groups for DBL3 and DBL 5 coated beads, suggesting that DBL2 domain of VAR2CSA is an important target of protective immunity.

In chapter five, we investigated the ability of IE to induce NET formation *in vitro*. Human neutrophils were cocultured with purified IEs or merozoites. SYTOX green was used to assess DNA release from neutrophils; confocal microscopy to assess changes in neutrophil morphology; and ELISA was used to quantify myeloperoxidase (MPO) release as a measure of neutrophil degranulation. Incubation of *P. falciparum* IEs with neutrophils did not cause significant DNA release. By confocal microscopy, neutrophils stimulated with IEs displayed normal lobulated nuclei and showed only very limited NET formation. The ability of IE to inhibit NET formation was investigated. For this purpose, neutrophils and IEs or purified merozoites were cocultured, with or without potent NETs stimulators phorbol 12-myristate 13-acetate (PMA) or lipopolysaccharide (LPS). The formation of NETs increased substantially upon stimulation with PMA, or LPS, but IEs were effective in inhibiting PMA-induced, but not LPS-induced, NET formation. IEs also inhibited neutrophil degranulation, decreasing PMA-induced MPO release. By contrast, *P. falciparum* merozoites did not inhibit NET formation or neutrophil degranulation. We report, for the first time, that IEs inhibit NET formation and myeloperoxidase (MPO) release as a means of neutrophil degranulation. Inhibition of NET formation might help the malaria parasite escape host immune responses, contributing to parasite survival. Identification of the mechanism by which *P. falciparum* inhibits NET formation could contribute to the development of new treatment strategies for malaria.

In chapter six, we hypothesized that neutrophil infiltration to the placenta is increased during malaria in pregnancy and leads to the formation of NETs in the IVS of the placenta. Sections of paraffin-embedded placental tissues were evaluated following staining with Giemsa and neutrophil specific markers (immunohistochemistry and immunofluorescence). Our findings showed that more neutrophils migrate into the IVS of

placentas infected with malaria compared to uninfected controls. The presence of NETs was significantly higher in malaria infected placentas compared to controls.

Overall, this Ph.D. thesis provides understanding of the essential role of neutrophils in the immune response to malaria including in opsonic phagocytosis of malaria-infected cells, and formation of NETs in response to malaria parasites in human tissues and *in vitro*.

Declaration

This is to certify that:

I. This thesis comprises only my original work towards the Ph.D. except where indicated in the statement of contribution.

II. Due acknowledgment has been made in the text to all other materials used.

III. The thesis is less than 100,000 words in length, exclusive to tables, figures, references, and appendices.

.....

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Statement of contribution

I would like to appreciate the contributions of all those who helped me get this Ph.D. going forward. Without the following people, this doctoral thesis cannot be done.

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List of abbreviations

ADRB	antibody-dependent respiratory burst
ALI	acute lung injury
ANA	antinuclear antibodies
APCs	antigen-presenting cells
ARDS	acute respiratory distress syndrome
ATS	acidic, terminal sequence
CD36	Cluster of Differentiation 36
CIDR	cysteine-rich interdomain region
CR 1	complement receptor 1
CSA	chondroitin sulphate A
DBL	Duffy-binding-like domains
DC	domain cassettes
EBA175	erythrocyte binding antigen – 175
EDTA	ethylenediaminetetraacetic acid
EPCR	endothelial protein C receptor
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
ICAM1	intercellular adhesion molecule 1
IEs	<i>P. falciparum</i> -infected erythrocytes
IL	interleukin
IFN	interferon

LBW	low birth weight
LPS	lipopolysaccharide
MPO	myeloperoxidase
MBL	mannose binding lectin
MAC	membrane attack complex
MASP-1	mannose binding lectin associated serine protease-1
MASP-2	mannose binding lectin associated serine protease-2
NADPH	nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil Extracellular Traps
PAMPs	pathogen-associated molecular patterns
PECAM1	platelet endothelial cell adhesion molecule 1
PfEMP1	<i>P. falciparum</i> erythrocyte membrane protein 1
PKC	protein kinase C
PRRs	pattern recognition receptors
RBC	red blood cell
ROS	reactive oxygen species
TGF- β	transforming growth factor - β
TM	trans-membrane domain
TNF	tumor necrosis factor
UPS	upstream promoter sequence
IVS	intervillous space
WHO	World Health Organization

1 Chapter 1: Literature review

1.1 Introduction to malaria

Malaria is a parasitic disease caused by different *Plasmodium* species and transmitted from person to person by infected female *Anopheles* mosquitoes. Of the many *Plasmodium* species, *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax*), *Plasmodium ovale* (*P. ovale*) and *Plasmodium malariae* (*P. malariae*) are responsible for human malaria worldwide. It has been recently documented that *Plasmodium knowlesi* (*P. knowlesi*), previously considered the causative agent of monkey malaria, causes human malaria. Although *P. vivax* and *P. knowlesi* can cause severe malaria, *P. falciparum* is the most important species responsible for most severe malaria (1, 2). It has been documented that *P. falciparum* is the most prevalent *Plasmodium* parasite in Africa, South-East Asia, the Eastern Mediterranean and the Western Pacific, whereas, the predominant *Plasmodium* parasite is *P. vivax* in America (3).

1.2 Global distribution of malaria

As stated by the World Health Organization (WHO), malaria cases had reduced to 219 million in 2017, compared to 239 million cases in 2010 (3), figure 1.1. In 2017, the highest burdens of malaria cases were found in sub-Saharan Africa (200 million, 92%), followed by Asia and the WHO Eastern Mediterranean Region with 5% and 2% of cases respectively (3). Disproportionately, India and 15 countries in sub-Saharan Africa carried almost 80% of the global malaria burden: Nigeria (25%), the Democratic Republic of the Congo (11%), Mozambique (5%), India (4%) and Uganda (4%) were home for nearly half of all malaria cases worldwide. Compared with 2016, in 2017 increased cases of malaria were reported in Nigeria, Madagascar and the Democratic Republic of the Congo, whereas Ethiopia, Rwanda and Pakistan noted reductions in cases of malaria in 2017 compared to 2016 (3).

Since 2000, the estimated malaria-related deaths were dramatically decreased in sub-Saharan Africa. The estimated number of malaria-associated deaths globally, in all ages, were 435,000 in 2017, compared with 451,000 estimated deaths in 2016, and 607,000 in 2010. In 2017, the highest percentages (93%) of all malaria-associated deaths occurred in

WHO African Region, and almost 80% of malaria-related deaths worldwide were concentrated in India and 17 countries in the WHO African Region (3).

Globally, the estimated number of deaths from malaria in under 5 years' children accounted for 61% (266,000) of all malaria deaths. Children aged under 5 years are the most vulnerable group affected by malaria (3) together with pregnant women, and in the latter, it is especially serious in the first pregnancy (4).

As previously reported, in endemic African countries, 25–35% of all outpatient visits, 20–45% of hospital admissions and 15–35% of hospital deaths are related to malaria (4, 5). The most serious and fatal type of malaria is caused by *P. falciparum*. *P. falciparum* is an enormous problem in Africa and is endemic in most malarious regions of the world (4, 5)

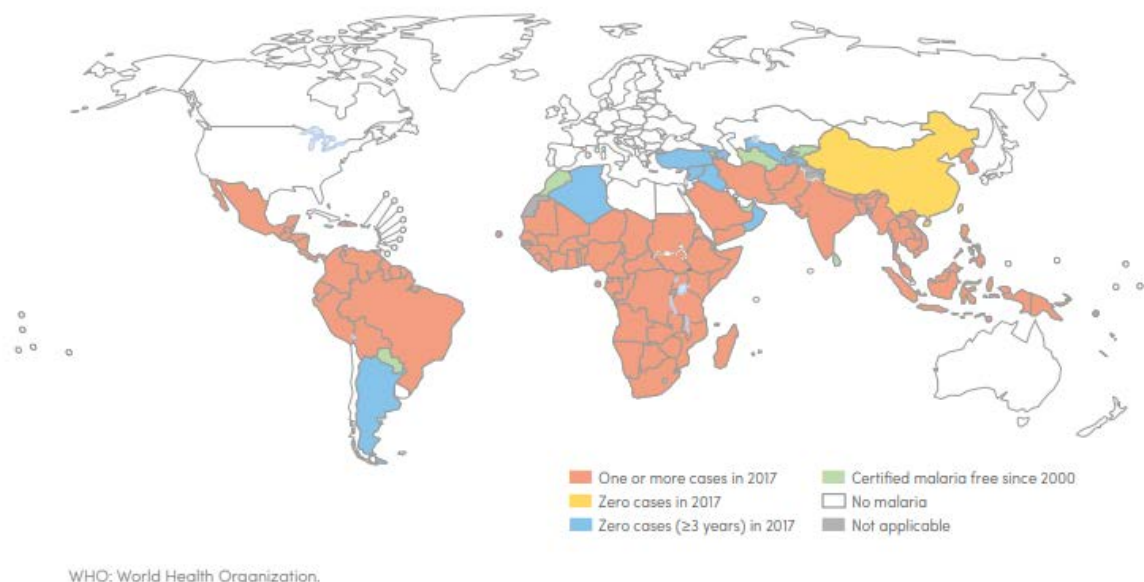


Figure1. 1 Countries with indigenous cases in 2000 and their status in 2017. The map shows how malaria cases have changed since 2000. According to the World Malaria Report 2018, there were 219 million cases of malaria globally in 2017 and 435 000 malaria deaths. Countries are considered malaria free when there are no reported cases of malaria for 3 consecutive years or beyond. Areas colored in red represent the highest percentage of the population at risk, and were also found to be mainly infected with *Plasmodium falciparum* species. Taken from (3).

1.3 Transmission and life cycle

Malaria is a protozoan parasitic disease which is most frequently introduced with the bite of an infected female *Anopheles* mosquito which injects the sporozoites into the susceptible host, however, it may be acquired congenitally from the mother by baby across the placenta, by blood transfusions and from the use of shared needles (Figure 1. 2). Sporozoites rapidly reach the liver within 30 min of infection and invade hepatocytes. Then sporozoites develop into liver schizonts within the infected hepatocytes over 5–16 days, depending on the parasite species, and this stage contains merozoites that fill the hepatocyte. The mature schizonts then rupture the cell. Please note that a dormant stage [hypnozoites] in *P. vivax* and *P. ovale* can remain in the liver and cause relapses by occupying bloodstream weeks or even years later. After merozoites are released from hepatocytes, they invade red blood cells and over a period of 2 or 3 days, develop into erythrocytic schizonts, eventually destroying the erythrocyte, and releasing more merozoites and cellular debris into the bloodstream, and then develop into either trophozoites or gametocytes in the circulation. Then, the female *Anopheles* mosquitoes take up the gametocytes. Within the mosquito fertilization occurs, forming a zygote. Then zygote changes into the ookinete, which penetrates the wall of the stomach to develop into a spherical oocyst between the epithelium and basement membrane, which contains sporozoites inside it. Oocysts rupture and sporozoites are liberated in the body cavity and migrate everywhere particularly to the salivary glands (6-9).

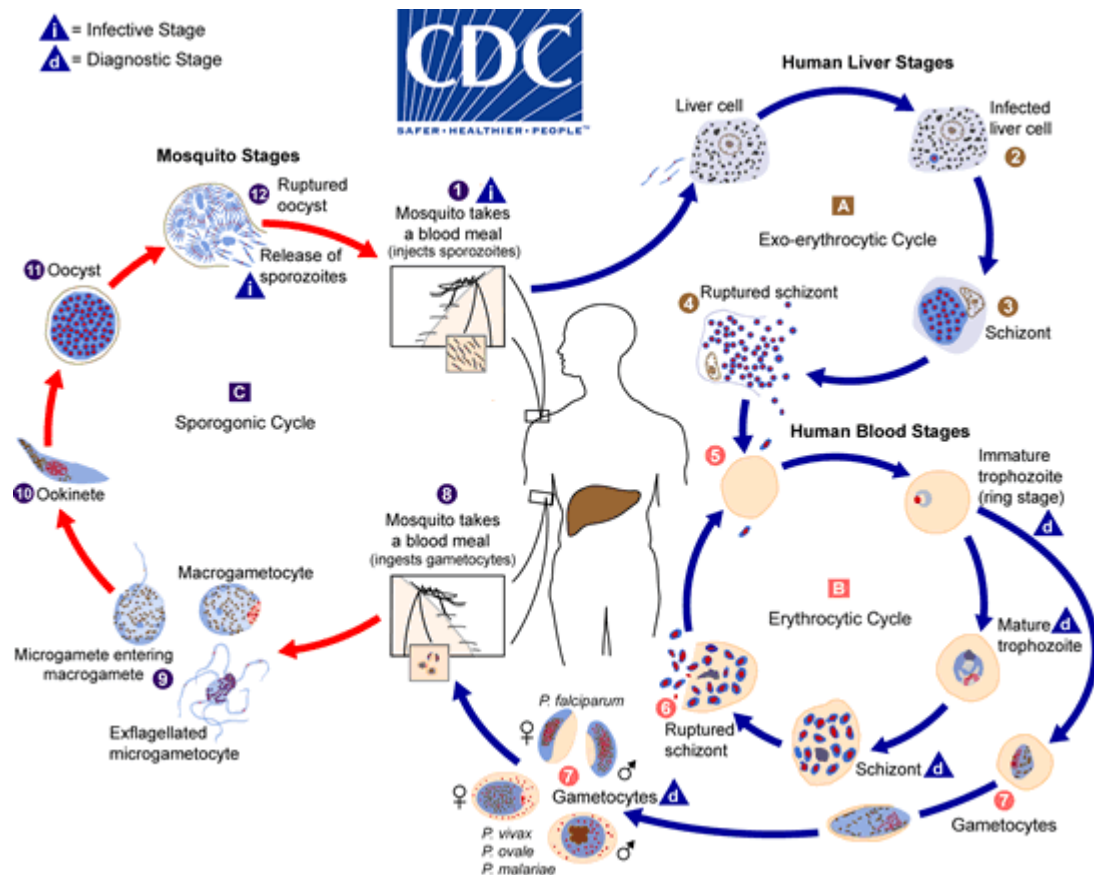


Figure1. 2 **Malaria life cycle.** This figure demonstrates the life cycle of the human malaria parasite *P. falciparum* with definitive (mosquito) and intermediate (human) hosts. Following the inoculation of sporozoites by infected female Anopheles mosquito into the susceptible host, sporozoites infect hepatic cells and merozoites are released from the mature hepatic schizont into the bloodstream causing invasion of red blood cells, leading to disease. In the case of *P. vivax*, some sporozoites form dormant hypnozoites which may cause relapses. Matured erythrocytic schizonts rupture and release merozoites, which will develop into male and female gametocytes that will be taken up by mosquitoes, completing the parasite life cycle. The male and female gametocytes fuse and form a zygote and then develop into a motile ookinete and into an oocyst. Matured oocyst ruptures and releases sporozoites that migrate to the insect's salivary glands, ready to infect a new vertebrate host. (Taken from CDC: <https://www.cdc.gov/malaria/about/biology/> (6)).

1.4 Clinical symptoms of malaria

Disease pathology and symptoms of malaria are a result of parasite development in the human blood (blood-stage parasites) and the release of cellular mediators. The first and most common clinical manifestation of malaria is irregular fever, which is mediated by cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, and interferons (INF) that are released into the bloodstream (10). Schizont rupture and erythrocyte destructions cause gradual or fulminant and nonspecific clinical symptoms of malaria, which look like most viral infections; this may lead to a delay in diagnosis (11, 12).

1.4.1 Why are some malaria infections asymptomatic, and some infections can progress to severe, potentially life-threatening disease?

Why some *P. falciparum*-infected individuals experience asymptomatic infection whereas some infections can progress to a severe and life-threatening illness that may cause lifelong disability has long been debated and, unusually, is still not understood clearly (13, 14). The causes are believed to include: (i) host factors (including genetic factors, age, the level of immunity), (ii) parasite factors (such as the dose of inoculum of parasite sporozoites, or strains of the malaria parasite that might vary in their virulence), and (iii) environmental factors (14, 15). The presentations of malaria infection can be classified as asymptomatic infection, severe and complicated, and uncomplicated malaria (15, 16).

1.4.1.1 Asymptomatic parasitaemia

In asymptomatic infections, there are no overt clinical symptoms but malaria parasites and/or their products are detectable by laboratory techniques such as microscopy, rapid diagnostic test or molecular methods. Asymptomatic infections are still poorly understood and there are no standard diagnostic criteria to define asymptomatic infections. Mostly, the existence of asexual or sexual stages of the parasites in peripheral thick blood smears, and absence of acute malaria-related symptoms (commonly fever $>37.5^{\circ}\text{C}$) are the common criteria to define asymptomatic malaria (16).

Host factors, parasite factors, and environmental factors are related to asymptomatic malaria infection. Immunity is the factor that most strongly determines whether a malaria

infection produces symptoms. Previous exposure to malaria parasites and the age of the person determines the level of immunity to infection (16), suggesting asymptomatic *P. falciparum* parasitemia is common among immune populations, and high levels of immunity to the parasite reduce parasite multiplication resulting in low parasite density which in turn reduces the severity of clinical presentations (16). More than 20 years ago, researchers have shown two forms of malaria immunity namely anti-disease immunity and anti-parasite immunity which may lead to asymptomatic infections. The anti-disease immunity can lead the infected individual to carry high parasite load without overt clinical presentation; whereas anti-parasite immunity may play a role in reducing parasite density (17). Asymptomatic infection, the major source of malaria transmission, is common in malaria-endemic regions (17).

1.4.1.2 Severe malaria

According to the diagnostic criteria established, the clinical presentation of severe malaria includes at least one of the following: “cerebral malaria, convulsions, severe malarial anaemia, haemoglobinuria, hypoglycaemia, metabolic acidosis (associated with respiratory distress), acute pulmonary oedema, acute renal failure, jaundice, circulatory collapse, hyperparasitaemia, high fever, and/or spontaneous bleeding” (17-19). The severity and complication of the disease may be variable within different populations. For instance, severe malaria anaemia, cerebral malaria, respiratory distress (acidosis) and hypoglycaemia are the commonest complications of *P. falciparum* infection in children. Metabolic acidosis, jaundice, cerebral malaria, severe malaria anaemia, acute renal impairment, pulmonary oedema, and hypoglycaemia are some of the common complications of *P. falciparum* infection in adults (20). Poor pregnancy outcomes such as abortion, stillbirth, premature delivery, low infant birth weight, and neonatal death are related to *falciparum* malaria infection in pregnancy. Severe maternal anaemia, hypoglycemia, and pulmonary oedema are more frequent complications among non-immune pregnant women with *falciparum* malaria (20).

1.4.1.3 Uncomplicated malaria

Fever, headache, chills and sweats, anaemia, jaundice, splenomegaly, vomiting, and watery diarrhoea are clinical presentations associated with uncomplicated malaria infections (17).

1.5 Malaria pathogenesis

1.5.1 The basis of malaria pathogenesis

The two distinct processes of malaria pathogenesis are sequestration and inflammation, and the process of malaria pathogenesis is dependent on host and parasite factors. These host and parasite interactions mainly happen during the blood-stage infection (21). The asexual stages of *Plasmodium* parasites are responsible for all disease pathology (22). In *P. falciparum*, only ring stage (young parasite) infected erythrocytes (IEs) are present in the peripheral circulation of the infected person, whereas erythrocytes infected by mature stages of the parasite sequester in the deep capillaries of various organs 18-20 hours after erythrocyte invasion and multiply asexually while sequestered in the tissues, reviewed in (23). Erythrocytes containing the mature stage of *P. falciparum* parasites produce protuberances or knobs on their surface, not existing on the surface of ring-infected red cells (18). These protuberances extrude high molecular weight and highly variable antigens, particularly the clonally variant adhesion protein called *P. falciparum* erythrocyte protein 1 (PfEMP1) that mediates attachment to endothelial surface receptors such as intercellular adhesion molecule 1 (ICAM1), platelet endothelial cell adhesion molecule 1 (PECAM1), cluster of differentiation 36 (CD36), endothelial protein C receptor (EPCR), complement receptor 1 (CR 1), heparin sulphate and chondroitin sulphate A (CSA) (9, 23, 24). Adherence of erythrocytes infected with the mature stages of *P. falciparum* leads to sequestration of IEs in the vasculature of various organs such as lungs, brain, liver, and placenta, which is not noticeable in infection with other human malaria parasites such as *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (9).

1.5.2 Mechanism of sequestration

The pathogenesis of *P. falciparum* malaria is mainly dependent on the sequestration of IEs in the microvasculature, as researchers reported (25-27), which is mostly determined by the expression of specific PfEMP1 and adhesion molecules, reviewed in (23). The interaction between PfEMP1 and receptors on the surfaces of endothelial cells of infected individuals leads to cytoadherence, reviewed in (28). For instance, ICAM1 contributes to sequestration in the brain, CSA is the main ligand for IEs in the placenta, and CD36 is most important for parasite sequestration in most other organs (9, 23, 29). The adherence and sequestration

of IEs causes organ-specific disease complications like cerebral malaria and placental malaria (28). The sequestration of IEs, which have reduced deformability, in the microvasculature leads to disruption of microvascular blood flow, and also allows the parasite to avoid splenic clearance (23, 28). In addition, sequestration of IEs damages the endothelial barrier integrity and causes localized endothelial dysfunction and induces pro-inflammatory, pro-adhesive and coagulation pathways, reviewed in (28), which could be associated with the severity and outcome of the disease (figure 1.3). Production of high levels of pro-inflammatory cytokines like IFN- γ and TNF- α suppresses renal erythropoietin production and consequently erythropoiesis that leads to malaria related anaemia. Malaria related anaemia is also aggravated by the removal of both uninfected and infected erythrocytes and leads to hypoxia, as reviewed in (27, 29).

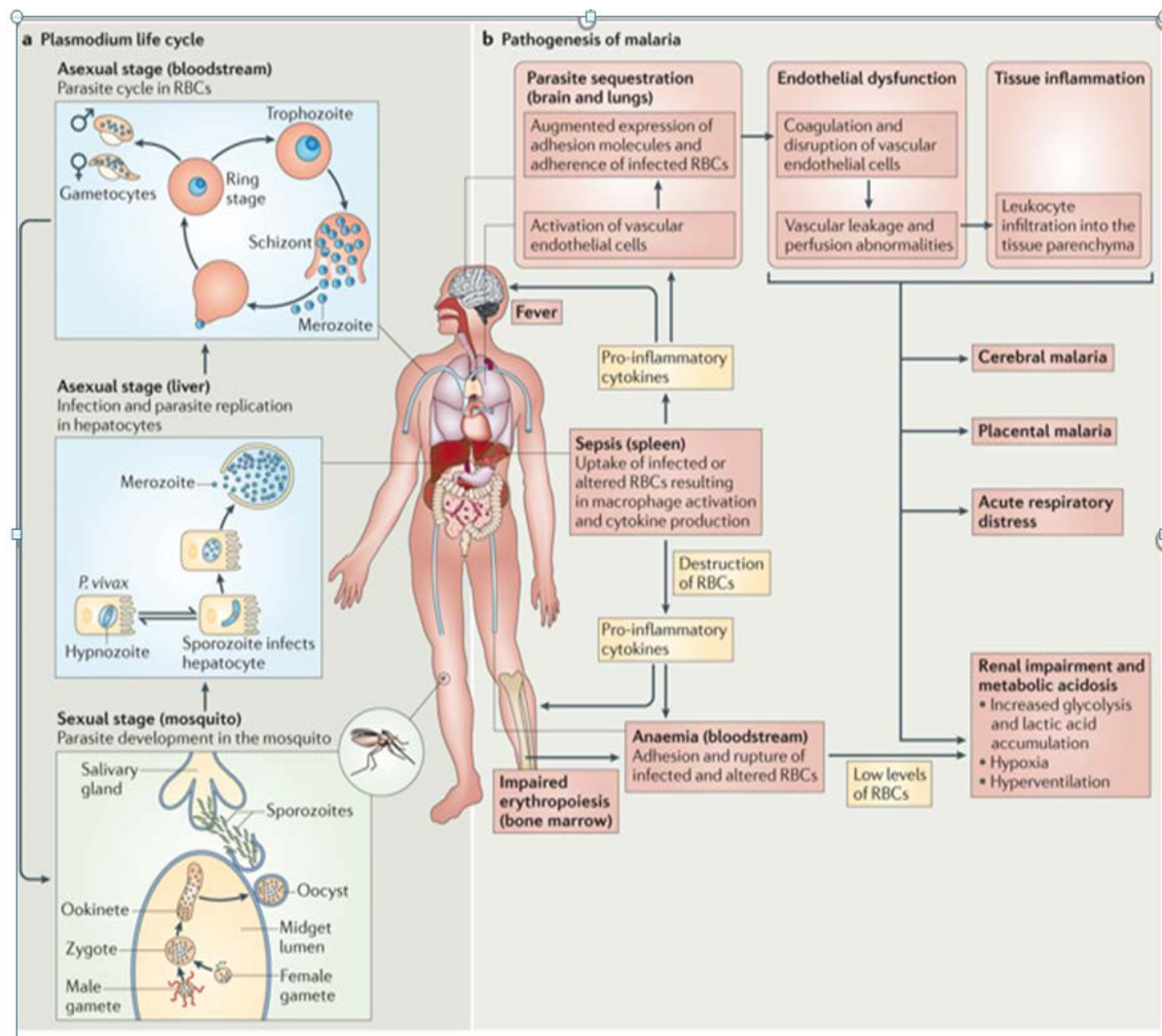


Figure1. 3 This figure illustrates the pathophysiology of malaria. Following inoculation of sporozoites, sporozoites infect hepatic cells and merozoites are released from mature hepatic schizonts into the bloodstream causing invasion of red blood cells which leads to disease. The clearance of IEs and hemozoin by splenic macrophages leads to cytokine secretion. Fever is mediated by the cytokines. The IEs sequestered in the microvasculature affect the flow of blood, cause coagulation disturbances, damage endothelial cells and may result in rupture of vascular walls. Together, these features and other aspects of host response may result in local tissue inflammation that causes acute respiratory distress, cerebral malaria or placental malaria. Adapted from Nature Reviews (9).

1.5.3 Overall PfEMP1 structure (DBL, CIDR domains)

1.5.3.1 *var* genes.

Various experimental studies confirmed that the PfEMP1 family of proteins is encoded by the *var* gene family and expressed on the surface of IEs, reviewed in (26). In 3D7 *P. falciparum* culture strain, this clonally variant adhesion protein, PfEMP1 is encoded by approximately 60 different *var* genes per genome, each approximately 150-450 kD (kilodalton) (23, 26), and switching between *var* genes facilitates parasite immune evasion and modifies IE binding specificity (23, 26). As reviewed by Pasternak and Dzikowski, PfEMP1 is the main virulence factor responsible for both cytoadherence and antigenic variation (30). *Var* genes have a common structure that constitutes two exons, namely exon 1 and exon 2, reviewed in (23).

1.5.3.2 Organization of *Var* genes

Var genes are categorized into three main types (groups A, B, and C) and two intermediate groups (B/A and B/C), as reviewed by Lavstsen *et al.*, in 2003 (31). This classification of *var* genes is based on the chromosomal position, upstream promoter sequence (ups), and direction of transcription of the *var* genes (figure 1.4). Groups A, B, and B/A *var* genes are present in the subtelomeric regions of all chromosomes, however, they are transcribed in opposite directions. Group A *var* genes are mostly telomeric genes that are transcribed to the telomere, group B and B/A *var* genes transcribed to the centromere. In comparison, the *var* genes of groups C and B/C are usually located in internal chromosome regions (31-34).

1.5.3.3 PfEMP1 adhesion molecules

PfEMP1 protein molecules are divided into Duffy-binding-like (DBL) domains and Cysteine-rich interdomain regions (CIDR), reviewed in (23). DBL domains are adhesion proteins present on PfEMP1 and primarily identified by homology to merozoite proteins *P. knowlesi* and *P. vivax* Duffy binding proteins or *P. falciparum* and *P. vivax* erythrocyte binding antigens-175 (EBA-175) (35). The number and size of PfEMP1 extracellular domains are variable. Almost all of the PfEMP1 proteins, with the exception of var3 and VAR2CSA, have a head structure at their N-terminus, consisting of DBL α domain and a

CIDR region of PfEMP1 has shown to mediate adherence to multiple host receptors (32, 36), whereas *var3* and *VAR2CSA* lack CIDR domains (32). In most PfEMP1 proteins belonging to groups B, B / C and C, the head structure is accompanied by a second, more diverse, DBL-CIDR pair. Group A and B/A PfEMP1 proteins contain extra DBL domains upstream and/or downstream of the second DBL-CIDR pair. Although the number of DBL and CIDR domains differs between PfEMP1, the architecture of the PfEMP1 domain is not random (23, 37)(figure 1.4), making domain cassettes (DCs) (23, 35). DCs are conserved sequence elements defined by grouping genes that share a similar order of domain architectures, reviewed in (23). Recently it has been recorded that, given the great diversity of PfEMP1 proteins, comparatively few endothelial surface molecules serve as host receptors for adhesion of *P. falciparum*-IEs, reviewed in (23). It, therefore, seems that several different PfEMP1 proteins have affinity to the same receptor and recent studies show that the specificity of the receptor is related to the presence of certain DCs, reviewed in (23).

Certain PfEMP1 contains multiple adhesive domains with different receptor specificity (38), which would allow multiple host receptors to bind to a given IE (39). Different subtypes of the DBL β and CIDR α domains have been correlated with endothelial receptor binding such as ICAM-1, EPCR and CD36 (40). Genome analysis of *P. falciparum* has shown that ICAM-1-binding PfEMP1s also interact with the EPCR which allows infected erythrocytes to bind both receptors synergistically (41, 42). It has been recently described that Parasites expressing elevated levels of *var* genes encoding PfEMP1 containing DC8, and DC13- were associated with severe malaria in children (23, 28, 43, 44). Group A and B/A and a subset of A and B/A *var* genes named CD8 and CD13 mediate cerebral binding and have been associated with severe malaria infections, as researchers reported (45).

Placental infection is associated with parasites that expresses the conserved variant VAR2CSA PfEMP1, which facilitates the adherence to a placental receptor called CSA. VAR2CSA is discussed separately in **section 1.9**.

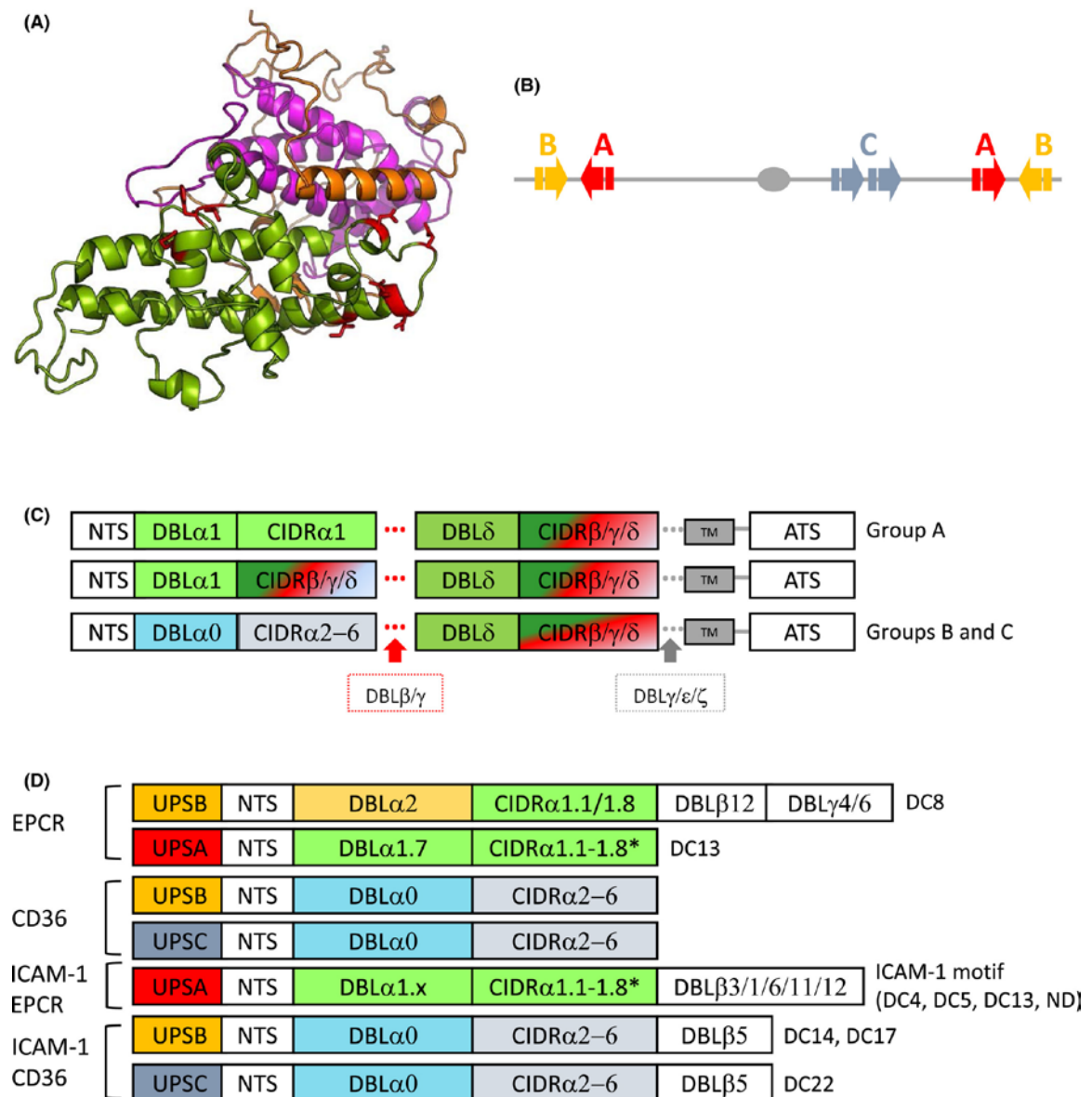


Figure1. 4 *Var* genes and PfEMP1 structure. schematic presentation of *Var* genes organization and protein domain designs of PfEMP1. (A) Modeled structure of PFD1235w DBLβ. (B) *Var* genes are located in sub-telomeric regions of all chromosomes (Group A and B) and in internal chromosomal regions (group C). Group A *Var* genes transcribed towards the telomer, while Group B *var* genes are transcribed in opposite directions (towards the centromere). (C) Duffy-(DBL and CIDR domains are the dissimilar subtypes of PfEMP1 proteins. Small PfEMP1 mainly has a four-domain structure, while group A PfEMP1 proteins have added DBL domains following the first or second DBL-CIDR domains. (D) Genomes from *P. falciparum* encode tandem domain called domain cassettes; DC). These DCs are related, as indicated to the right, to distinct recognized adhesion phenotypes. DC8 is a gene chimeric between group A and group B *var* gene. Taken from Hviid L et al.(46).

1.5.4 Cytokines

Cytokines are soluble mediators that are small proteins (~5–20 kDa) produced and secreted by immune cells, mainly by T cells, neutrophils and macrophages, that are important in cell signaling. Cytokines that can facilitate the migration of cells in the body are called chemotactic cytokines. Interleukins, interferons, chemokines, and tumor necrosis factors are examples of cytokines. It has been reported that cytokines act as endogenous pyrogens and antipyretic agents. IL-1, IL-6, and TNF are the pro-inflammatory cytokines reported as pyrogenic whereas the anti-inflammatory cytokines such as interleukin-1 receptor antagonist (IL-1ra) and IL-10 have been most investigated for their antipyretic action (47).

A study conducted in Bandiagara, Mali, demonstrated the complex associations between inflammatory cytokines and disease in children with severe *P. falciparum* malaria and matched uncomplicated malaria or healthy controls (48). For instance, as compared to severe malaria cases, very low levels of IL-6, IL-10, TNF- α , and IL-12 (p70) were reported in the serum of healthy controls. As compared to uncomplicated malaria cases, the levels of IL-6 and IL-10 were significantly raised among severe malaria and cerebral malaria cases. Compared to severe malaria cases without cerebral manifestations, a significantly higher level of IL-6 and IL-10 was reported in cerebral malaria (48).

A study has shown that IFN- γ plays a role in parasite clearance and in the protection against severe malaria anaemia (49). In this same study, as compared to uninfected controls, the levels of IFN- γ , IL-6, IL-10, and IL-13 were significantly higher in infected children. Moreover, severe malaria anaemia and hyperparasitemia were associated with higher levels of IL-6 and IL-10, whereas the levels of IFN- γ were significantly lower among children with uncomplicated malaria and higher in those with parasitemia (49). Acute malaria is associated with the production of high levels of IL-1 β , IL-6, and TNF- α by monocytes (50). However, only a handful of studies show the role of neutrophils and cytokines in malaria infection. The detail of neutrophils and cytokines are described in **section 1.9.2.2.2.2**

1.6 Immunity to *P. falciparum* malaria

In high malaria transmission areas, immunity to malaria is acquired slowly with exposure and age, and this immunity could be anti-parasite immunity that limits *plasmodium* burden

in the blood stage or clinical (anti-disease) immunity that protects against symptoms of the disease, reviewed in (51). The *plasmodium* parasite has a complex life cycle and during its development, the parasite has different localizations and infects different cell types suggesting various innate and adaptive immune mechanisms are involved in parasite control and elimination, reviewed in (52) (figure 1.5).

Innate immunity, the first line of defense, to *P. falciparum* is initiated when the sporozoites are injected by the mosquito into the dermis (51), and the deposited sporozoites spread from injection site within several hours and most of the sporozoites enter into the blood circulation (53). In *plasmodium* infection, CD8⁺ T cells are the important immune player, and CD8⁺ T cell kills intrahepatic parasites by producing interferon (IFN)- γ (54). During the pre-erythrocytic stage (figure 1.2) immune response is targeted on free sporozoites and infected hepatocytes (54). The sporozoites migrate through the skin and travel into the liver, where they differentiate and replicate into merozoites, which egress into the circulation and initiate blood stage infection (51).

During this blood stage (figure 1.2) of the parasite development, the innate phagocytic cells control parasite growth via phagocytic clearance during the early stage of malaria infection (55). In addition, the phagocytic innate immune cells ingest IEs leading to the induction of pro-inflammatory cytokines and chemokines (51), which are important for clearing infection by promoting appropriate cell-mediated and humoral immunity (55). However, prolonged and elevated responses of such cytokines may lead to severe malaria (55).

Although innate and adaptive immune cells are unable to eliminate the infections in isolation they can reduce circulating IEs, limit the peak of parasitaemia, and prevent severe pathology (56, 57). Repeated exposure to malaria parasites is required for the production and maintenance of protective immune responses. Malaria immunity is stage, species, and variant-specific and complex interactions occur between humoral and cell-mediated responses, but there is no sterile immunity (56). During the blood stage of the parasite life cycle, adaptive immunity primarily targets extracellular merozoites, intraerythrocytic stages, and soluble toxins (51).

The adaptive immune responses produced help to eliminate microorganisms in the late stage of infection (56, 57). For example, Renia L and Goh YS reviewed the role of

antibodies for the liver and blood stage parasites (52). According to their review, antibodies during the liver stage can (1) inhibit sporozoite motility in the dermis or in the liver, (2) mediate phagocytosis by spleen or the liver phagocytes, (3) block sporozoite invasion into hepatocytes by preventing the sporozoite ligand from interacting with the hepatocyte receptor(s), (4) inhibit sporozoite growth within the hepatocytes, and (5) induce an antibody-dependent cell-mediated parasite killing in the liver likely involving Kupffer cells and natural killer cells. For erythrocytic stage (for merozoites), antibodies can (1) prevent invasion of erythrocytes by merozoites, (2) prevent egress of merozoites from erythrocytes, (3) agglutinate released merozoites, (4) facilitate phagocytosis of merozoites, and (5) mediate IE clearance by phagocytic cells through a mechanism called antibody cell-dependent inhibition (ADCI). For surface antigens, anti-malarial antibodies can prevent cytoadherence of IE, promote IE phagocytosis by phagocytic cells, or cause IE agglutination (52).

The innate immune response to *P. falciparum* is described in detail in **section 1.9.3**.

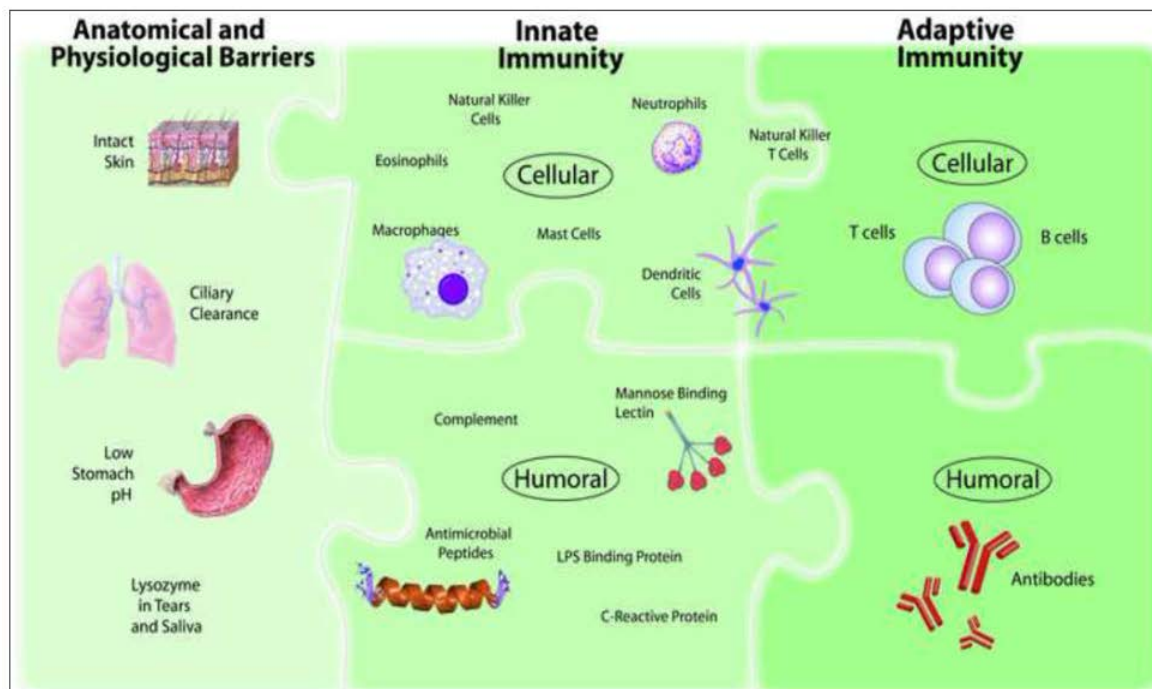


Figure1. 5 The different levels of the human immune defense system. This figure illustrates the levels of human immune system such as (i) innate immunity including innate cellular and humoral; and (ii) adaptive immunity such as antibodies, T cells and B cells. Adapted from (58).

1.7 Malaria during pregnancy

Malaria during pregnancy is a massive burden in tropical and subtropical regions (59). In Africa, pregnant women are more at risk of developing malaria than their non-pregnant counterparts (60), and this is caused by a number of factors, including physiological changes, such as increased body temperature and increased release of volatile substances from the skin surface, that happened during pregnancy and attracted more *Anopheles* mosquitoes to pregnant women than their non-pregnant counterparts (61). Importantly, primigravidae are particularly susceptible to *P. falciparum* malaria relative to women of other gravidities (62) and this susceptibility is characterized by high parasite densities, and placental sequestration of IEs (63), and lack of immunity towards placental binding parasites explains some of the pregnancy-associated malaria susceptibility (64).

Placental binding *P. falciparum* can express a unique protein, VAR2CSA protein, on the IE surface that mediates specific binding to CSA (65), a sulfated glycosaminoglycan found on the surface of placental trophoblasts. It was reported previously that IEs obtained from human placentas bound to purified CSA but IEs collected from nonpregnant donors did not bind to CSA (66), suggesting CSA is an important receptor for a subpopulation of parasites that causes malaria in pregnancy. In addition to CSA, *P. falciparum*-IEs have also been reported to bind to the non-sulfated glycosaminoglycan, hyaluronic acid (HA), which is found in abundance on the placental syncytiotrophoblasts (67, 68). The accumulation of IEs in the intervillous space (IVS) of the placenta by binding to CSA causes placental malaria (PM)(69, 70).

The diagnosis of malaria infection is challenging in pregnancy. This could be because of the sequestration of higher densities of IEs in the IVS of the placenta (probably few parasites in the circulation) increases the chance of negative test results from the peripheral blood (71). Compared to peripheral parasitemias, a strikingly noticeable concentration of IEs in the placenta was reported (69), suggesting that placental histology is the most sensitive method of diagnosing PM compared to peripheral microscopy (72). Frequent migration and activation of phagocytic cells particularly monocytes into the IVS of the placenta might cause inflammatory pathology. Ordi J *et al* demonstrated the association of high numbers of monocytes and macrophages in the IVS and poor pregnancy outcomes

such as low birth weight (LBW) (73). Syncytial knotting, syncytial rupture, thickening of the placental barrier, necrosis of villous tissue and intervillitis are some of histological lesions found in PM (74).

1.8 Consequences associated with malaria during pregnancy

Placental malaria is a condition associated with severe outcomes for mother and child (74, 75). Some poor pregnancy outcomes associated with malaria in pregnancy include maternal anemia, spontaneous abortions and intrauterine growth restriction, leading to LBW babies (75-77). It was previously reported that malaria in pregnancy causes severe anaemia and is responsible for ~900,000 deliveries of LBW babies each year in Africa (78), and an estimated 100,000 infant deaths.

The deposition of hemozoin and increased numbers of maternal phagocytic cells (mainly monocytes) sequestered in the IVS are also associated with pregnancy-related malaria (76). Rogerson S *et al* reviewed the relation between placental histological findings and birth weight, suggesting that chronic PM infection has been most closely related to reduced birth weight, lower maternal haemoglobin or severe anaemia, whereas the presence of high parasite load in the blood during acute infection has been more closely associated with preterm delivery (76). Infiltration of monocytes into the IVS is linked to pathogenesis in pregnancy malaria (76), while the role of neutrophils in PM pathogenesis is still unclear.

1.9 Immunity to *P. falciparum* during pregnancy

Naturally acquired antibody is important in protection against malaria, as has been demonstrated by passive transfer experiments (79). The key targets of this naturally acquired immunity have been hard to identify as the parasite expresses many different proteins, some from multigene families, and with extensive allelic diversity. Uniquely, pregnancy malaria is associated with a specific deficiency in antibody to VAR2CSA (63). The association of a specific lack of immunity to VAR2CSA with susceptibility to pregnancy malaria provides the opportunity to define the functional and structural characteristics of a protective antibody response in malaria.

VAR2CSA is a member of the PfEMP1 family of proteins (discussed in more detail in **section 1.5.3**), encoded by *var* genes. PfEMP1 proteins mediate the sequestration of IE in

the vasculature, avoiding clearance by the spleen, but predisposing to organ-specific morbidity (23). VAR2CSA is a relatively conserved, multi-domain protein, with six DBL domains (DBL 1-6) expressed on the IE surface. Of these, strong evidence implicates DBL2 and its flanking sequences (the minimum binding domain) in placental sequestration, and the PRIMVAC vaccine, based on the minimum binding domain, has entered Phase 1 trials (80). VAR2CSA is abundant within the placental space and is important in the pathogenesis of pregnancy malaria (23), its expression leading to *P. falciparum*-IEs sequestration in the placenta (76).

1.9.1 Immuno-epidemiology of Malaria in pregnancy

Environmental and maternal factors can be considered as a risk factor in PM. For instance, in low malaria transmission areas, women of all gravidities have little or no immunity to malaria (81) and are at a 2 to 3-fold higher risk of developing a severe disease compared to non-pregnant adults living in the same area (82). In the areas of high malaria transmission, antibodies against to VAR2CSA protein develop over subsequent pregnancies and are partially protective; this tends not to happen in areas of low transmission (81). Gravidity and maternal age can also be associated with the severity of pregnancy malaria outcomes. In sub-Saharan Africa, multigravid women developed immunity to placental binding parasites compared to primigravidae and secundigravidae (83). Compared to older mothers, younger mothers are at greater risk for malaria infection (81).

1.9.2 Adaptive immunity in malaria during pregnancy

The importance of antibody functions and antibody subclasses for malaria immunity have been highlighted in a recent study (84). Antibodies against the malaria parasite target all stages of the parasite and are stage-specific in both definitive and intermediate hosts and immunological response contributes to the pathophysiology of human disease (85).

1.9.2.1 Antibody functions in pregnancy malaria

Antibodies to placental binding IE acquired over successive pregnancies are protective from high parasitaemias (71), and help women become relatively resistant to PM (86).

1.9.2.1.1 Antibodies targeting IEs

The functions of antibodies that target parasite antigens on IEs include rosette disruption (87), blocking erythrocytes invasion by parasites (88), inhibition of parasite adhesion to host receptors (89), enhancing the killing of infected cells by phagocytic cells (88), and opsonic phagocytosis of IEs (90), and each of these antimalarial antibodies is correlated with protection from malaria infection (84, 91). PfEMP1 is a major target of antimalarial antibodies, antibodies against variant surface antigens (VSA) on IEs are variant-specific, and antibodies specific to certain PfEMP1 types are associated with decreased risk of severe malaria (92). Antibodies against VAR2CSA antigens expressed by placental adhering parasites are largely confined to pregnancy, although very low levels of such antibodies found in men and children (93, 94).

1.9.2.1.2 Antibodies that block binding of IE to CSA

Antibodies against malaria antigen can block adhesion of IE to host cell receptors. IEs adhere to CSA on the surface of syncytiotrophoblasts lining the maternal blood space in the placenta (66), and acquisition of anti-adhesion antibodies block the accumulation of parasites in the placenta on multigravid women, which improves pregnancy outcomes (89). In secundigravid women, anti-adhesion antibodies were associated with a higher birth weight of infants compared to women without these antibodies (95). Antibodies against placental binding parasites develop with gravidity and block placental adhesion, and are associated with protection against malaria in pregnancy and its consequences (89). Several studies have reported that IEs express VAR2CSA antigen on their surface and anti-adhesion antibodies produced against this antigen help protect individuals from symptomatic and severe malaria outcomes (95-97).

1.9.2.1.3 Antibodies that promote opsonic phagocytosis of IE.

Phagocytic cells play an important role in the clearance of opsonized IEs (84), and antibodies to VSA2CSA antigens opsonize IEs for phagocytic clearance, helping to prevent maternal anemia and LBW in infants (96). In the second trimester of pregnancies, high birth weight was associated with opsonizing antibodies (98, 99), which is associated with less maternal anemia in multigravid women (96). High levels of opsonizing antibodies are

also correlated with protection in pregnant women from recrudescence of infection (96). Lambert *et al* demonstrated that DBL2 χ , DBL3 χ , and DBL5 ϵ , were the primary targets of opsonizing antibodies from multigravid women (100). Hommel *et al.*, reported that antibodies generated against the DBL5 and DBL3 domains of VAR2CSA effectively promoted the opsonic phagocytosis of IEs by human monocytes (90). However, antibody-dependent neutrophil-mediated phagocytosis of IEs is not investigated well.

In summary, antibodies against IEs are associated with protection from malaria infection by blocking adhesion, facilitating phagocytic clearance of IEs, reviewed in (88). Opsonic phagocytosis of IEs by monocytes has been reported (97-99, 101), however, the antibody-dependent neutrophils phagocytosis is not investigated. Therefore, it is important to establish and validate new high throughput antibody-dependent neutrophils phagocytosis assays in order to understand whether neutrophil responses are an important part of the immune response to malaria in pregnancy.

1.9.2.2 Systems serology

Systems serology is a novel and powerful approach to identify antibody features associated with protection and/or control of an infectious disease. This technique uses a combination of high throughput experimental assays that examine a broad spectrum of functional and structural aspects of antibody responses to a specific antigen or pathogen. Chung and colleagues assessed >60 antibody functions and characteristics and used computational tools to identify components of antibody response associated with HIV vaccine efficacy, or that differentiated individuals with latent from active *Mycobacterium tuberculosis* (TB) (102, 103). Using Systems serology, she identified novel and, most importantly, definable aspects of the humoral response associated with control of TB (103), or with protection from HIV vaccination (102). Systems serology is ideally suited to malaria in pregnancy, because there is a very circumscribed hole in antibody response to VAR2CSA, making the identification of specific protective responses highly feasible. Identification of the specific features of a protective immune response can guide vaccine design and can be used to develop a small toolbox of assays to prospectively identify women at greatest risk of placental malaria.

1.9.3 Innate immune response to *P. falciparum*

The innate immune response does not require any prior recognition and is not specific to a particular pathogen in the way that the adaptive immune response are. Turvey and Broide, in 2010, reviewed that innate immunity relies on a rather unspecific type of response orchestrated by a cellular component and a humoral component (58). Phagocytic cells and antigen-presenting cells, such as granulocytes, monocytes, macrophages, natural killer cells, and dendritic cells facilitate the innate immune response (104). These phagocytic cells are responsible for controlling the early progression of the disease through phagocytosis and/or production of inflammatory mediators (29). Some less frequent cells that also belong to the innate arm are the eosinophils, basophils and mast cells (29).

Pattern recognition receptors (PRRs) like Toll-like receptors, an important element of the innate immune system, recognize microorganisms by engaging pathogen-associated molecular patterns (PAMPs) which are limited to microbes and are thus not present in humans (27).

The complement system is one component of the humoral innate immune systems. Pathways of complement activation include the classical, mannose-binding lectin, and alternative (105), all leading to the cleavage of C3 into surface deposited C3b (that also gets fragmented to iC3b) and release of the soluble mediator C3a (106) (figure 1.6). According to Biryukov *et al*, “the main effector fragment (C3b) plays a role in opsonization that leads to phagocytosis of invading pathogens. Other fragments known as anaphylatoxins (C5a, C4a, and C3a) activate neutrophils and macrophages. The end result is lysis through the formation of the membrane attack complex or terminal complement complex, composed of C5b-9” (105).

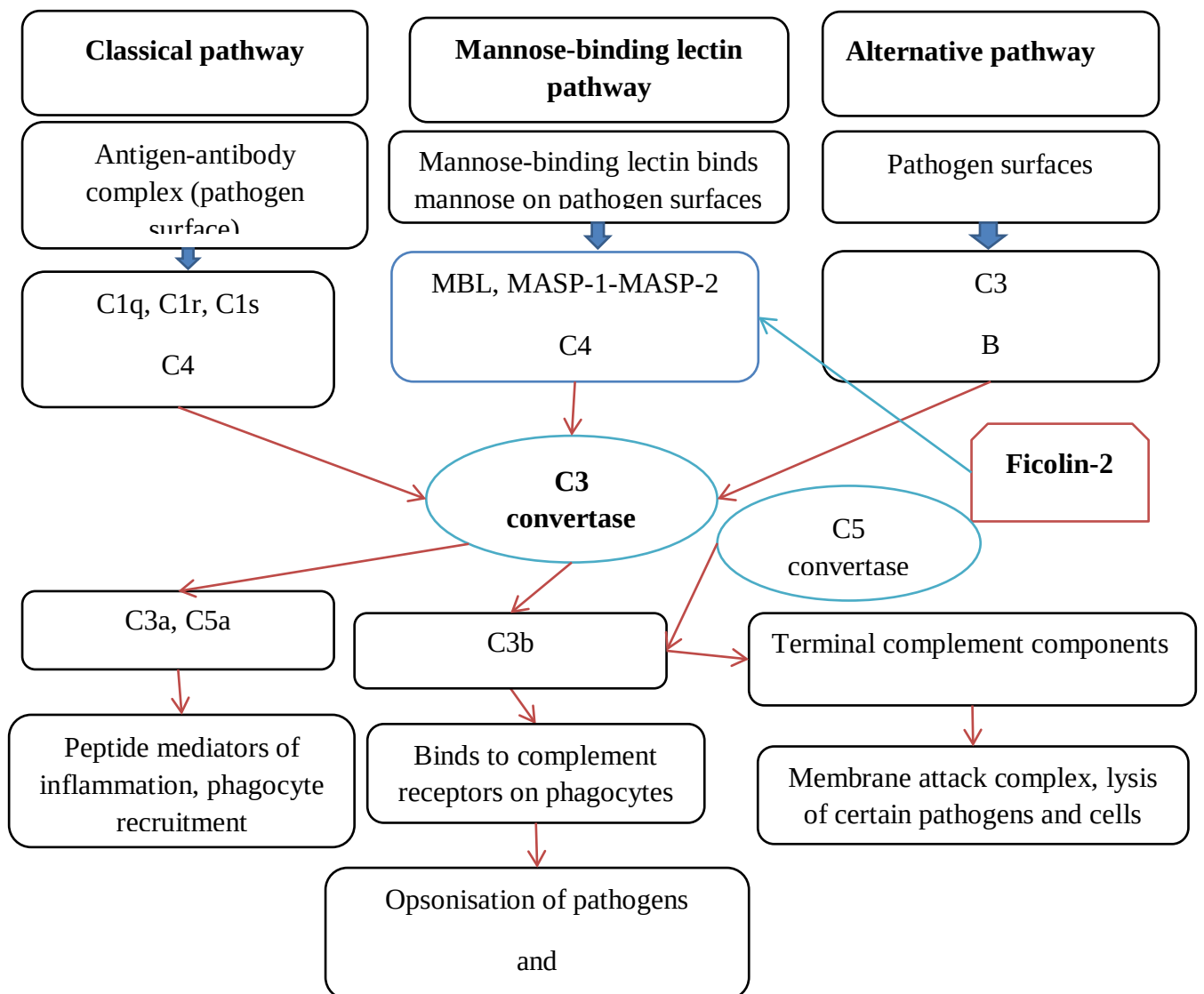


Figure1. 6 Schematic presentation of the three complement pathways: the classical pathway, which is triggered by an antibody or by direct binding of complement component C1q to the pathogen surface; the mannan-binding lectin (MBL) pathway, which is triggered by a mannan-binding lectin, a normal serum constituent that binds some encapsulated bacteria; and the alternative pathway, which is triggered directly on pathogen surfaces. Ficolin-2 activates the lectin pathway. C1s cleaves C4 forms C4a and C4b. C4b binds to C2 (which is bound to the pathogen) to produce C2a and C2b, which results in C3 convertase. MBL is bound to MBL-associated serine proteases 1 and 2 (MASP-1 and MASP-2), that cleave C4 (similar to C1). The activation of the three complement cascades involves a chain of cleavage reactions that end in the formation of a C3 convertase, which cleaves complement component C3 into C3b and C3a. The formation of the C3 convertase is the point at which the three pathways unite and the main effector functions of complement are produced. C3b bound to the C3 convertase to form the C5 convertase, which in turn cleaves C5 to C5a and C5b. C3b binds to complement receptors on phagocytes and opsonizes the pathogen. C3a and C5a are peptide mediators of local inflammation and phagocyte recruitment. C5b activates the terminal membrane-attack complex resulting lysis of certain pathogens and cells. Taken and modified from Mathern DR and Heeger PS (107).

1.9.3.1 Monocyte/Macrophage response to malaria

Monocytes are produced from haematopoietic stem cells in the bone marrow, circulate in the peripheral blood circulation and then differentiate into tissue macrophages. Resident macrophages in the liver (Kupffer cells), spleen (splenic macrophages), and bone marrow are directly involved in the interaction with *P. falciparum*-IEs, in malaria-infected hosts (108). Phagocytosis of free parasites or IEs in the spleen is an important mechanism for the clearance of parasites from the circulation (109). Macrophages protect against *P. falciparum* infection either by phagocytosis of IEs or by the production of cytokines (51). However, unbalanced production of pro-inflammatory cytokines by activated macrophages suppresses erythropoiesis and leads to severe malaria anaemia (15). Studies have shown that macrophage function is inhibited following phagocytosis of IEs containing malaria pigment (also called hemozoin) (110). Ingestion of hemozoin-containing IEs by macrophages inhibits oxidative burst activity, which results in inhibition of parasite killing (111). The role of monocytes in placental malaria is discussed in **section 1.7 and 1.8**.

1.9.3.2 Neutrophils

Neutrophils are the most abundant blood leukocytes comprising around 50-70% of the white blood cells, the first line of defense against infections, and they play a vital function in innate immunity by protecting the host against invading pathogens. They have a short lifespan and usually live for 6 - 8 hours in the circulatory system before entering apoptosis (112, 113). A review focuses on neutrophil responses to human malaria, summarising information that helps us understand where neutrophils are, what they do, how interact with parasites, and their possible role in vaccine-mediated immunity is annexed (appendices).

1.9.3.2.1 How the neutrophil is formed and its structural composition

The neutrophils are derived from hematopoietic stem cells in the bone marrow. Large numbers of neutrophils, approximately 10^{11} cells, are produced per day. Neutrophils are belonging to the family of myeloid cells. The fully differentiated and matured neutrophils are released from bone marrow and rapidly migrate to the sites of infections or inflammation (112), where they complete their functions and are eliminated by macrophages. In the circulation mature neutrophils have an average diameter of 7- 10 μ m, their nucleus is segmented and their cytoplasm is enriched with granules (114).

Structurally neutrophils are different from most other white blood cells. The presence of granules and their multi-lobulated and oddly shaped nucleus (called polymorphonuclear cells) are probably the most distinctive feature of neutrophils. The presence of several lobes (2-4 nuclear lobes connected by chromatin strands) helps neutrophils to squeeze easily out of capillaries (increase flexibility), when extravasating to an inflammation site (115), and the other probable function is the release of neutrophil extracellular DNA in the form of neutrophil extracellular traps (NETs) as a strategy to trap, restrict dissemination and kill pathogens. NETs are web-like structures composed of nuclear DNA, histone and granular proteins (116) (discussed in more detail in section 1.9.2.2.2.5).

Neutrophils engulf and kill potential microbial pathogens such as bacteria, fungi, and parasites via phagocytosis and by releasing granular enzymes and proteins. Neutrophils contain three different subsets of granular proteins such as: [1] primary (azurophil) granules (store most toxic mediators like myeloperoxidase, MPO, cathepsins, and defensins); [2] secondary (specific) granules (lactoferrin) and [3] tertiary (gelatinase granules, containing matrix metalloprotease 9, also called gelatinase) (114, 117, 118). These granules contain proteins that enable neutrophils to attack microbes.

1.9.3.2.2 How neutrophils attack microorganisms

Neutrophils, a specialized innate immune cell, spend most of their lives racing through the bloodstream, patrolling for invading pathogens or other foreign particles. Since neutrophils are equipped with toxic granular molecules, the activation of neutrophils is tightly controlled (119). During infection, however, neutrophils are highly mobile and the first cell types to be recruited to inflammatory sites, where they have a crucial role in the clearance of microorganisms (119) directly by acting as phagocytes, or killing them by producing reactive oxygen species (ROS) or secretion of other antimicrobial products via degranulation (116, 120), and formation of NETs (116), figure 1.8. Through these and other activities, neutrophils play a role in the activation and regulation of the immune response and in parasite clearance (121). Neutrophils also release cytokines extracellularly to kill extracellular pathogens (118, 122). Each strategy is discussed below.

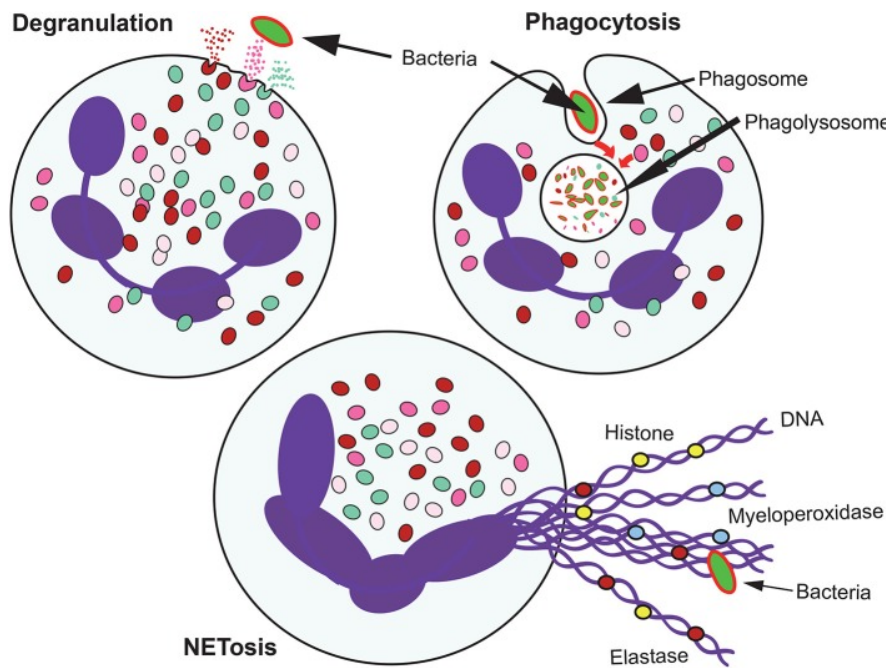


Figure1. 7 Strategies of pathogen killing operated by neutrophils. When microbial pathogens are identified, neutrophils use different mechanisms to kill them. Neutrophils engulf microbial pathogens and form phagosomes which fuse with lysosome and form phagolysosomes (phagocytosis). Neutrophils often degranulate and release their granules (degranulation) into the environment. If neutrophil cannot ingest microbial pathogens because of its size or toxicity, they form and release NETs (NETosis). Adapted from (123).

1.9.3.2.2.1 Neutrophil-mediated Phagocytosis

Phagocytosis is defined as the uptake of microbial pathogens (bacteria, fungi, and parasites), dead host cells and foreign debris, which is the main strategy of neutrophils for capturing and killing of invading pathogens. The interaction of opsonizing molecules with receptors on the surface of neutrophils stimulates phagocytosis, reviewed in (121). There are phases in phagocytosis including attachment of opsonizing particles to specific receptors and completion of engulfment. Neutrophils release antimicrobial granules intracellularly that bind to parasite proteins, and form phagosomes and then phagolysosomes. Following the fusion of phagosomes with granules, neutrophils release antimicrobial proteins, peptides, and enzymes into the phagosome (112, 116), where they

destroy pathogens, together with ROS which are produced by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase also termed as a phagocytic oxidase (116).

Neutrophils are professional phagocytic cells that play an important role in the clearance of *Plasmodium* parasites via phagocytosis, both free forms of *Plasmodium* parasites (merozoites) and IEs from the host circulation. Neutrophils express surface receptors (Fcγ receptor I (FcγRI) (when activated), FcγRII and FcγRIII as well as complement receptors complement receptor 1 (CR1) and CR3) that interact with opsonizing antibodies that can facilitate phagocytosis, reviewed in (121).

Previously, it has been reported that neutrophils can phagocytosis opsonized IE *in vitro* (124). Phagocytosis of malaria parasites *in vivo* by neutrophils has been observed in blood films from a child in Nigeria (125), and another in the Gambia (126). Microscopy findings from bone marrow aspirates from patients with cerebral malaria also show neutrophils, and bone marrow neutrophil metamyelocytes appeared to have phagocytosed merozoites and, occasionally, trophozoites (127).

Five decades ago, Trubowitz S and Masek B, have documented that both neutrophils and monocytes phagocytosed free forms of *P. falciparum* (merozoites) but not normal erythrocytes or IEs (128). In another study, microscopic examination revealed that neutrophils isolated from malaria-infected children phagocytose schizont-IEs *in vitro* (129). Years later, it has been reported that human neutrophils can recognize and ingest the free forms of *P. falciparum* as well as IEs (130). It has been reported that neutrophils from healthy blood donors phagocytosed IEs in preference to normal erythrocytes (124, 131), and the phagocytic activity of neutrophils is highly increased by the addition of immune sera, suggesting opsonization of IE by IgG enhances *P. falciparum* clearance by human neutrophils (124, 131). Another study also demonstrated that neutrophils phagocytosed more merozoites opsonized with Ghanaian plasma IgGs than merozoites opsonized with European plasma IgGs (132). In the presence of high-titered antibodies against *P. falciparum*, merozoites were phagocytosed by neutrophils (133), however, phagocytosis of both IEs and merozoites by neutrophils in nonimmune serum was not observed (133).

A study done in the Solomon Islands and Papua New Guinea demonstrated that sera from malaria-immune individuals promoted phagocytosis of merozoites by neutrophils (134). The authors have also shown that neutrophil-mediated phagocytosis of merozoites

significantly increased in the presence of cytokines IFN- γ , TNF- α , IL-1 β , and GM-CSF (134), and treatment of neutrophils with cytokines and opsonization of merozoites have a synergistic effect in neutrophil-mediated phagocytosis (135).

In summary, though it is clear that neutrophils phagocytose both trophozoites and merozoites *in vivo*, it is still uncertain how important neutrophil phagocytosis is in the defence from malaria disease. The adaptive immune system and factors that activate neutrophils are likely to play a role in the extent of phagocytosis. Studies looking at associations between antibody levels that induce phagocytosis of parasites (of multiple life cycle stages) by neutrophils and patient outcomes could help determine a role for neutrophil phagocytosis in protection from disease. Employing systems serology provides a powerful method to identify antibody features and functions required for protection from disease, and antibody-dependent neutrophil response is one mechanism investigating functional antibodies protective from PM.

1.9.3.2.2.2 Cytokine production

Neutrophils secrete cytokines in response to a variety of inflammatory stimuli to recruit additional immune cells and contribute to microbial clearance (136). Although neutrophils produce low amounts of cytokines per cell compared to macrophages or lymphocytes, they are so abundant at inflammatory sites that their contribution to total cytokine levels is significant (137). TNF and IL-1 β and IL-8 are the most abundantly produced cytokines by neutrophils. It was previously reported that immunoregulatory cytokines like IFN- γ , IL-4, IL-10, and IL-13 can regulate cytokines secretion by activated neutrophils (137).

In an experimental cerebral malaria model, after infection neutrophils expressed cytokines IL2, IL12p40, IL18, IFN γ , and TNF as well as chemoattractive-chemokines monokine-induced by gamma (MIG), macrophage inflammatory protein-1 α (MIP1 α) and IFN γ induced- protein 10 (IP10) suggesting a role for neutrophils in cytokine and chemokine secretion (138). During *Plasmodium* infections the levels of TNF and IL8 are increased (139), suggesting TNF is important in immunity to *P. falciparum* infection. For instance, Kumaratilake *et al* reported that TNF increases *P. falciparum* killing by neutrophils by increasing release of granular constituents and toxic reactive oxygen intermediates on

neutrophils (140), suggesting it is an important component of the immune phagocytic effector mechanisms which are involved in the destruction of the malarial parasite (140).

Immune serum and cytokines such as TNF, IFN- γ , and lymphotoxin enhanced parasites killing by neutrophils (140, 141). In addition, TNF has very little effect on the macrophage-induced anti-plasmodial activity, but significantly increases the damage of parasites by neutrophils (142). GM-CSF significantly enhanced phagocytosis and killing of asexual blood forms of *P. falciparum* by human neutrophils. Furthermore, neutrophil treatment with a combination of GM-CSF and TNF resulted in a synergistic increase in phagocytosis and killing of the parasite (143). In summary, cytokines secreted by neutrophils help to recruit additional immune cells and increased phagocytosis and killing of *P. falciparum*.

1.9.3.2.2.3 ROS production/Respiratory burst

Activated neutrophils produce reactive oxygen species (ROS) in a process called the respiratory burst. Neutrophils produce ROS, including superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), hypochlorite (OCL^-), and singlet oxygen (144), which is mediated by NADPH oxidase that catalyzes the production of superoxide ($O_2^{\cdot-}$) by the one-electron reduction of oxygen, using NADPH as the electron donor (145). ROS are important for neutrophil antimicrobial activity, and play a role in the killing of intracellular and extracellular parasites (121). Production of ROS by neutrophils is associated with parasite clearance and protection from clinical malaria (146), and antibodies that opsonize merozoites to promote neutrophil antibody-dependent respiratory burst have been associated with protection from malaria (122).

1.9.3.2.2.4 Releasing antimicrobial proteins/degranulation

Neutrophil granules are a specialized storage organelle that serves to store their antimicrobial repertoire until it needs to be released. Both primary and secondary neutrophil granules contain a wide range of antimicrobial compounds, and the degranulation of these granules contributes to the creation of an antimicrobial milieu at the inflammatory site which kills invading pathogens (136). Neutrophils engulf the microbes to the phagosome and the granules are fused with the phagosome and forming phagolysosome. Within the phagosome, invading pathogens are killed by locally high concentrations of antimicrobial proteins and ROS released by activated neutrophils. The oxygen radicals are converted into

hydrogen peroxide and the antimicrobial granular protein, myeloperoxidase (MPO), converts hydrogen peroxide to hypochlorous acid (bleach) (136, 147), which readily kills almost any microorganism.

1.9.3.2.5 *Neutrophil Extracellular Traps (NETs)*

Beyond phagocytosis, neutrophils can release NETs into the extracellular environment (116). The structures were named NETs, and the process of the cell death pathway was termed NETosis. Neutrophils form NETs by releasing their genomic deoxyribonucleic acid (DNA) and its granular proteins into the extracellular environment, leading to cell death or NETosis, to trap invading microorganisms. NETs are a network of mesh-like extracellular structures containing decondensed chromatin (chromatin fibers with diameters of 15–17 nm) and histones, heavily decorated with different antimicrobial granular proteins such as neutrophil elastase (NE), MPO, cathepsin G, leukocyte proteinase 3 (PR3), lactoferrin, gelatinase, lysozyme C, calprotectin, neutrophil defensins, and cathelicidins that capture, neutralize and kill a diversity of microbes (148-150) (figure 1.9). NETs have been characterized in several mammals including humans (148), mice (151), goats (152) sheep (153), and cattle(154-156). As neutrophils constitute the largest population of circulating leukocytes, they are expected to play an important role in *Plasmodium* parasite clearance. However, the roles of NETs in malarial infection remain poorly understood.

Morgado *et al* (149) identified two patterns of NET formation, namely small homogeneously distributed webs and large structures, and unfixed or highly concentrated neutrophils form clusters of cells that leads to the large NET formation. NETs are commonly made by activated neutrophils. however, NET-like structures can also be formed by monocytes and macrophages, eosinophils, basophils and mast cells in which the process is named ETosis.

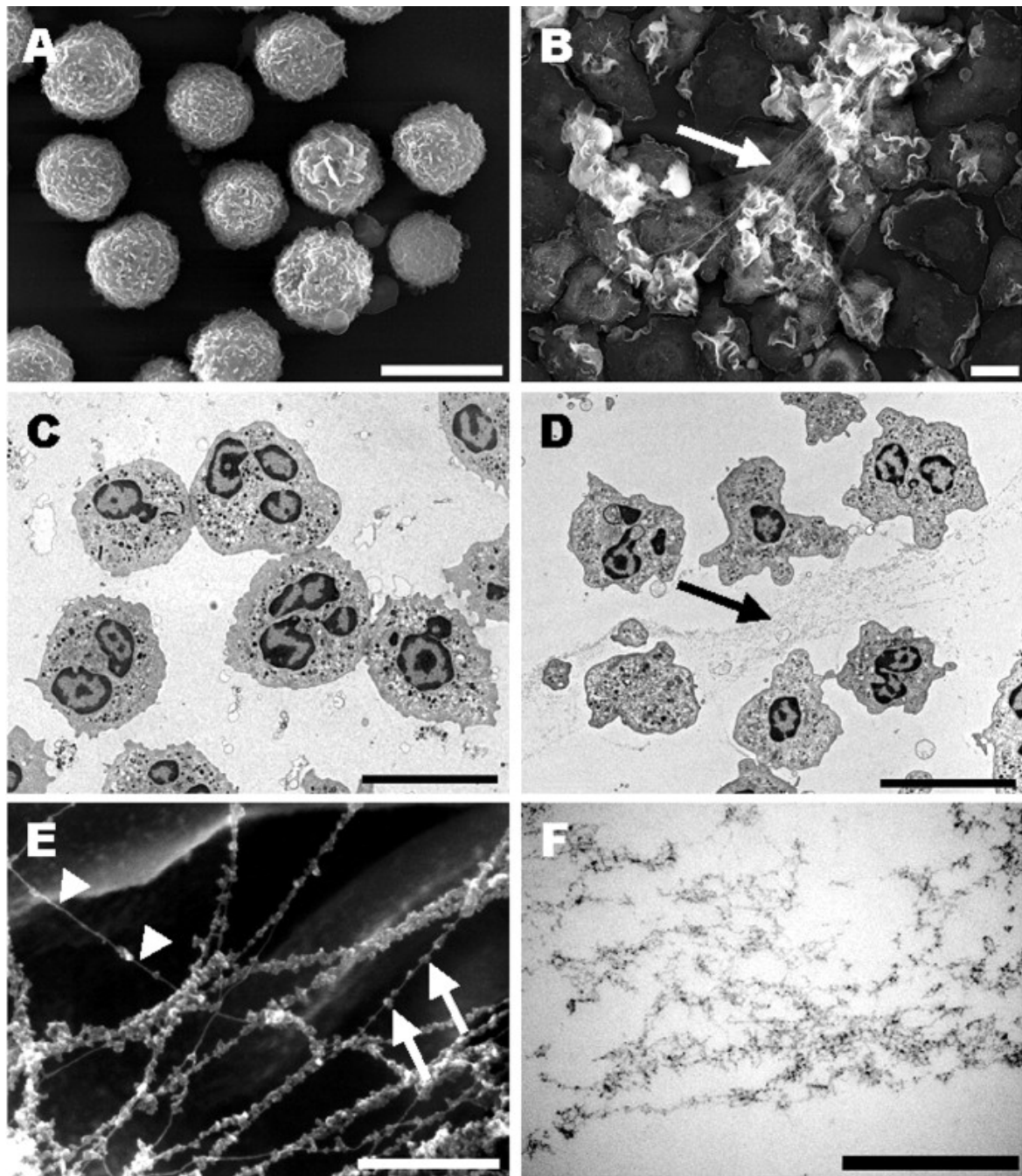


Figure1. 8 Microscopic images of the neutrophil extracellular traps (NETs) liberated *in vitro*. (A) unstimulated neutrophils are round and with no fibers. (B) PMA stimulated neutrophils are flattened, make many protrusions and form NETs. The white arrows in (B) and black arrows in (D) point out web-like chromatin structures. (C)Transmission electron microscopy analysis of naïve neutrophils. (D) Neutrophils activated with IL-8 have many pseudopods and show NETs (black arrow). (E) High-resolution scanning electron microscopy analysis of NETs (arrowheads). (F) Ultrathin sections of NETs released by IL-8 stimulated neutrophils show that they are not membrane-bound. Adapted from (116).

1.9.3.2.2.5.1 How NETs form

When neutrophils are stimulated, the nuclei of neutrophils lose their shape, and the nuclear envelope and the granule membranes disintegrate, allowing the mixing of NET components, which is also called NETosis (148). NETosis is a kind of neutrophil-specific purposeful active cell death that is distinct from either apoptosis or necrosis (157). ROS promote disruption of the cytosolic granules membranes and release antimicrobial proteins and enzymes, which together with ROS cause disintegration of the nuclear membrane, and chromatin decondensation is mediated NE and MPO. Following chromatin decondensation and nuclear membrane disruption nuclear and granule antimicrobial components are mixed in the cytoplasm forming a matrix that is released extracellularly as NETs (158). The specific mechanism involved in the formation and release of NET is not clearly understood. However, researchers have identified various mechanisms by which NETs are formed, named lytic NETosis and vital NETosis (157, 158).

Lytic NETosis, also called suicidal NETosis, has been described as an active cell death that is distinct from either necrosis or apoptosis, which is characterized as a slow mechanism and leads to lytic cell death within 2–3 h (157, 158). Vital NETosis is a rapid mechanism that releases nuclear contents rapidly (within minutes), independent of cell lysis (157, 158). Clark *et al.* in 2007 reported production of vital NETosis by lipopolysaccharide (LPS)-stimulated neutrophils within just 30 minutes involving TLR4 on platelets (159) Unlike lytic NETosis, the process of NET release leaves the nuclear and plasma membrane intact in vital NETosis and allows neutrophils to continuously perform their normal functions such as protease release and phagocytosis (160). As reviewed in (161), neutrophils may retain their chemotactic and phagocytic functions and viability during vital NETosis.

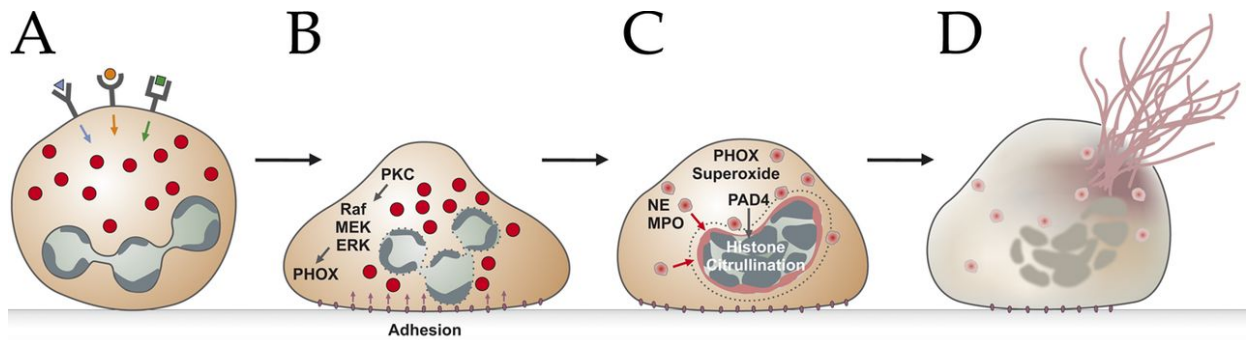


Figure1. 9 Mechanism of neutrophil extracellular trap formation. Granular enzymes and PAD4 plays an important role in the formation of NETs. (A) Stimulation of receptors leads to endoplasmic reticulum calcium store release and increased cytoplasmic calcium. (B) Elevated calcium ions stimulate the activity of protein kinase C (PKC), NADPH oxidase, leading to the formation of ROS. (C) ROS cause nuclear membrane disintegration and mobilize granule components, such as neutrophil elastase (NE) and myeloperoxidase (MPO). Neutrophil elastase and MPO facilitate chromatin decondensation and peptidylarginine deiminase 4 (PAD4) mediates histone citrullination. Finally, granular and nuclear contents mixed, cell membrane ruptures, and the mixture of cytoplasm and nucleoplasm get expelled to form NETs (D). Adapted from (162)

1.9.3.2.2.5.2 Triggers of NET formation

Both biological and chemical stimuli induce NETs formation in human neutrophils. For example, previous studies have shown that the mitogen phorbol myristate acetate (PMA), LPS, glucose oxidase, and nitric oxide all promote NETs release by enhancing the formation of ROS (161, 163, 164). Inflammatory cytokines and chemokines including TNF, IL-8, IL-23, and IL-1 β can also promote the generation of NETs (163, 164). Neutrophils have been shown to release NETs in response to fungal, bacterial, viral, and protozoan infections such as *Candida albicans* and *Aspergillus fumigatus* (165, 166), *Mycobacterium tuberculosis* (167), *Haemophilus influenzae* (168), human immunodeficiency virus (HIV-1) (169), *Toxoplasma gondii* (170), *Eimeria bovis* (171), and *Leishmania* species (172, 173); however, little is known on NET formation in response to *P. falciparum* parasites (120, 174).

ROS as well as granular protein are required for NET formation. A study has shown that neutrophils from malaria patients are highly activated leading to increased release of ROS and MPO (175), in turn, ROS and MPO initiate NETs formation. NETs have been reported

in vivo from the blood of children with uncomplicated malaria, without neutrophil-specific markers such as neutrophil elastase and MPO (120), and from symptomatic malaria-infected adults (176). However, the authors of these research papers didn't evaluate *in vitro* the capacity of *P. falciparum* to induce NETs formation. The presence of NETs in the blood might not be induced by the parasite alone as *in vivo* NETs formation could be induced by other factors like free heme and cytokines released during infection. In addition, concomitant infection with other invading pathogens might be the cause of the formation of NETs *in vivo*, suggesting a need for an *in vitro* study to investigate the ability of *P. falciparum* to induce NET formation by human neutrophils.

1.9.3.2.2.5.3 Inhibition of NETS formation.

It was previously reported bacteria, fungi, viruses, and protozoan parasites induce NET formation in neutrophils (177), while an inhibitory effect on NET formation is rarely reported (178, 179). It was reported previously that PMA, LPS, calcium ionophore or interleukin (IL)-8 activate neutrophils and form NETs (116, 180, 181). Neutrophil responses can be affected by several mosquito and parasite antigens, reviewed in (121). For instance, *P. falciparum* infection increases the secretion of agaphelin protein in the salivary glands of the mosquito, and agaphelin inhibited PMA induced NET formation (182). It has been reported that pathogens such as *P. falciparum* (183) and *Streptococcus* (184) release deoxyribonuclease (DNase) to counteract NETs, suggesting *P. falciparum* may possess escape mechanisms enabling it to evade several immune responses. A stimulatory or an inhibitory effect of *P. falciparum* on NET formation is needed to be investigated.

Previous studies have shown that NET formation in response to PMA or bacteria could be prevented by inhibiting NADPH oxidase or MPO (148). Recently, it has been reported that *A. baumannii* inhibits NET formation *via* the action of PMA-induced neutrophils (185). Another study also demonstrated that both PMA-and *Staphylococcus aureus*-induced NETs formation from murine bone marrow-derived neutrophils and the neutrophil-differentiated human myeloid cell line HL-60 is inhibited by *Lactobacillus rhamnosus* strain GG (186). The inhibitory effect of Propofol, an intravenous anesthetic agent diluted in a lipid emulsion with rapid onset of action after the infusion was tested and results showed that both propofol and lipid emulsion inhibited PMA induced NET production (187).

It was reported that NET formation is inhibited by microorganisms like HIV-1 (169), *Bordetella pertussis* (188), *Lactobacillus rhamnosus* strain GG (178), *dengue virus* serotype-2 (189), and *Cryptococcus neoformans* and the capsular component GXM (190). A mosquito salivary protein, agaphelin, is also indicated to inhibit PMA-induce NET formation (182). However, the inhibitory effect of *Plasmodium* parasites is not investigated. NET is one of the innate strategies for killing extracellular pathogens (118), but inhibition of NET formation by *Plasmodium* parasite may lead to parasite survival. Thus, it is important to investigate the inhibitory effect of *Plasmodium* parasites on NET formation using different NET stimulants that may lead to developing new malaria treatment strategies.

1.10 Placenta and malaria

The interface between mother and fetus is the placenta, which plays a crucial role in successful pregnancy outcomes (191). Placenta, the largest fetal organ, is essential for the health of both the mother and the fetus, and has a lifelong impact on their future wellbeing (192). A defect in the development of the placenta is associated with complications of pregnancy including pre-eclampsia, fetal growth restriction, stillbirth, abortion, and abruptio placentae (192).

Placental malaria (PM) is correlated with severe pregnancy outcomes including maternal anemia, abortion, stillbirth, premature delivery, LBW, and congenital malaria (193). PM is characterized by sequestration of *P. falciparum* IEs in the IVS of the placenta (191), and VAR2CSA is responsible for sequestration by binding to CSA in the placenta. The binding of IE to CSA in the placental stimulates syncytiotrophoblasts and cells in the placenta to release various chemokines, which mediates the migration of phagocytic cells into the placenta (194).

Neutrophils are the first phagocytic cells recruited to the site of infection, and there they play a vital role in pathogen clearance through phagocytosis, intracellular killing and release of NETs into extracellular space to trap and kill pathogens (195), however, the release of different inflammatory mediators and NETs by neutrophils could contribute to tissue injury (195).

1.10.1 Neutrophils and NETs in the IVS of the placenta

In vivo NETs are disintegrated cells with a decondensed chromatin structure. NETs were detected from frozen placental tissues collected from normal deliveries and pregnancies affected by pre-eclampsia (196). Immunofluorescence staining has indicated that NETs are observed both in the normal placenta as well as pre-eclamptic placental sections (196, 197), and significantly increased numbers of NETs were observed infiltrated in the IVS of pre-eclamptic placental sections compared to normal placentas. The presence of NETs in the normal placental sections might be associated with syncytiotrophoblast microparticles (STBM) that deport in the third trimester of pregnancy, and that causes NET formation, usually located near the syncytiotrophoblast layer (196, 197). Gupta A *et al*, in 2005, also reported that placentally-derived IL-8 and STBM triggered NETs formation by stimulating neutrophils (180). In addition, high numbers of NETs were found directly in the IVS of pre-eclamptic placentae and appear to fill entire areas of the IVS (confirmed by the overlay of DAPI, histone, and elastase immunofluorescence staining) (180, 197). In another study, a significantly higher number of neutrophils and NETs were identified by immunohistochemistry (IHC) using MPO staining in the IVS from pre-eclampsia and pre-eclampsia plus lupus pregnancies compared to normal pregnancies, which is confirmed by immunofluorescence staining with anti-MPO and DAPI (MPO-DNA overlap) (198).

Neutrophils form NETs *in vivo* in response to parasite infections. In American tegumentary leishmaniasis, neutrophil elastase IHC stained cutaneous lesions demonstrated variable sized NETs throughout the cutaneous lesions analyzed, which were in close proximity with neutrophils (149), and NET formation patterns were classified as small homogeneously distributed networks observed in all lesions; and large web-like structures (149). In an animal model of gut inflammation, strong neutrophils mucosal infiltration was seen in sections of *Eimeria bovis*- and *Eimeria arloingi*-infected intestinal tissue samples, where neutrophils were found attached with parasite-infected host cells resulting in small as well as diffused NET materials (199), suggesting migration of neutrophils to the site of infection and NETosis plays a role against apicomplexan parasites. In the same study components of NETs were confirmed by co-localization of extracellular DNA, neutrophil elastase and histones in *Eimeria*-infected tissue samples (199). Likewise, NET formation was reported

in vivo from a mouse intranasal infection model in another apicomplexan parasite, *T. gondii* (170).

In animal model, a significant reduction in circulating neutrophil counts has been reported in *Plasmodium* infections during pregnancy (200), and one explanation could be reduction in the formation of NETs in the course of the disease (201). Boström *et al*, also indicated that pregnant women exposed to *P. falciparum* had decreased numbers of circulating neutrophils (202), suggesting, partly it may be migration and accumulation of neutrophils in the IVS of the placenta to phagocytose the sequestering parasites (202) or it could be explained by induction of NETs. Malaria infections during pregnancy induce significantly increased expression of IL-8 by maternally derived hemozoin-laden placental macrophages, which may activate NET formation in the IVS (203). In summary, during malaria in pregnancy neutrophils could be infiltrated into the maternal blood space of the placenta and may form NETs to limit dissemination and kill the parasite, therefore, it is important to assess the presence of NETs in the IVS of malaria-infected placentas.

1.11 Beneficial and harmful effects of NETs

The role of NETosis is probably a balance between antimicrobial defense and host tissue damage. Neutrophils have several strategies to control invading pathogens, and one of them is their capability to form NETs that control infection by trapping microorganisms, killing them, or preventing their dissemination (204). The concept of NETs-mediated extracellular killing of microbes was reported in 2004 by Brinkmann and his colleagues (116). However, recent evidence has emerged suggesting uncontrolled or excessive production of NETs is associated with the exacerbation of inflammation and the development of non-infectious diseases including lung injury, systemic lupus erythematosus, rheumatoid arthritis, diabetes, atherosclerosis, thrombosis and cancer (205, 206).

A recent review hypothesized that intravascular NET formation in response to malaria infection contributes to the vasculopathy, driving severe malaria (201). The formation of NETs limits capillaries and post-capillary venules bore diameter, which restricts end organ perfusion. The accumulated intravascular NETs block the vessels or induce disseminated intravascular coagulation, a disorder involving haemorrhages and microthrombi, which can

impact microcirculation and facilitate end-organ ischemia (201), suggesting NETs formation might be harmful in the context of malaria. It has been reported that the digestion of NET-DNA with DNase or NETs clearance by macrophages reduces NETs related tissue damage (207, 208).

1.12 NETs clearance mechanisms

Although NETs are important to trap and disarm microbes in the extracellular environment, the excessive NET formation has been implicated in several pathological conditions. NET removal is thought to be accomplished mainly by extracellular DNase I degradation (209). During inflammation, neutrophils are the first cell to arrive at the site of infection and then macrophages are recruited immediately to assist in the clearance of pathogens and cellular debris. A previous study by Farrera *et al.* (208) reported that macrophages are capable of clearance of NETs and that this occurs in an immunologically silent manner. In addition, authors of the same study have documented that macrophages enhance DNase I NETs clearance activity (208).

1.13 Immunopathology in malaria: Role of neutrophils

1.13.1 Hemozoin

The phagocytic cells, monocytes and neutrophils, acquire hemozoin through phagocytosis of IEs (210-212). Hemozoin is also known as malaria pigment and is the end product of haemoglobin digestion by *Plasmodium* parasites, and can be found either within IEs or insoluble form (213). Hemozoin has been reported to bind *plasmodium* but not human DNA, which can then be recognized by the TLR 9 (214). Studies demonstrated that the carrier of *Plasmodium* DNA wrapped around histone molecules (215, 216).

Higher numbers of malaria pigment containing phagocytes were reported in severe malaria cases than uncomplicated malaria (217, 218). *Plasmodium* parasites do not directly replicate in the neutrophils, but neutrophil functions can be affected by the associated parasitic products. It has been shown that ingestion of excessive hemozoin destroys neutrophils, which could impair the immune system (219). Another study showed prolonged persistence of hemozoin in phagocytes in the bone marrow could cause prolonged HO-1 induction (220) and abnormal development of neutrophils (221). Another

study showed that the release of heme either at erythrocyte rupture or upon destruction of IEs in the spleen seems to activate neutrophils, thus inhibiting human neutrophil apoptosis (222), and heme stimulates neutrophils to release NETs *in vitro* and *in vivo* (223).

1.13.2 Malaria pigment containing neutrophils and malarial disease severity

During malaria infection, malaria pigment containing neutrophils can sometimes be seen in the peripheral blood. A study compared the distribution of intraleukocytic malaria pigment in children with asymptomatic malaria, uncomplicated malaria, and no malaria, indicating higher numbers of pigment-containing neutrophils in the uncomplicated malaria group, followed by the asymptomatic malaria group with the no malaria group having the least numbers (224). A cross-sectional study conducted in Ethiopia reported pigment-containing neutrophils in 58.9% of the study participants (malaria-infected children aged between 1 and 15 years) (225). A study from Ibadan, Nigeria demonstrated that the proportion of neutrophils containing malaria pigment is increased in children with cerebral malaria followed by uncomplicated malaria and asymptomatic malaria as compared to no malaria group (median values 27.0%, 9.0%, 6.5%, and 2.0%, respectively) (210). Another study has shown that adult patients with severe *falciparum* malaria who died had significantly higher proportions of neutrophils containing malaria pigment on admission compared to survivors (226). Lyke KE *et al.*, also demonstrated that pigmented neutrophils are associated with cerebral malaria and with death in children with severe malaria (218). A strong association between pigment-containing neutrophil count and the risk of disease severity was reported by earlier studies (225, 227, 228). Studies have shown the number of malaria pigment containing neutrophils and other phagocytic cells in the circulation is associated with malaria disease severity (210-212, 218).

An increased number of neutrophils containing malaria pigment is associated with severe anemia (229), and the number of circulating pigmented neutrophils are negatively correlated with birth weight (77). A study has shown that reporting pigment-containing neutrophils may be a better indicator of disease prognosis than the peripheral parasite count (226). White NJ, in 1996, reported that the prognosis of the disease worsens as counts of neutrophils containing pigment increase (230). In conclusion, all the aforementioned studies suggest that the number of pigmented containing neutrophils in circulation may

serve as a simple, rapid and informative tool for the better prognostic value of malaria infection.

1.13.3 Neutrophil counts and malaria

Hematological changes are the most common complications in malaria infection and play a major role in disease outcomes (225). Neutrophil count during malaria infection is affected by disease severity, parasite density, parasite species as well as the stage of the parasites. It has been reported that neutrophil counts were significantly higher in patients with *P. falciparum* infection compared to those with *P. vivax* infection. (231). Studies have documented that neutrophil count was significantly higher in patients with high parasitemia compared to those with low and moderate parasitemia (231), in malaria-infected than uninfected children (232), and an increased neutrophil count is associated with severe malaria (233), however, decreased numbers of circulating neutrophils have been reported from *P. falciparum*-infected pregnant women (202).

In controlled human malaria infections (CHMI) in health non-immune volunteers, neutrophil numbers are stable during the liver stage development of the parasite, whereas during the subsequent blood stage infection, a significant decrease in neutrophil counts occurs (227). Neutrophil to lymphocyte count ratio was found as an early indicator of bloodstream infection particularly in the emergency department (228), which helps predict malaria. For instance, the neutrophil to lymphocyte count ratio (NLCR) was significantly correlated with the parasite load and the NLCR reached a maximum of 4.0 (227), the normal NLCR values in an adult population in good health are between 0.78 and 3.53 (234). Together with higher neutrophil counts, pigment containing neutrophils are considered as a diagnostic marker of malaria disease severity.

1.13.4 Anti-malarials and neutrophils

Previous studies have investigated the effect of antimalarial drugs on neutrophil activities. The targets for most antimalarial drugs are designed focusing on the major pathways of *P. falciparum*, including nucleic acid metabolism, heme detoxification, oxidative stress and fatty acid biosynthesis (235). The generation of ROS and neutrophil degranulation is a vital component of the human neutrophil response to malaria infections, however, the mechanisms by which neutrophils help antimalarials to kill the parasite or vice-versa are

far from clear. Infections by *Plasmodium* parasites activate the natural host defense mechanisms with the involvement of neutrophils that generate high amounts of ROS which triggers oxidative stress (236).

Results from previous studies reported that antimalarial drugs dampen neutrophil functions associated with their antimicrobial activity (237-239). For example, Ferrante A and his colleagues mentioned that all antimalarial drugs studied strongly influenced neutrophils' iodination reactions (a measure associated with phagocytosis and oxidative burst) and locomotion (237). However, Percario and his colleagues reported that antimalarial drugs such as chloroquine increase neutrophil functions by preventing hemozoin formation, promoting the accumulation of free heme and increasing oxidative stress in *Plasmodium* (236).

Kharazmi A has shown that the anti-malarial drugs studied (chloroquine diphosphate, quinine HCl, mefloquine, proguanil HCl, cycloguanil, pyrimethamine, sulphadoxine, and tetracycline HCl), at clinically obtainable concentrations, had no inhibitory effect on neutrophils functions (neutrophil oxygen consumption, glucose oxidation, superoxide production, and chemiluminescence), although neutrophil oxidative burst was inhibited by high concentrations of chloroquine, quinine, and mefloquine (240). Ferrante A also reported that mefloquine affected neutrophil respiratory burst and probably degranulation, which is closely associated with the NET formation (237). Mefloquine also inhibits human neutrophil functions by inhibiting the action of cellular phospholipid-dependent protein kinase C (PKC) (241), which is linked to activation of the respiratory burst (242). Likewise, another study indicated that chloroquine has been found to prevent NET formation (243).

It has been reported that chloroquine has a dose-dependent inhibitory effect on neutrophil phagocytosis as well as on the morphology of neutrophils (244). Incubation of neutrophils with 50 µg/ml chloroquine for 1 hour resulted in slight swelling of some granules and evidence of degranulation, and by 2 hours the swelling of neutrophil granules had become much more widespread, and after 4 hours' exposure, 50 µg/ml chloroquine caused neutrophil granule vacuolation (244).

A clinical study conducted among uncomplicated malaria patients in Cambodian, who were allocated 1 of 3 oral artesunate monotherapy regimens (2 mg/kg, 4 mg/kg, or 6 mg/kg/day for 7 days) and were observed for 42 days, demonstrated that patients who received

artesunate developed neutropenia (245). Although, after 3 doses, the absolute neutrophil counts were reduced in all groups, and the counts were significantly lower at 6 and 14 days among patients who received high-dose treatment than the other groups who received 2 mg/kg or 4 mg/kg per day (245). Another study demonstrated that the antimalarial drug combination artesunate-fosmidomycin is associated with transient grade I or II neutropenia for the first 2 days of treatment (absolute neutrophil counts of 750 to 1,200/ μ l and 400 to 749/ μ l, respectively) (246). In Malawi, a randomized trial showed a marked fall in neutrophil counts in the amodiaquine and sulfadoxine-pyrimethamine combination than the sulfadoxine-pyrimethamine alone group (247).

2 Chapter 2. Research Questions, Hypothesis and Aims

2.1 Chapter 4 (Aim 1)

Hypothesis: We hypothesized that antibodies to VAR2CSA in plasma of malaria exposed pregnant women increase antibody-dependent neutrophil phagocytosis (ADNP) of beads coated with recombinant DBL antigens or IEs and consequently protects from placental malaria

Aim: The aim of the current study is to measure ADNP of beads coated with recombinant DBL antigens or IEs and investigate its association with protection from malaria infection

Specific Aims:

- 2.1.1** To establish an ADNP assay using IEs and antigen-coated beads
- 2.1.2** To evaluate ADNP of individual recombinant DBL domains of VAR2CSA coated on beads in association with protection against placental malaria.
- 2.1.3** To evaluate ADNP of CSA-binding IE expressing VAR2CSA in association with protection against placental malaria.

2.2 Chapter 5 (Aim 2)

Hypothesis: Human neutrophils generate neutrophil extracellular traps (NETs) in response to *P. falciparum* *in vitro*

Aim: To investigate whether *P. falciparum* induce NET formation *in vitro*

Specific Aims:

- 2.2.1** To examine the capacity of *P. falciparum* to induce NET formation by human neutrophils
- 2.2.2** To quantify NET formation (microscopically and fluorometrically)
- 2.2.3** To measure MPO release as result of NET formation or neutrophil degranulation in supernatants collected from iRBCs and merozoite stimulated human neutrophils
- 2.2.4** To investigate the inhibitory effects of IEs or merozoites on PMA-induced NET formation

2.3 Chapter 6 (Aim 3)

Hypothesis: Infiltration of neutrophils and formation of NETs in the intervillous space is
a feature of malaria-infected placentas

Aim: To assess infiltration of neutrophils and the presence of NETs in the
intervillous space of malaria-infected placentas

Specific Aims:

- 2.3.1** To evaluate total leukocyte populations in the intervillous space of malaria-infected and non-infected placentas
- 2.3.2** To evaluate neutrophils infiltrations into the intervillous space of malaria-infected placentas
- 2.3.3** To evaluate NETs in the intervillous space of malaria-infected placentas

3 Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Buffers and solutions

Neutrophil isolation recommended media	1× Phosphate-buffered saline (PBS) containing 1 mM EDTA, without calcium chloride/magnesium chloride. Store at 4°C
Neutrophil media	RPMI 1640, 0.5% (v/v) FBS, 1% Penicillin-Streptomycin-Glutamine
Microscopy blocking buffer	PBS, 10% (v/v) normal goat serum, 1% (w/v) bovine serum albumin, 0.05% (v/v) Tween 20
Sodium citrate buffer	10 mM Sodium Citrate Buffer: 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) to 1 liter dH ₂ O. pH to 6.0. with 2 M HCl
FACS buffer	Cold 1x PBS supplemented with 2% FBS
FACS lysing solution	2 ml FACS in 18 ml Milli-Q water
<i>P. falciparum</i> complete growth medium	RPMI - HEPES (500 ml) supplemented with 25 ml of Albumax (5% in RPMI) and 25 ml of heat-inactivated human serum
Phosphate buffered saline	1 PBS tablet make up in 500 ml Milli-Q water
Sterile phosphate-buffered saline	1× phosphate-buffered saline (PBS), pH 7.4. Filter and Store at 4°C.
BSA	Bovine serum albumin. Stored at 4°C.
Trypan blue	Trypan blue 0.4% solution
ELISA wash buffer	0.05% Tween 20 in PBS, pH 7.2-7.4
ELISA sample/ reagent diluent	1% BSA in PBS, pH 7.2-7.4, 0.2 µm filtered
Gelatin from porcine skin, G1890-500g	0.75 g gelatin to 100 ml of RPMI 1640-HEPES
Sorbitol	D-sorbitol (Ajax Finechem, 1585-500G) in water as a hypotonic solution to lyse trophozoite and schizont IE

Percoll	90% Percoll stock: 450 ml percoll (pharmacia) + 50 ml 10 X PBS. Percoll in PBS were prepared using RPMI HEPES
Paraformaldehyde (PFA)	prepared at 2% concentration in PBS, filtered and stored at 4°C.

3.1.2 Cell culture reagents

Reagents	Product number /name	Manufacturer
Percoll	17-0891-01	GE Healthcare
High-molecular-weight dextran	10173452	Sigma-Aldrich
RPMI 1640 medium 1x	2187076	Gibco
Roswell Park Memorial Institute (RPMI) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)	RPMI-HEPES	Gibco
Red blood cells		Australian Red Cross Blood Service
Albumax II		Invitrogen
Heat-inactivated human serum (HIHS)		Australian Red Cross Blood Service
Fetal bovine serum (FBS)		Gibco
Penicillin/Streptomycin		Gibco
Phorbol 12-myristate 13-acetate (PMA)	P 8139	Sigma Aldrich
Lipopolysaccharide (LPS), 0111:B4 <i>E.coli</i>	L4391-1mg	Sigma Aldrich
E64 protease inhibitor	E3132-5MG	Sigma Aldrich
EasySep™ Direct Human Neutrophil Isolation Kit	19666	STEMCELL technologies
0.5 M EDTA	15575038	Invitrogen by life technology

3.1.3 ELISA kits

Reagents	Product number/name	Manufacturer
Human Myeloperoxidase ELISA kit	DY3174	R&D Systems

3.1.4 Deparaffinisation and rehydration agents

Reagents	Product number /name	Manufacturer
Xylenes histological grade	534056-4L	Sigma Aldrich
Ethanol alcohol		Chemo-Supply

3.1.5 Antibodies & Dyes

Reagents	Product number/name	Manufacturer
SYTOX green Nucleic Acid Stain - 5 mM solution in DMSO	S7020	Thermofisher scientific
4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI), 5 mg/ml	D1306	Life Technologies
Myeloperoxidase (MPO), polyclonal rabbit anti-human MPO primary antibody	A0398	DAKO
human neutrophil elastase, monoclonal mouse IgG (500 µg/ml), clone # 950317	ELA2 ab	R & D systems
Goat anti-rabbit IgG secondary antibody, AlexaFluor 488	ab150077	Abcam
Goat anti-mouse IgG secondary antibody, AlexaFluor 647	A21235	Life Technologies
Biotinylated Goat anti-rabbit IgG antibody, 1.5 mg/ml	BA-1000	Victor Laboratories
Biotinylated goat anti-mouse IgG antibody, 1.5 mg/ml	BA-9200	Victor Laboratories

3, 3' Diaminobenzidine (DAB)	ab64238	Abcam
Giemsa stain improved R66 solution	350865P	VWR chemicals
Hematoxylin	MHS1-100 ml	Sigma Aldrich
Organo/Limonene mount	1002148843	Sigma Aldrich
Fluorshield mounting medium	F6182-20 ml	Sigma Aldrich

3.2 Methods

3.2.1 Neutrophil-mediated phagocytosis

3.2.2 Ethics, study site, and study populations

Pregnant women were enrolled between November 2009 and February 2013 at nine antenatal clinics in the Madang and Sumkar districts of Madang Province, Papua New Guinea (PNG). Following written informed consent, pregnant women between 14 and 26 weeks of gestation and aged 16–49 years were included and women were followed until delivery (248, 249). From each participant, venous blood (10 ml) was collected at enrolment into Lithium Heparin vacutainers (BD, USA), and plasma was separated and stored at –80°C until further analyses. Light microscopy and quantitative polymerase chain reaction of peripheral blood at delivery were done at the PNG Institute of Medical Research. Placental biopsies were collected from the maternal side of the placenta. Placental malaria was staged based on the presence/absence of infected erythrocytes in the placenta (248). Ethical approval was obtained from the PNG Institute of Medical Research (IMR) Institutional Review Board, PNG Medical Research Advisory Committee and Melbourne Health Human Research Ethics Committee, Australia (248, 249).

Samples were selected for a case control study based on the presence of parasites in the periphery and placenta at delivery. In this study, groups were frequency-matched for age, gravidity, intermittent preventive treatment in pregnancy (IPTP) regime, rurality and bed net use. In this study, women with placental malaria (PM; ‘susceptible’) and infected no PM (protected) were compared to investigate the possible protective and functional roles of antibodies. Pregnant women with placental malaria (PM, n = 50), pregnant women infected with *P. falciparum* but not placental malaria (NPM, n =27) and non-infected pregnant women (NI, n = 50) were included. The study subjects were categorized into

three groups based on the placental histological results and PCR and light microscopy of peripheral blood at delivery.

3.2.3 Primary Human Neutrophils

Ethical approval was obtained from the Melbourne Health Human Research Ethics Committee. Neutrophils were obtained from healthy volunteers and were used for the neutrophil experiments. Fresh whole blood samples were collected from informed, consenting healthy volunteers. Participants were recruited via a general request in the form of an advertisement on bulletin boards within the department and within Doherty Institute where the laboratory is located. Each participant was recruited and interviewed in accordance with Research Ethics Committee approval (Human Research Ethics Committee of Melbourne Health). Once signed informed consent has obtained a sample of peripheral whole blood (5-10 ml) was collected and assigned a sample number. All peripheral blood samples were collected by a trained clinician or phlebotomist into 6 ml plastic heparin blood collection tubes with a green cap (BD Vacutainer LH 102 IU ref 367885). Samples were immediately used in experiments. Neutrophils were purified using EasySep direct human neutrophil isolation kit (Stem Cell Technologies) according to the manufacturer's instructions as detailed in **section 3.2.7.2**.

3.2.4 Parasite lines

P. falciparum parasite line CS2 was grown in the laboratory in group O red blood cells provided by the Australian Red Cross Blood Service.

3.2.5 Culture Media

A 50:50 culture media was made by supplementing 500 ml Roswell Park Memorial Institute (RPMI) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) media with 0.2% w/v sodium bicarbonate and with 25 ml of heat-inactivated human serum and 25 ml of Albumax II (5% in RPMI), and was stored at 4°C. Culture media were always warmed up at 37°C at a minimum of 30 minutes before use.

3.2.6 Parasite culture and synchronization

3.2.6.1 Parasite culture

P. falciparum parasite line CS2 was cultured in 10 ml or 25 ml Petri-dishes (Falcon, Corning) in a 50:50 culture medium at 4% hematocrit. Cultures were then incubated at 37°C (Forma Scientific, incubator) and maintained in a gas mixture of 1% O₂, 4% CO₂ and 95% N₂ in airtight boxes (Coregas, Australia). Each day, thin smear was prepared from cultures. Thin smears were prepared by pipetting 3.5 µl of culture from the culture plate onto a microscope slide and spread forward or backward using spreader (second) slide, dried and fixed with 100% methanol approximately for about 30 seconds and stained with 10% Giemsa staining solution to check the parasitaemia. Cultures were maintained at $\leq 7\%$ parasitaemia and diluted using uninfected erythrocytes as required.

3.2.6.2 Gelatin treatment

The trophozoite stages IEs that express knobs were selected every 2 weeks by gelatin. Gelatin (0.75%) for the selection of trophozoites/knobby lines is a mixture of 0.75 g gelatin to 100 ml of RPMI 1640-HEPES. The medium was removed from the dishes and red blood cells were re-suspended in pre-warmed (37°C water bath) gelatin mixture, 6 ml for 10 ml Petri dishes and 12 ml for 25 ml Petri dishes, and transferred to 50 ml centrifuge tube. The mixture was incubated at 37°C for 40 minutes. The supernatant, which contains ‘knobby’ parasites, was collected (pellet discarded) and spun down, the supernatant discarded, and the pellet washed once in warm RPMI -HEPES at 1500 rpm for 5 minutes. The pellet was re-suspended in culture medium in the 50 ml test tube and transferred to a new petri dish and uninfected red blood cells were added to maintain the haematocrit.

3.2.6.3 Sorbitol treatment

Sorbitol (5%) treatment was also performed every 2 or 3 weeks as needed to select IEs infected with the ring stage. The ring stage of parasites was synchronized by sorbitol treatment. Sorbitol treatment uses 5% D-sorbitol (Ajax Finechem, 1585-500G) in water as a hypotonic solution to lyse trophozoite and schizont IE. In brief, parasite culture was centrifuged at 1500 rpm for 5 minutes and the supernatant discarded. Packed cells were resuspended in 10x pellet volumes of 5% sorbitol (6 ml for 10 ml Petri dishes and 15 ml for 25 ml Petri dishes) and incubated at 37°C for 5 minutes. After the incubation, the cell

suspension was centrifuged (1500 rpm, 5 minutes) before discarding the supernatant (lysate). The cell pellet (containing ring-stage parasites and uninfected erythrocytes) was washed twice in RPMI-HEPES, resuspended in 50:50 culture media and transferred into an appropriate petri dish.

3.2.6.4 Percoll purification

Trophozoite IEs were isolated by the Percoll purification technique (a density gradient synchronization approach). For assays that need a mature stage of IE cultures were always purified using Percoll purification techniques because the parasite surface protein PfEMP1 is expressed by matured stages of the parasite. Percoll in PBS were prepared using RPMI media. A bulk of Percoll in PBS (GE Healthcare, 17-0891-01), 80%, 60%, and 40% were prepared in RPMI-HEPES, filter sterilized and stored at 4°C. A decreasing gradient was carefully layered in a 15 ml tube by slowly pipetting each layer on top of each other without creating any air bubbles using pre-warmed 80% (3 ml), 60% (2 ml), and 40% (2 ml) Percoll respectively. The entire parasite culture was resuspended and collected in a 50 mL falcon tube. Synchronized IEs culture at 5-8% parasitemia of mid to late trophozoite stages were collected by centrifugation at 1500 rpm for 5 minutes. Supernatants were discarded and sediments were resuspended in 2 ml of RPMI-HEPES and carefully layered over the Percoll gradient, centrifuged at 1600 x g for 15 minutes. Using a new 15 ml test tube, mature trophozoite IEs were collected from the interphase between the 60% and 40% percoll gradients and washed twice in 10 ml of RPMI-HEPES. Haemocytometer was used to determine total cell count and purity estimated by thin smear microscopy prepared after the second wash. Routinely, >85% purities were observed.

3.2.6.5 Merozoite purification

P. falciparum (CS2) was cultured as described above in **section 3.2.7** of this thesis. Repeated synchronizations using 5% sorbitol solution were performed to have the parasites in highly synchronized culture. Merozoite purification was performed as described elsewhere (132, 250), with some modifications.

In brief, trophozoites were isolated when most parasites were in the trophozoite stage by Percoll gradient purification method. Trophozoites were washed twice using the wash medium (RPMI- HEPES). After resuspension in a known volume of culture media, they

were returned to the incubator. Then, the parasite growth was monitored by Giemsa staining of microscope slides and observing the slides using light microscopy. At the late pigmented trophozoite stage, 10 μ M E64 (Epoxy succinyl-L-leucylamido (4-guanidino) butane (E64) was added and cells were cultured for a further 6-12 hours until >90% of them were highly matured schizonts (formed membrane-enclosed merozoites). The E64- treated schizonts were pelleted at 2,000 $\times$ g for 10 min when at least 90% of the schizonts had membrane-enclosed merozoites. To remove plasma and E64, treated schizonts were washed with wash medium, and resuspended in 2-5 ml wash buffer before being passed through a 1.2 μ m/32 mm syringe filter. The filtrate was collected into a 50 ml falcon tube and centrifuged at 4,000 $\times$ g (4300 rpm) for 10 min to pellet the merozoites. The supernatant was discarded and the merozoites were resuspended into a known volume of RPMI for immediate use or to freeze at -80°C for further assay.

3.2.7 Isolation of Human Neutrophils from Venous Blood

Different neutrophil isolation methods have been optimized, namely (1) Ficoll and dextran gradients; and (2) EasySep direct human neutrophil isolation kit.

3.2.7.1 Isolation of neutrophils from venous blood by Ficoll and dextran gradients

Neutrophils were isolated by Ficoll and dextran gradients, as described previously (164), with some modifications. Briefly, fresh venous blood from a healthy donor (5-10 ml in a BD Vacutainer heparin) was collected. Immediately after collection, the whole blood was carefully layered over 5 ml of Ficoll-Hypaque (Sigma 1077) in a 15 ml tube using a sterile plastic pipette and centrifuged at 400 $\times$ g for 25 min at room temperature, with no brake on the centrifuge. All layers above erythrocytes were removed (important to completely pipette out PBMCs layer as best as possible) while leaving the RBC layer intact. The pellet which consists of neutrophils plus erythrocytes was resuspended in 3 mL of 3% solution of high-molecular-weight dextran (dextran 450,000-650,000; Sigma-Aldrich life science from *Leucostoc spp.*) in PBS. The tube was mixed thoroughly by pipetting up and down (ten times) to ensure adequate mixing. The tube was stood upright for 18-25 minutes at room temperature to sediment erythrocytes. The upper leukocyte layer was aspirated with a sterile plastic pipette and transferred to a new 15 ml tube and then the aspirate centrifuged at 500 $\times$ g for 10 min at 4°C. The pellet was resuspended in 2 ml of endotoxin-free, sterile

water (pipetted up and down for 25 seconds) and tonicity was promptly restored by adding an equal volume of 1.8% NaCl (2 ml) followed by 5 ml of 0.9% NaCl, at room temperature, and centrifuged at 500 x g for 5 min at 4°C, supernatant discarded. The lysis step was repeated as needed.

Cell concentration was determined by manually counting using a haemocytometer after re-suspending with 2-5 ml of RPMI plus 10% fetal bovine serum (FBS). Cells were resuspended at 1×10^6 cells/ml and placed at room temperature until use. To count the cell concentration, a suspension was prepared containing 5 μ l of cells (isolated neutrophils) and 95 μ l of 0.4% trypan blue solution (1:20 dilution), then 10 μ l of the suspension was charged into a haemocytometer and counted (both live and dead cells). Cells viability was assessed using the trypan blue exclusion test. The protocol produced 95% viable neutrophils. The cell purity was determined by performing a differential blood cell count of smear after staining the cells with 10% Giemsa stain (Sigma) for 10 min or Diff. Quick stain for 15 seconds. The purity of the isolated neutrophils was 90%. Lymphocytes and monocytes have been the identified contaminants.

3.2.7.2 Preparation of neutrophils using EasySep direct human neutrophil isolation kit

Neutrophils were isolated by negative selection from whole blood collected from healthy donors using EasySep direct human neutrophil isolation kit (Stem Cell Technologies; Catalog #19666) strictly following the manufacturer's instruction. Briefly, 5-10 mL of whole blood was collected by BD vacutainer test tube, REF.367885, from a healthy donor, and EDTA was added to the whole blood sample to a final concentration of 1 mM. The whole blood sample was transferred to a 50 ml conical tube. Isolation Cocktail and Rapid Spheres (50 μ l/ml of the sample) were added to whole blood, mixed (pipetting up and down) and incubated at RT for 5 minutes. Neutrophil isolation recommended medium (PBS containing 1 mM EDTA which is free of Ca^{++} and Mg^{++}) was added to top up the sample to the 50 ml, mixed again by gently pipetting up and down 2-3 times, and the tube was placed (without lid) into the magnet (Easy 50, Catalog #18002) and incubated at RT for 10 minutes.

The enriched cell suspension was carefully pipetted out into a new 50 ml conical tube, and then Rapid Spheres (50 μ l/ml of sample) were added, mixed and incubated at RT for 5

minutes. The new tube was placed (without lid) into the magnet and incubated for a second separation for another 5 minutes at RT. The clear fraction of the enriched cell suspension was carefully pipetted out into a new 50 ml conical tube. The old tube was removed from the magnet and a new tube (without lid), containing the enriched cells, was placed into the magnet and incubated for a third separation at RT for 10 minutes. Then, the clear fraction of the enriched cell suspension was transferred into a new tube. Isolated cells are ready for use.

However, to remove serum in the suspension, which might have an effect on the assay, the enriched cell suspension was centrifuged at 500 x g, 4°C for 5 minutes. The sediment was resuspended with the medium at 1×10^6 cells/ml and placed at room temperature until use.

3.2.7.2.1 Neutrophil viability assessment

Neutrophils were routinely analyzed for cell viability by light microscopy under 10X magnification. Viability of neutrophils was determined by Trypan Blue exclusion assay which identifies the fraction of neutrophils with an intact outer membrane (not permeable to the blue dye) as being viable and neutrophils stained blue (dead or dying due to outer membrane damage).

3.2.7.2.2 Neutrophil purity determination

The cell purity was determined before addition to the assay in order to meet basic quality control standards (after staining with 10% Giemsa stain (Sigma) for 10 min). The purity of neutrophils was confirmed in samples by Flow Cytometry targeting the cell marker cluster of differentiation 66b (CD66b) expressed on the human neutrophil surface. The protocol produced $\geq 99\%$ neutrophils purity (both in microscopy and Flow Cytometry).

3.2.8 Antibody-dependent neutrophil-mediated phagocytosis (ADNP) assay

3.2.8.1 ADNP assay towards IEs

U-bottom 96-well plates were coated with 0.1% BSA in PBS (170 μ l/ well) for at least 1 hour. Plates were gently flicked, and the remaining blocking solutions were removed by tapping the plate against centre fold handtowel. Plasma was placed (3.3 μ l) in the coated wells and kept at -20°C overnight. The positive and negative controls were plated fresh on the day of the experiment. Positive control came from pooled positive plasma and negative

controls from non-malaria exposed pooled plasma. In addition, rabbit anti-human antibodies (Cappel) were also used as an additional positive control to assess the phagocytic ability of the neutrophils.

Plates were kept aside, and the parasites were prepared by Percoll density gradient purification. Parasite preparation was performed as described in section 3.2.6.4. A smear was prepared from the pellet obtained and checked for trophozoites purity using Giemsa stain (1 μ l of the pellet taken and a smear was very slowly prepared, fixed and stained in 10% Giemsa). Then, the purified parasites were incubated with dihydroethidium (DHE) (25 μ g/ml of DHE was added to the resuspended parasite suspension, diluted 1:400) in the dark for 30 minutes. After 4 times wash at 1500 rpm for 3 minutes each with wash buffer (RPMI-HEPES), the pellet of IEs was mixed with 1-2 ml volume of RPMI-HEPES. To count cells, 5 μ l of IEs mixture was further diluted with 995 μ l of RPMI- HEPES (1:200 dilution), and then 10 μ l of these diluted parasites suspension was charged into the haemocytometer. After counting on the haemocytometer, the final concentration was adjusted to 1.65×10^7 cells/ mL with RPMI- HEPES. The calculated 30 μ L of the IEs (approximately 5×10^5 cells) was added in each well of the previously prepared plate (master plate) containing the test and control serum/plasma samples. The cells were agitated by gentle tapping with the side of the plate and incubated for 1 hour in dark at RT. During this incubation time, neutrophils were purified by using the EasySep direct human neutrophil isolation kit as detailed in section 3.2.7.2. After the previously standing parasite with plasma incubation time, cells were washed three times with wash buffer at 1500 rpm for 3 minutes each. The parasites were resuspended in 50 μ l of neutrophils media per well, and aliquots of this mix were dispensed into duplicate wells, 25 μ l/well (containing 2.5×10^4 cells) in a new U-bottom 96-well plate coated with 0.1% BSA in PBS. Then, 50 μ l volume containing 2.5×10^4 neutrophils were dispensed into each of the duplicate wells prepared and cells agitated gently tapping the side of the plates and incubated for 1h at 37°C, 5% CO₂. During the incubation period, the centrifuge was pre-set to 4°C, and FACS lysing solution (BD Biosciences, 1:10 dilution), was prepared in Milli-Q water.

Phagocytosis was stopped by spinning plates at 4°C, 1500 rpm for 5 minutes. To lyse the unphagocytosed infected erythrocytes, supernatants were discarded and 75 μ l of lysing solution was added into each well for 10 minutes at room temperature in the dark. After centrifugation at 1500 rpm for 3 minutes at 4°C supernatants were discarded and three

washes were performed with freshly prepared FACS buffer (2% FBS in cold PBS) at 1500 rpm for 3 minutes each (100 µl volume per well each time). Last, the cells were fixed in 100 µl cold 2% PFA in PBS and kept at 4°C in the dark for at least 2 hours or overnight before acquisition by flow cytometry. Test samples were acquired using flow cytometry (Beckman Coulter, CytoFLEX S) on plate reader mode.

3.2.8.2 *Bead-based ADNP assay*

3.2.8.3 *Antigen biotinylation*

A vial of 1 mg micro tube sulfo NHS-LC-Biotin was removed from the freezer and placed at RT. Sterile ultrapure water (180 ml) was added into 1 mg microtube to prepare a 10 mM solution of biotin reagent. The DBL protein domains were adjusted to a working concentration of 1mg/ml, biotinylated with 50 µl/ml of biotin stock solution (sulfo NHS-LC-Biotin) and incubated for 30 minutes at RT. Non- reacted biotin was removed with desalting column (Zebra spin desalting columns 0.5 ml). The column's bottom closure was removed and the top cover was untightened. The column was placed in a 1.5 - 2.0 ml collection tube, centrifuged at 1500 x g for 1 minute to remove storage solution. The column was washed three times with 300 µl of PBS (1500 x g for 1 minute). Then, the biotinylated protein was pipetted into the desalting column and centrifuged for 2 minutes at 1500 x g, and the flow-through was collected. A mark was placed on the side of the column where the compacted resin was slanted upward and the column was placed in the microcentrifuge with the mark facing outward in all subsequent centrifugation steps.

To concentrate protein, protein concentration column Millipore, 0.5 ml (UFC501024) was used. The AmiconR Ultra 0.5 device was inserted into microcentrifuge tubes and 100 µl of the sample was added to the AmiconR Ultra filter device and the filter device was capped, placed into the centrifuge rotor, aligning the cap strap toward the center of the rotor, centrifuged at 14,000 x g for 15 minutes. The assembled device was removed from the centrifuge and the AmiconR Ultra filter device separated from the microcentrifuge tube.

To recover the concentrated solute, the AmiconR Ultra filter device was placed upside down in a clean microcentrifuge tube, placed in the centrifuge, aligning open cap towards the center of the rotor, spun for 2 minutes at 1,000 x g to transfer the concentrated sample

from the device to the tube (the ultra-filtrate could be stored in the centrifuge tube) and resuspend at 1 mg/ml in PBS.

3.2.8.3.1 Coating of beads with biotinylated protein

The amounts of beads (1%V/V Neutravidin 1 μ m green, Thermofisher) desired for the assay were calculated: 0.1 μ l beads/well. Beads and biotinylated proteins were placed in an Eppendorf tube in a ratio of 1:6 (e.g. 1 μ l beads: 6 μ l protein, 1 mg/ml) and incubated overnight at 4°C in a rotor. The following day, the solution was washed twice with 0.1% BSA in sterile PBS by spinning it at 14000 x *g* for 5 minutes. Finally, the beads coupled with biotinylated DBL domains were resuspended in 0.1% BSA and 0.02% of sodium azide was added. The preparation was wrapped in foil and placed in the fridge at 4°C. The antigen covered beads were used within 1-4 weeks of preparation.

3.2.8.3.2 ADNP assay

Sterile 96 well U-bottom plates (Corning Costar, treated, polystyrene) were blocked with high salt PBS (1% BSA /0.5 M NaCl in PBS) for 30 minutes at RT. For opsonization, aliquots (6 μ l/well) of the DBL protein-biotinylated beads were dispensed and incubated with plasma samples (12 μ l) at RT (1:100 dilution with high salt PBS). Pooled plasma samples (PPS) positive controls and negative controls (Melbourne controls, MC) were also diluted similar to the samples. After 1 hour incubation, the opsonized protein coated beads were spun down at 4700 x *g* for 3 minutes and the supernatants were flicked off. Antibody opsonized beads and human neutrophils were co-cultured at a density of 1.2×10^6 beads/well and 3×10^4 cells/well respectively. The plates were left in the incubator for 1h at 37°C, 5% CO₂. The phagocytosis was stopped by centrifugation at 4°C for 3 minutes at 350 x *g*. After that, cells were washed once at 350x *g* for 3 minutes, supernatants removed and cells were fixed with cold 2% PFA, 100 μ l/well for at least 2 hours. The samples were analyzed by flow cytometry within 24 hours.

3.2.9 Gating strategy for antibody-dependent neutrophil phagocytosis assay

In this study, both *P. falciparum* whole infected erythrocytes and individual *P. falciparum* recombinant DBL antigens were tested for ADNP assay. ADNP was measured by flow cytometry (Beckman Coulter, CytoFLEX S) as described in chapter 4, section 4.3.5 (as illustrated in Figure 1 in this chapter and figure 4.1 in chapter 4).

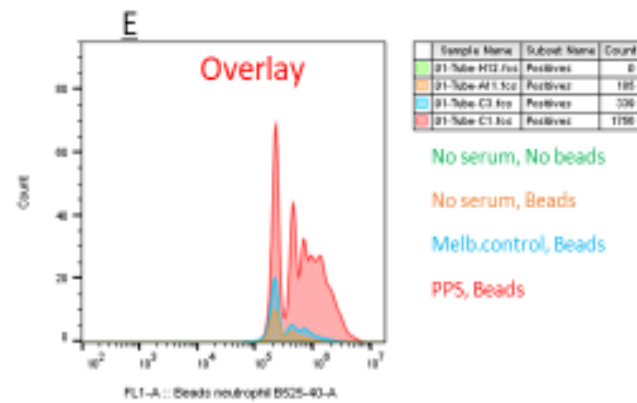
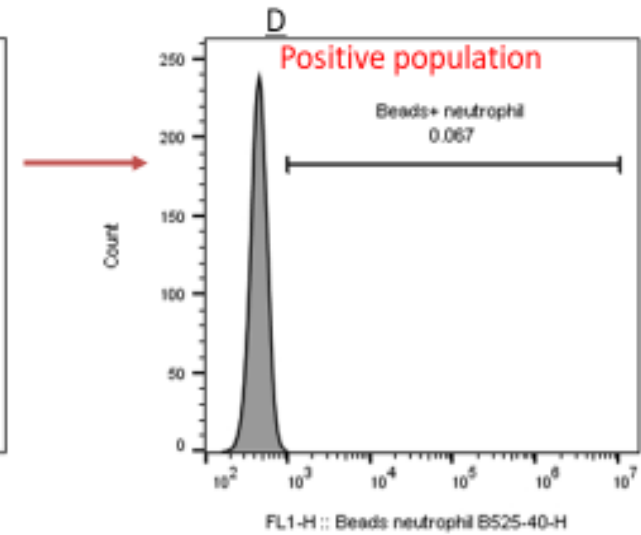
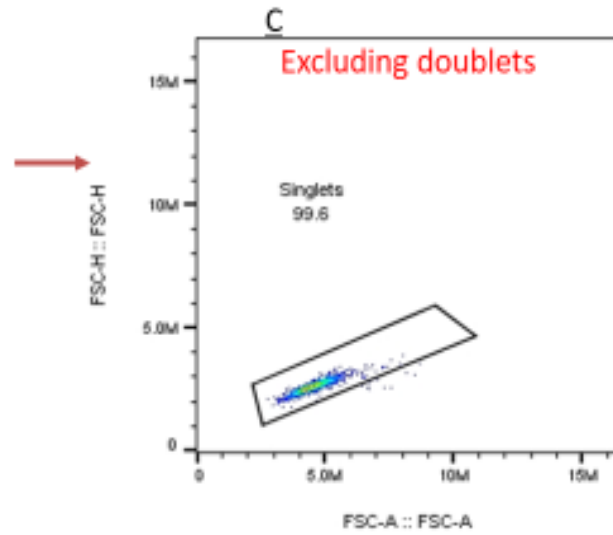
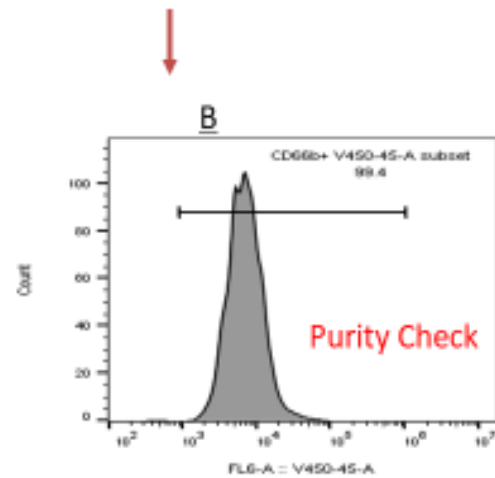
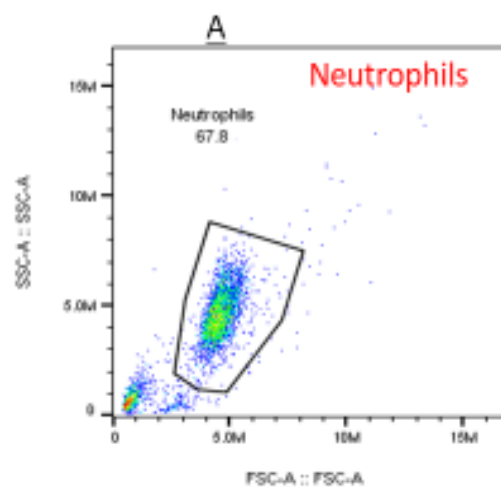


Figure 3 1 Neutrophil gating strategy. Schematic representation and gating strategy used for antigen-coated beads in the antibody-dependent neutrophil phagocytosis assay. Beads coated with recombinant VAR2CSA protein (DBL3 in this example) were opsonized with pooled plasma from positive and negative controls. Isolated neutrophils were cocultured with opsonized beads and then were fixed, and stained for CD66b flurophore. Cells were analyzed by flow cytometry and data were analyzed using FlowJo. Neutrophils were gated based on SSC-A and FSC-A (A) and the neutrophil purity was determined using a neutrophil-specific marker, CD66b- BV421 (B). To identify a single population, doublets were excluded by SSC-H and FSC-A (C). Uptake of beads by neutrophils was quantified by measuring the percentage and gMFI (geometric mean fluorescence intensity) of AlexaFluor 488+ cells within the neutrophil population (D) and expressed in phagocytosis and phagocytic score. Phagocytic score was calculated by the percent of neutrophils that had taken up beads multiplied by the fluorescent signal of beads taken up by the neutrophils (gMFI) of bead-positive neutrophils. In the overlay histogram (E), neutrophils alone are depicted (green), neutrophils incubated with unopsonized beads (orange), neutrophils incubated with beads opsonized with Melbourne control (blue) and neutrophils incubated with beads opsonized with the positive control (red).

3.3 Neutrophil extracellular traps (NETs)

3.3.1 NET induction assay

In this study, we use DAPI (to stain for both intracellular and extracellular DNA), SYTOX green (to stain dead cells or extracellular DNA), and neutrophil granular protein markers of NETs. NET induction was evaluated by microscopy and fluorometrically.

3.3.1.1 Microscopic visualization of NETs with DNA stain (DAPI and SYTOX green)

Neutrophil extracellular trap induction assays were performed in costar 24-well cell culture plates (Corning Incorporated, USA) containing 13 mm round glass coverslips, poly-L-lysine-uncoated, (Deck-glass, Germany). Neutrophils (1×10^5 /well in 100 μ l) were seeded onto the coverslips and incubated for 1h at 37 °C in a humidified atmosphere of 5% CO₂ allow attachment to coverslips. Following preincubation, neutrophils were treated with stimulants (IEs or merozoites). Neutrophils incubated with a final concentration of 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) were used as positive controls, and unstimulated neutrophils in isolation medium as a negative control (to check whether neutrophil isolation affected membrane integrity) for 3 hr in a humidified incubator at 37°C, 5% CO₂.

Next, the cells were fixed with 200 μ l of 4% paraformaldehyde (PFA) for 30 min at room temperature. The PFA was gently aspirated out from each well and washed once with 200 μ l PBS (all the solutions in the well were never aspirated directly over the coverslip. Thus, the plate was always tilted and aspirated from the side of the well, touching the wall of the well). The DNA was stained by adding 200 μ L of DAPI and 25 μ L SYTOX green for 15 min at room temperature. The DAPI and SYTOX green were removed and wells were washed twice with PBS for 5 min each at room temperature and coverslips were mounted on slides using a drop of fluoroshield histology mounting medium (Sigma-Aldrich, USA).

The coverslips were placed upside down on the fluoroshield histology mounting medium, being careful to avoid bubbles and medium was allowed to dry overnight at 4°C, protected from light. Fluorescent microscopic images were captured using 63x magnification in different fields on Zeiss LMS700 and Zeiss LMS710 confocal microscopes, and NETs were quantified using Fiji software. Three separate experiments were performed using

neutrophils from 3 different donors, with duplicates assays performed within each experiment.

3.3.1.2 Immunofluorescence staining of NETs markers

Fluorescent image analysis of NETs was performed according to previous studies with some modifications (251-254). NET assays were performed in costar 24-well cell culture plates (Corning Incorporated, USA) containing 13 mm round glass coverslips, poly-L-lysine-uncoated, (Deck-glass, Germany). Neutrophils (1×10^5 /well in 100 μ l) were seeded onto the coverslips and incubated for 60 minutes at 37 °C in a humidified atmosphere of 5% CO₂ allow attachment to coverslips. Following preincubation, neutrophils were treated with IEs for 3-4 hr in a humidified incubator at 37°C, 5% CO₂. Neutrophils incubated with a final concentration of 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) were used as positive controls and unstimulated neutrophils in isolation medium as a negative control (to check whether neutrophil isolation affected membrane integrity). The cells were then fixed with 200 μ l of 4% PFA for 30 min at room temperature. The PFA was gently aspirated out from each well and washed two times with 200 μ l PBS (all the solutions in the well never aspirated directly over the coverslip, always tilt the plate and aspirate from the side of the well, touching the wall of the well).

To stain NETs markers, the nonspecific binding was blocked with 200 μ l blocking buffer (10% goat serum plus 0.05% Tween 20 in PBS) for 1h at room temperature. Primary antibody was prepared, 1:50 dilution of mouse anti-human neutrophil elastase (5 μ g/ml), and rabbit anti-human myeloperoxidase, MPO (DAKO /Denmark) (1:200) in blocking buffer. A drop of (20- μ l) diluted antibody was placed on a sheet of parafilm in a humidified chamber. Using curved needle and forceps, coverslips were transferred from the 24-well plate to the humidified chamber, putting each coverslip face down on a drop of the antibody for 60 min at room temperature, in the dark. The blocking buffer in the 24-well plate was replaced with PBS, 200 μ l/well. The coverslips were returned back face up to the 24-well plate and washed for 5 min, without rotation, for a total of two washes. Then, secondary antibody (goat anti-mouse AlexaFluor 647 (Life Technologies, A21235, and goat anti-rabbit AlexaFluor 488, Abcam, ab150169) was prepared in blocking buffer (1:500) and placed 100- μ l on glass coverslips in a 24-well plate for 1h at 37°C, 5% CO₂. After that, the coverslips were washed two times with PBS as before and the PBS was replaced with 200 μ l DAPI (1: 10,000 in PBS as needed to make a 300 nM DAPI stain solution) 10 min at

room temperature. The counterstain was removed, washed twice with PBS for 5 min each at room temperature, and coverslips were mounted on slides using a drop of fluoroshield histology mounting medium (Sigma-Aldrich, USA).

As described in section 3.3.1.1, the coverslips were carefully placed upside down on the fluoroshield histology mounting medium and images were captured using confocal microscope.

3.3.1.3 Fluorometric quantification of NETs formation

The NET release was also quantified fluorometrically by measuring extracellular DNA release using the membrane-impermeable fluorescent dye, SYTOX green (Thermo Fisher, S7020), as described previously (116, 255-257). Briefly, freshly isolated human neutrophils (1×10^5 cells/well) in 100 μ l RPMI containing 0.5% FBS were seeded onto black-walled 96-well culture plates and SYTOX green was added to a final concentration of 10 μ M. After 10 min incubation at 37 °C, 5% CO₂, 25 μ l of IgG-opsonized or unopsonized IEs were added to the appropriate well (Table 1) and incubated at 37 °C, 5% CO₂ for up to 4 h. In this assay, PMA and LPS were used as a positive control and unstimulated neutrophils as a negative control. To determine the effect of uninfected erythrocytes (uRBCs), neutrophils cocultured with uRBCs were also used as additional negative controls. The signals of SYTOX green were read in a microplate reader (FLUOstar Omega plate reader, OMG LabTech) with fluorescence excitation at 485-12 nm and with emission measured at 520 nm. The first SYTOX green signal measurement was taken immediately after the addition of stimulants (0 minute) and repeated 30 minutes later. After that, in every one hour difference measurements were continued up to 240 minutes. During each measurement step, samples were incubated back at 37°C, CO₂ 5%. The rise in fluorescence was referred to as “extracellular DNA release” and expressed as arbitrary fluorescence units (AFU). Three separate experiments were performed using neutrophils from 3 different donors, with triplicate assays performed within each experiment. To evaluate NETs formation in response to IEs across time, time course assay was performed.

Table 3 1 Treatment conditions used for the quantification of neutrophil extracellular DNA (NETs) releases

	RPMI (μl)	Neutrophils (1x10 ⁵ /100 μl)	Triton X-100 1% (μl)	PMA(μL) (100nM)	IEs (1x10 ⁶) suspension (μl)	IEs with IgG (1x10 ⁶) (μl)	SYTOX green (μL) (50 μM)
Neutrophils	25	100	-	-	-	-	25
Neutrophils + Triton X-100		100	25	-	-	-	25
Neutrophils + PMA	25	100	-	25	-	-	25
Neutrophils + IEs	25	100	-	-	25	-	25
Neutrophils + IgG opsonized IEs	25	100	-	-	-	25	25

*Total reaction volume is 175 μl. IgG = Immunoglobulins; PMA = Phorbol 12-myristate 13-acetate; IEs = Infected erythrocytes

PMA(μL) (100nM is final concentration. IEs were opsonized with purified IgG for 30 minutes at room temperature

100nM PMA is the final concentration

3.3.2 NET inhibition assay

In the current study, we developed a protocol to measure the inhibitory effect of *P. falciparum* parasite on neutrophil extracellular trap formation. To do this, neutrophils, 1×10^5 cells /100 μ l, were cocultured with IEs and merozoites in the presence of PMA or LPS (Table 2). NET inhibition effect of IEs was evaluated by microscopy and fluorometric methods. PMA and LPS, a potent inducer of NETs formation, was used as a positive control. For observation of NET formation inhibition microscopically the DNA was stained with DAPI and SYTOX green. For fluorometric quantification, SYTOX green was added and the signals of SYTOX green quantified on a fluorescence microplate reader at different time points.

3.3.2.1 Microscopic quantification of NETs formation inhibition

For semi-quantitative analysis of NETs, neutrophils were stimulated with the different stimulants as described above for 3 hr and stained with DAPI (blue) and SYTOX green. Fluorescent microscopic images were captured using 63x magnification on Zeiss LMS710 confocal microscope, nine images were acquired (tile scan 3x3 and Z-stack with four steps of 1.5 microns) from different areas of each coverslip to accurately represent the effect of the stimulus. Every image had the same X, Y, and Z dimensions. In the Green channel (SYTOX green-stained) and blue channel (DAPI), an intact nucleus and NETs were counted to give the total amount of DNA. When counting NETs, an attempt was made to identify the cell body of the cells that extruded the DNA. NET structures and total cells were counted using the Fiji software counting tool (drew a circle on the cell→click ‘T’ on the keyboard→drew the number in the circle to the second cell→click ‘T’ on the keyboard and continue until you finish counting). Image analysis algorithm was developed, by Dr. Ellie Hyun-Jung Cho, and NETs were evaluated based on the total cell area. To explain the elongation of the cell, the Feret’s diameter (the longest diameter of the cell) was also determined. The NET formation was also evaluated by measuring the length of the cell boundary (perimeter) which detects shape differences better if cells are not just round but are in complex shape). Three separate experiments were performed using neutrophils from 3 different donors, with duplicates assays performed within each experiment.

3.3.2.2 Fluorometric quantification of NETs formation inhibition

In this experiment, PMA and LPS were used as a positive control and unstimulated neutrophils as a negative control. The inhibitory effect of IE on PMA or LPS induced NET release was quantified fluorometrically using the membrane-impermeable fluorescent dye, SYTOX green (Thermo Fisher, S7020). Briefly, freshly isolated neutrophils (1×10^5 cells/well) in 100 μ l RPMI containing 0.5% FBS were seeded onto black-walled 96-well culture plates and SYTOX green were added to a final concentration of 10 μ M. After 10 min incubation at 37°C, 5% CO₂, 25 μ l of IEs and merozoites were added to the appropriate wells. An inhibitory effect of IEs and merozoites on extracellular DNA release as a measure of NETs was tested. To do this, neutrophils were cocultured with IEs in the presence of PMA or LPS, and merozoites in the presence of PMA or LPS. After that, the samples were incubated at 37°C, 5% CO₂ for up to 5 h. To evaluate the NETs inhibition effects of IEs, the SYTOX green signals were fluorometrically measured up to 5h. The SYTOX green signals were read in a microplate reader with fluorescence excitation at 485-12 nm and with emission measured at 520 nm.

A time course assay was performed to evaluate PMA induced NETs formation inhibition across time. The first signal measurement was taken immediately after stimulants were added and repeated 30 minutes later, then in every one hour difference measurements were continued up to 300 minutes. During each measurement step, samples were incubated back at 37°C, CO₂ 5%. The amount of fluorescence corresponds to the amount of extracellular DNA release as a result of NET formation and expressed as arbitrary fluorescence units (AFU). Three separate experiments were performed using neutrophils from 3 different donors, with triplicate assays performed within each experiment.

To determine whether the IE have inhibitory effects on the NET formation or were able to degrade the already formed NETs, IEs and merozoites were added after 4 hr of neutrophils treatment with PMA or LPS in a separate assay.

Table 3 2 Treatment conditions used for the quantification of PMA induced neutrophil extracellular DNA (NETs) formation inhibition

	RPMI (μl)	Neutrophils (1x10 ⁵ /100 μl)	Merozoite suspension (μl)	PMA (μL) (100 nM)	IEs (1x10 ⁶) (μl)	LPS 1 mg/ml (μl)	SYTOX green (μL) (50 μM)
Neutrophils	50	100	-	-	-	-	25
Neutrophils +PMA	25	100	-	25	-	-	25
Neutrophils + PMA +IEs	-	100	-	25	25	-	25
Neutrophils + PMA + merozoites	-	100	25	25	-	-	25
LPS		100	-	-	-	25	25
Neutrophils + LPS +IEs	-	100	-	-	25	25	25
Neutrophils + LPS + merozoites	-	100	25	-	-	25	25
PMN + IEs	25	100	-	-	25	-	25
PMN + merozoites	-	100	25	-	-	25	25

*Total reaction volume is 175 μl. IgG = Immunoglobulins; PMA = Phorbol 12-myristate 13-acetate; IEs = Infected erythrocytes;
LPS = Lipopolysaccharides

3.3.3 Neutrophil supernatant collection for ELISA

Human neutrophils and *P. falciparum*-IEs or purified merozoites were cocultured, with or without potent NETs stimulators PMA or LPS, and ELISA was used to quantify myeloperoxidase (MPO) release as a measure of neutrophil degranulation from supernatants of neutrophils. To do this, neutrophils (1×10^5 /well in 100 μ l) were seeded onto 96 well plates in duplicates (Thermofisher, NUNC), before stimulation with IE, merozoites, PMA +IE, PMA + merozoites, LPS +IE, LPS + Merozoite. PMA and LPS were used as positive controls and unstimulated neutrophils as a negative control. Cells were co-incubated for 4 h at 37 °C, 5% CO₂. Cell supernatants were collected from duplicate wells separately. Then, the cell supernatant was centrifuged for 5 min at 5,000 x g to remove parasite and debris, aliquoted to a smaller volume, and stored at -80°C for further analysis or used immediately. The cell-free supernatant was used to quantify MPO release as a measure of neutrophil degranulation by ELISA assay using DuoSet ELISA kits. Each supernatant was diluted 1:50 for MPO ELISA.

3.3.4 Myeloperoxidase (MPO) ELISA.

To measure human MPO as a measure of neutrophil degranulation using sandwich ELISA kits (R&D system, USA & Canada, Catalog Number: DY3174), the capture antibody was diluted to the working concentration in PBS. The diluted capture antibody was immediately coated in a 96-well microplate with 100 μ l per well, sealed and incubated at room temperature overnight. Then, the capture antibody was decanted from each well and washed with Wash Buffer (0.05% Tween 20 in PBS, pH 7.2-7.4), repeating the process twice for a total of three washes and after the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels (as complete removal of liquid at each step is essential for good performance). Nonspecific bindings were blocked by adding 300 μ l of reagent diluent (1% BSA in PBS, pH 7.2-7.4, 0.2 μ m filtered) to each well for 1 hour at room temperature, washed twice and 100 μ l of detection antibody was added for 2 hours. After washing as before, 100 μ L of sample or standards in reagent diluent were added per well for 2 hours at room temperature (plates were cover with an adhesive strip). The washing procedure was repeated as described before, 100 μ l of the working dilution of Streptavidin-HRP was added to each well for 20 minutes, followed by 100 μ l of substrate solution for another 20 minutes at room temperature, plates

were covered and placed in the dark. Then, stop solution (50 µl) was added to each well and thoroughly mixed by gentle tapping of the plates. Finally, the optical density of each well was determined immediately, using a microplate reader set to 450 nm. Since wavelength correction is not available in this microplate reader (FLUOstar Omega plate reader, OMG LabTech), readings at 540 nm or 570 nm were subtracted from the readings at 450 nm. This subtraction corrects for optical imperfections in the plate. Two separate experiments were performed using neutrophils from 2 different donors, with duplicates assays performed within each experiment. The concentration of MPO in samples was calculated by extrapolation from a standard curve.

3.4 Placental infiltration of neutrophils and placental NETs

3.4.1 Ethics, study site, study populations, and sample collection

Written informed consent was obtained from all participants, and the study was approved by the College of Medicine Research Committee, University of Malawi. The study populations included pregnant women living in Blantyre, Malawi. From January 1998 to November 2000, women attending the labour ward of the Queen Elizabeth Central Hospital, Blantyre, Malawi, for routine delivery were tested for peripheral, placental, and cord blood *P. falciparum* infection, by microscopy of Giemsa-stained thick blood films (258). Placental tissues, together with age- and gravidity-matched controls, were collected from different women who underwent labour at Queen Elizabeth Central Hospital, Blantyre, Malawi. Full-thickness placental biopsies obtained from a healthy pericentric area were placed into 10% neutral buffered formalin at delivery.

3.4.2 Placental examination and development of a classification system.

Fixed placental biopsies were wax embedded, and sections were stained with Giemsa and/or hematoxylin and eosin (258). Slides were examined by two observers who were blinded to other patients' data. The presence of *P. falciparum* IEs and malaria pigment in fibrin and monocytes was noted. Blood cells were counted in the intervillous under oil immersion objective and were classified as uninfected erythrocytes (UE), infected erythrocytes (IEs), or leukocytes (258).

In the current study, 10 age and gravidity matched malaria-infected and 10 noninfected paraffin-embedded placental tissues were selected. Placental tissues were cut into a water

bath (5 μ m thickness) using a Leica HM 325 microtome following standard histological techniques. The slices were transferred to Superfrost slides from the water bath and dried overnight at 37 °C oven. The slides were kept in a slide box at RT until use. After deparaffinization and rehydration neutrophil infiltration into the intervillous space (IVS) and the presence of NETs in the IVS were quantified by conventional Giemsa microscopy, immunohistochemistry, and immunofluorescent staining.

3.4.3 Giemsa staining

Giemsa staining of placental sections was optimized by staining them for 2 hr and 30 minutes at 37°C in a water bath. Both conditions work fine for the present study but sections stained for 2 hours produced more clear images compared to 30 minutes staining. Thus, all samples were stained for 2 hours at 37°C unless and otherwise specified in this study.

Deparaffinization and rehydration were performed with standard xylene-to-ethanol washes. In brief, slides were deparaffinized and rehydrated (in fume hood) by dipping in 3 changes of xylene for 5 minutes each followed by 2 changes of 100% and 95% ethanol respectively for 10 minutes each, and then tissues were washed with distilled water for two 5 minutes' periods. Placental tissues were stained for 2 hours at 37°C by immersing slides in 10% Giemsa staining solution (filtered), which was prepared by adding 1 drop of stock solution/ml of water containing jar. After rinsed with distilled water, they were destained with 0.5% aqueous acetic acid for 30 seconds followed by another rinsing. Then, placental tissues were dehydrated by placing them in two changes of 95% and 100% ethanol respectively for 10 seconds each. After that, the tissue was cleared in two changes of xylene for 10 seconds each. Then, slides were mounted with Organo/limonene mounting media and coverslips were placed on the mounting media to prevent scratching.

Total leucocytes, neutrophils, and NETs-like structures were quantified. All slides were reviewed with two experienced microscopists, who were blinded to the status of infection. Neutrophils, NETs-like structures, and other leucocytes were differentially counted to determine the percentage of neutrophils and NETs in the IVS of the placenta. The criteria used to identify neutrophils were multilobulated nucleus (2 or more) whereas 'netting' cells were identified when extracellular DNA was detected in the presence of a disrupted, decondensed nucleus. Cells with unclear nuclear-cytoplasmic integrity were counted as other types of leucocytes. Fields of Giemsa stained placental sections were scanned with

40x magnification to check cell distribution and evaluated at 100× magnifications, with cell counts obtained in 30 consecutive fields, where the maternal IVS was identified. Results were presented as number per field.

3.4.4 IHC for the detection of placental NETs and neutrophil in the IVS

3.4.4.1 Myeloperoxidase (MPO) staining

Placental sections were prepared as described above and deparaffinisation and rehydration were performed with standard xylene-to-ethanol washes. In this assay, unless and otherwise specified placental sections were deparaffinised and rehydrated for 10 minutes each. In brief, placental sections were deparaffinised and rehydrated by dipping in 3 changes of xylene, 2 changes of 100% and 95%, 1 changes of 70% and 50% ethanol. Then, the sections were immersed with distilled water for two 5 minutes' periods.

Antigen unmasking was achieved by heating citrate buffer to 95-100°C in a beaker and boiling the samples for 90 minutes in sodium citrate buffer (10 mM sodium citrate, 0.1% Tween 20, pH 6.0). The beaker was removed from the hot plate and rest at room temperature (RT) to cool on the bench for 30 minutes. To quench endogenous peroxidase activity tissues were dipped in 3 changes of distilled water for 5 minutes each followed by 2 changes of 3% hydrogen peroxide (H₂O₂) for 10 minutes each. After 2 times wash with 2 changes of distilled water 5-minutes, tissues were washed with wash buffer (PBS plus 0.1% Tween 20) for 5 minutes. Using a hydrophobic pen, the area to be stained was surrounded and nonspecific binding was blocked with blocking solution (PBS plus 0.1% Tween- 20 plus 10% goat serum) for 1 hour at RT in a humidified chamber. Then, blocking solution was removed and primary antibody, rabbit anti-human myeloperoxidase, MPO (DAKO /Denmark) (1:200 dilution in blocking solution, 100 µl per section) was added and incubated in a humidified chamber in cold room overnight. On the next day, the primary antibody was removed and washed with 3 changes of wash buffer for 5 minutes each and sections were re-outlined with a hydrophobic pen. Then, the secondary antibody was added, 100 µl/section (biotinylated goat plus rabbit (Vector Laboratories Inc., Burlingame, CA, 94010, BA-1000; 1.5 mg/ml; 1:200 dilutions) and incubated in a humidified chamber at room temperature for 30 minutes. After washing 3 times with wash buffer for 5 minutes each, Streptavidin-Horseradish Peroxidase (strep-HRP Abcam (1:10000 in blocking solution) was added for 30 minutes, followed by three washes with wash buffer for 5

minutes each. The freshly prepared DAB (3, 3' Diaminobenzidine; eBioscience Inc. San Diego, CA; 100 µl per section (comprising 4 µl reagent A plus 96 µl reagent B) was added for 10 minutes. Color development was stopped by adding drops of water and then slides onto 2 changes of water for 5 minutes each. Sections were counterstained with haematoxylin for 5 minutes, washed twice in distilled water for 5 minutes each. Last, they were dehydrated by placing them into two changes of xylene and 50%, 70%, 95%, 100% ethanol respectively for 10 seconds each. Then, slides were mounted using aqueous mounting media and left to dry at RT. The distribution of neutrophil and NETs in the IVS were examined under the microscope (40 x magnification), and neutrophil and NETs were counted in 30 consecutive fields, where the IVS were found using oil immersion objective.

3.4.4.2 Neutrophil Elastase (ELA2) staining

Placental section preparations, deparaffinisation, antigen retrieval, quenching, and blocking were done as described above in MPO staining. Primary antibody, mouse anti-human ELA2 ab (in vitro technology, USA) (1/200 in blocking solution, 100 µl per section) was added and incubated in a humidified chamber in cold room overnight. On the next day, the primary antibody was removed and washed with 3 changes of wash buffer (PBS plus 0.1% Tween 20) for 5 minutes each. After the sections were re-outlined with a hydrophobic pen, 100 µl/section of the secondary antibody (biotinylated goat anti-mouse) was added and incubated in a humidified chamber at room temperature for 30 minutes. The secondary antibody was removed by gently tapping slide on paper towel followed by 3 washes with PBS/0.1% Tween-20 for 5 minutes each. Streptavidin-Horseradish Peroxidase (MabTech 3310-9 (1:1000 in blocking solution) was placed for 30 minutes at 4°C followed by three washes with PBS/0.1% Tween-20 for 5 minutes each. The freshly prepared DAB (3, 3' Diaminobenzidine; Abcam, ab64238; (30 µl or 1 drop of DAB chromogen with each 1.5 ml /50 drops of DAB substrate), 100 µl per section was added for 10 minutes followed by washing with 2 changes of water for 5 minutes each. Then the sections were counterstained with haematoxylin for 5 minutes. After washing with 2 changes of distilled water for 5 minutes each, they were dehydrated by placing them in two changes of 95% ethanol, 100% ethanol, and xylene respectively for 10 seconds each. Then, slides were mounted using aqueous mounting media and left to dry at RT. The distribution of neutrophil and NETs in the IVS were examined under the microscope (40 x magnification), and neutrophil and

NETs were counted in 30 consecutive fields, where the IVS were found using oil immersion objective.

3.4.4.3 Neutrophil and NETs quantification

Neutrophils and NETs were identified by neutrophil elastase IHC as well as by nuclear morphology. Fields of neutrophil elastase IHC stained placental sections were scanned with 40x magnification to check cell distribution and evaluated at 100× magnifications, with neutrophil counts obtained in 30 consecutive fields, where the maternal IVS was identified and results were presented as number per field.

To do this, neutrophils were categorized into three groups such as intact, indeterminate and netting. A neutrophil was defined as ‘**intact**’ if elastase staining was confined within the cytoplasm of the cell and if an intact, multilobulated nucleus was present based on the morphology of e stained cells. A neutrophil was defined as ‘**netting**’ when extracellular elastase was detected in the presence of a disrupted, decondensed nucleus. Finally, neutrophils were identified as ‘**indeterminate**’ when extracellular elastase was present, but with unclear nuclear-cytoplasmic integrity. For NET quantification, intact and indeterminate neutrophils were considered as non-netting neutrophils (198). A cell that presents decondensed nuclei and exceeds the normal average area of an unstimulated neutrophil (80 μm^2) can be considered as NETotic and counted as NETs. In addition, NETs that were already expanded to the extracellular environment were quantified (259).

3.4.5 Immunofluorescence detection of placental NETs

Fluorescence-based detection of placental NETs was performed according to previous studies (199, 251) with some modifications. Briefly, the samples were deparaffinized in 3 changes of xylene (Sigma, Aldrich, USA) for 10 minutes, dehydrated by 100% ethanol alcohol (Chemo-Supply, 2 × 5 min), 95% ethanol alcohol (2 × 5 min) and 70% ethanol alcohol (2 × 5 min). After rinsed with 2 changes of MilliQ water with gentle agitation for 5 minutes in total, antigen unmasking was achieved by heating citrate buffer to 60°C in a beaker and boiling samples for 30 minutes in sodium citrate buffer (10 mM sodium citrate, 0.1% Tween 20, pH 6.0). Beaker was removed from the hot plate and rested to cool on the bench for 30 minutes. After 2 times wash with wash buffer (PBS plus 0.1% Tween 20) for 5 minutes each, the areas to be stained were surrounded using a hydrophobic pen. Slides

were placed on a rack in an incubation chamber and a few drops of PBS were added to the sample within the enclosed circle. The barrier was allowed to dry for at least 2 minutes and initially blocked with cold glycine, 100 µl/ sample, 200 mM (751 mg in 50 mL milliQ water) for 1 hour in a humidified chamber in cold room. Following 2 times wash with wash buffer, nonspecific binding was blocked with blocking solution (PBS plus 0.1% Tween 20 with 10% goat serum) for 1 hour at RT in a humidified chamber. Blocking solution was removed by gently tapping slides on a paper towel and samples were exposed to the primary antibody, mouse anti-human elastase antibody (in vitro technology, USA) and rabbit anti-human myeloperoxidase, MPO (diluted 1:50 and 1:200 with blocking solution respectively, 100 µl per section) and incubated in a humidified chamber in cold room overnight at 4°C. On the next day, the primary antibody was removed and washed with 3 changes of wash buffer for 5 minutes each. The sections were re-outlined with a hydrophobic pen and incubated with secondary antibody solutions, 100 µl/section Alexa Fluor® 647 and 488 conjugated goat anti-mouse antibodies and goat anti-rabbit antibodies, 1 in 1000 dilution with antibody diluent with 10% goat serum) for 1 hour at room temperature, in a humidity chamber in the dark. After three times washing with wash buffer for 5 minutes each, the samples were mounted with Prolong Gold with DAPI (30 µl per sample) and coverslip and the edges were sealed with nail polish and stored at 4°C. The visualization of extracellular DNA and MPO and Elastase-positive signals was achieved using Zeiss LMS700 confocal microscopy.

3.5 Data analysis

Data were analyzed using FlowJo and GraphPad Prism (GraphPad Software version 5). The detailed data presentation and analysis methods are described in each result chapter (chapters 4, 5 and 6).

4 Chapter 4: Antibody-dependent neutrophil phagocytosis of *P. falciparum*-infected erythrocytes and antigen-coated beads

4.1 Background

Plasmodium falciparum malaria remains the major public health in young children and pregnant women (260). In *P. falciparum* malaria endemic areas, protective antibodies against clinical malaria are acquired with repeated exposure to *P. falciparum* malaria (261). Despite this acquired immunity, pregnant women are highly susceptible to *P. falciparum* malaria (262). Malaria infections during pregnancy can have serious consequences for the mothers and their fetuses, mainly for primigravid women (263), which is related to the lack of previous immunity against *P. falciparum*-infected erythrocytes (IEs) that bind chondroitin sulfate A (CSA).

This susceptibility is characterized by placental sequestration of IEs which express VAR2CSA on their surface, which results in placental malaria (PM) (63). A member of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family, VAR2CSA (350kDa) contains six Duffy Binding-Like domains (DBL domains 1-6), a cysteine-rich interdomain region between DBL2X and DBL3X, and a number of other interdomain regions (264) that play a vital role in adhesion and immunogenicity of the VAR2CSA protein (265). Pregnant women who had previous exposure to malaria produce antibodies to VAR2CSA that inhibit the attachment of IEs to CSA *in vitro*, which are associated with decreased malaria-associated complications (266), suggesting that antibody to VAR2CSA plays a significant role in protecting pregnant women from the adverse effects of PM.

Antibodies to VAR2CSA are specific to pregnant women (93), and the mechanism of protection may be either blocking IE binding to CSA or opsonizing VAR2CSA expressing IEs for phagocytosis, and these antibodies have been related to reduced PM (71, 89, 99, 101), suggesting naturally acquired functional antibodies protect against PM. VAR2CSA is the leading vaccine candidate to prevent placental malaria in pregnant women, but the expression of the full-length protein is difficult (267). A suitable solution is to identify the individual DBL domain(s) that mediate the adhesion to CSA and are recognized/blocked by protective antibodies. Recombinant multi-domain ID1–DBL2 fragment bound CSA with similar affinity as the full-length protein (265). The ID1-DBL2x region has been termed the minimum binding domain (267). In addition to blocking activities, antibodies

can facilitate recruitment of innate immune cells to eradicate antibody opsonized microbes through phagocytosis (268). Measuring the capacity of antibodies to induce opsonic phagocytosis of IE may be an essential correlate of protection in vaccine development. In a recent systematic review, antibody responses to VAR2CSA were often correlated with exposure to placental malaria, rather than protection from infection, although some data supported a possible association between antibody to IE and protection from low birth weight (262).

Previous studies focused mainly on monocyte interaction with IE, and it has been reported that antibodies produced against VAR2CSA promoted the opsonic phagocytosis of IEs by monocytes and THP-1 cell lines (71, 90, 99, 101), however, similar studies showing the interaction of neutrophils and *Plasmodium* parasites are lacking. Neutrophils are the most abundant innate immune cell in the circulation and the first cell to arrive into the sites of infection where they play an important role in anti-pathogen immunity through phagocytosis, degranulation and reactive oxygen species production, and the release of extracellular traps (figure 4.1a). Despite the relative predominance of neutrophils in circulating blood, ADNP of IE has been little studied in malaria, perhaps because primary cells are generally required, and are difficult to work with.

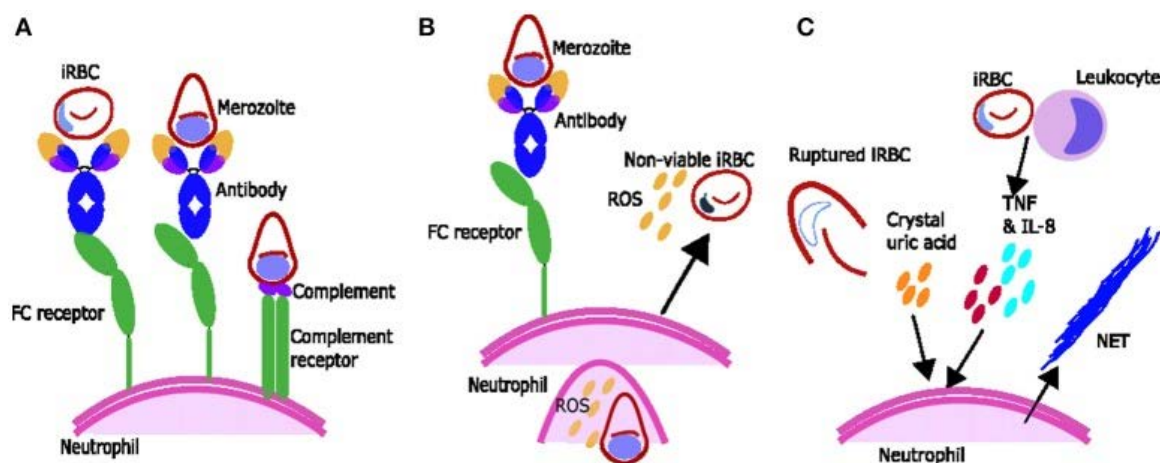


Figure 4.1a proposed mechanism of parasite clearance by neutrophils during *Plasmodium* spp. infection. (A) Neutrophils phagocytose infected red blood cells (iRBC) with the help of antibodies and probably with complement. (B) neutrophils exposed to merozoite antigen opsonised with antibody produce reactive oxygen species (ROS) which can inhibit intraerythrocytic parasite growth. (C) components associated with *Plasmodium* spp. infection including inflammatory cytokines TNF and IL-8 produced by white blood cells and crystal uric acid released up on infected red blood cells (iRBC) rupture may induce neutrophil extracellular traps (NETs). Taken from (121).

Recent studies have shown that functional characterisation of antibody responses has led to the identification of specific antibody determinants that correlate with vaccine-induced protection from HIV (102), control of latent tuberculosis infection (103), or transplacental transfer of antibody (269, 270). Antibody studies with respect to IE have generally concentrated on the amount of bound antibodies and their ability to inhibit IE binding with CSA (262), but there is growing interest in how IE-recognizing antibodies interact with innate immune cells (271).

In this study, we developed an ADNP assay to VAR2CSA recombinant proteins and IEs expressing VAR2CSA using plasma samples collected mid-pregnancy. Pregnant Papua New Guinean women participating in a trial of intermittent preventive treatment against malaria (248) were categorised as having placental malaria, non-infected, or having non-placental infection. ADNP is a novel high throughput assay developed to assess antibody mediated phagocytosis of parasites by human neutrophils, which is associated with protection from PM. ADNP is important to measure responses to CSA adhering CS2 IEs and explore how antibody to recombinant DBL domains and IE could activate neutrophils to identify innate immune cells that play important roles in parasite clearance or killing.

4.2 Hypothesis and aim of the project

4.2.1 Hypothesis: We hypothesized that high levels of opsonizing antibodies to VAR2CSA in plasma of malaria exposed pregnant women increase antibody dependent neutrophil phagocytosis (ADNP) of beads coated with recombinant DBL antigens or IEs and consequently protection from PM.

4.2.2 Aim: The aim of the current study is to measure ADNP of beads coated with recombinant DBL antigens or IEs and investigate its association with protection from malaria infection.

The specific objectives were:

4.2.2.1 To establish ADNP assays using IEs and antigen-coated beads.

4.2.2.2 To evaluate ADNP of individual recombinant DBL domains of VAR2CSA coated on beads in association with protection against placental malaria.

4.2.2.3 To evaluate ADNP of CSA-binding IE expressing VAR2CSA in association with protection against placental malaria.

4.3 Materials and methods

Two experimental approaches were used to test our hypotheses: (a) coculturing of neutrophils and IEs, and (b) incubation of neutrophils and recombinant DBL antigens. To explore donor to donor variability of neutrophil functions, three different neutrophil donors were used for the IE assay as well as for individual recombinant antigen. To minimize donor to donor variability of ADNP between the three DBL antigens (DBL2, DBL3, and DBL5) the assay was done the same day using the same neutrophil donor and repeated with different neutrophil donors three times in duplicates. In this study, IEs expressing VAR2CSA antigens and beads coated with DBL antigens were opsonized with pooled plasma samples (PPS) from Malawian malaria-infected pregnant women used as a positive control, or plasma from non-immune Melbourne control (MC) were used as a negative control.

For this ADNP assay, we optimized a 5-step protocol for beads and IEs based assays (figure 4.1). For beads, (1) isolation of human neutrophils, (2) coating of recombinant VAR2CSA proteins to fluorescent neutravidin beads, (3) incubating the beads with plasma (dilution 1:100), (4) incubating the opsonized beads with neutrophils and (5) measuring phagocytosis by flow cytometry. For IEs, (1) isolation of human neutrophils, (2) staining of purified trophozoite *P. falciparum* IEs with dihydroethidium (DHE), (3) opsonizing with plasma samples (dilution 1:10) (4) incubating the opsonized IEs with neutrophils for 1 h, and (5) measuring phagocytosis by flow cytometry.

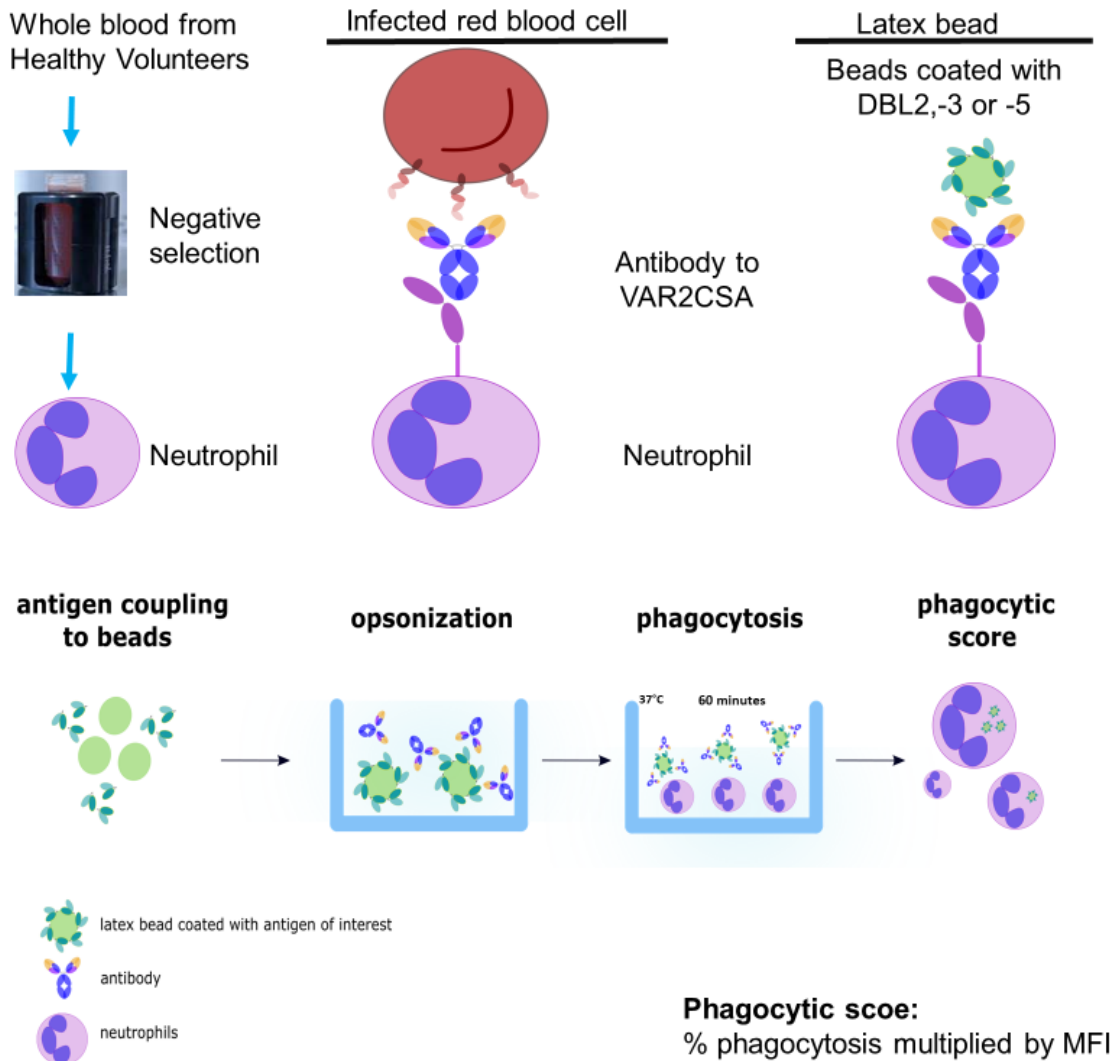


Figure 4 1 Schematic representation of ADNP (antibody-dependent neutrophil phagocytosis) assay. Whole blood collected from healthy volunteers and neutrophils were isolated using a direct neutrophil isolation kit following manufacturer's instructions. *P. falciparum* infected erythrocytes (CS2 *P. falciparum* strain express VAR2CSA in its native form) or latex beads coated with DBL-VAR2CSA single domains (DBL2, DBL3 or DBL5) were opsonised with (heat-inactivated) plasma. IgGs bind if domains are recognized. Next, opsonised IEs or beads were cocultured with human neutrophils isolated from healthy donors and left for phagocytosis at 37°C. Cells were fixed and analyzed by flow cytometry and data were analyzed using FlowJo. Phagocytosis (arbitrary units) was defined as the percentage (%) of beads/IEs positive events while the phagocytic score (arbitrary units) was calculated as: ((% of events positive for beads/IE) * (the geometric mean fluorescent intensity of the % of events positive for AF488)) or * (the median of fluorescent intensity of the % of events positive for DHE)).

4.3.1 Ethics, study site, and study populations

Ethical approval was obtained from the PNG Institute of Medical Research (IMR) Institutional Review Board, PNG Medical Research Advisory Committee and Melbourne Health Human Research Ethics Committee, Australia (248, 249). Groups were frequency-matched for age, gravidity, intermittent preventive treatment in pregnancy (IPTP) regime, rurality and bed net use as described in detail in **section 3.2.2**.

4.3.2 Parasite culture and preparations

Parasite cultivation was done as described in **section 3.2.6.1**, and mature trophozoite IEs were purified by Percoll gradient as explained in **section 3.2.6.4**.

4.3.3 Isolation of neutrophils

Human neutrophils were isolated from whole blood collected from healthy donors using EasySep direct human neutrophil isolation kit as described in **section 3.2.7.2**

4.3.4 ADNP assays

ADNP described in this study was done as previously described: for bead-based assay (97, 103, 272) and ADNP towards native VAR2CSA-expressing IE was done according to (99, 101) with modifications.

4.3.4.1 ADNP assay to recombinant VAR2CSA antigen

Antigen biotinylation and coating of beads with VAR2CSA proteins was done as described in the general methods of the thesis in **section 3.2.8.2.1 and 3.2.8.2.2**. Bead-based ADNP assays were done as described in **section 3.2.8.2.3**. In brief, recombinant VAR2CSA proteins were coupled with fluorescent neutravidin beads. Pooled plasma samples (PPS or MC) were serially diluted from 1:25 to 1:3,200 using high salt PBS (1% BSA /0.5 M NaCl in PBS). Then, DBL2 or DBL3-coated latex beads (bead: protein ratio of 1: 6) were opsonized for 1 h at RT. Opsonized beads were incubated with human neutrophils (bead: neutrophil ratio of 40:1). Next, we calculated the dilution of plasma at which the phagocytic score is half its maximal (Ec50). The activity of neutrophil was measured as the % phagocytosis and phagocytic score and the lowest dilution (1:25) was scored to 100. The Ec50 value corresponded to the log of the plasma dilution: -2.2, -2.4 which was at

approximately a 1:200 dilution of plasma, that is the dilution used in the rest of the experiments.

4.3.4.2 ADNP assay towards IEs expressing VAR2CSA antigen

For IE-based ADNP assays, mature Percoll-purified trophozoite *P. falciparum* IE were stained with dihydroethidium (DHE, 25 µg/ml), resuspended at 1.65×10^7 /ml, and opsonized with plasma samples (diluted 1:10) and cocultured with neutrophils for 1 h as described in section 3.2.8.1 in the general methods of the thesis. The ADNP of **IEs expressing VAR2CSA antigens** opsonized with plasma samples collected from PNG pregnant women with (n = 27) or without (n = 50) placental malaria and noninfected pregnant women (n= 50) were analyzed using flow cytometry (Beckman Coulter, CytoFLEX S) and data were analyzed by FlowJo, gating for neutrophils and determining the percentage of DHE positive cells.

To estimate the phagocytotic score for bead based ADNP, the percent neutrophils that had taken up beads (cells taking opsonized beads coated with the individual DBL domains of VAR2CSA) were converted into relative phagocytosis using the following equation: $100 * (\% \text{ phagocytosis of the sample}) / (\% \text{ phagocytosis of PPS (mean of PPS on different test plates)})$. Similarly, the fluorescent signal of beads that were taken up by the neutrophils was also normalized into relative geometric median fluorescent intensity (gMFI) using the following equation: $1,000,000 * (\text{gMFI of the sample}) / (\text{gMFI of PPS (mean)})$. Then, the phagocytic score was calculated by multiplying relative % phagocytosis (% of events positive for AF488) by the relative Gmfi (% of events positive for AF488) and divided by 100.

To estimate the phagocytotic score for IE based ADNP, the percent neutrophils that had taken up IEs (cells taking opsonized IEs) were converted into relative phagocytosis using the following equation: $100 * (\% \text{ phagocytosis of the sample}) / (\% \text{ phagocytosis of PPS (mean of PPS on different test plates)})$. Similarly, the fluorescent signal of IEs that were taken up by the neutrophils was also normalized into relative median fluorescent intensity (MFI) using the following equation: $1,000,000 * (\text{MFI of the sample}) / (\text{MFI of PPS (mean)})$. The phagocytic score was calculated by multiplying relative % phagocytosis by the relative MFI and divided by 100.

4.3.5 Gating strategy for ADNP assay

The gating strategy was implemented using unstained and single colour controls. As illustrated in Figure 4.1, neutrophils were gated using forward (FSC-A) and side scatter (SSC-A) and doublets were excluded by SSC-H and FSC-A. The purity of isolated neutrophils was determined using a neutrophil-specific marker (CD66b). By applying this gating strategy on the test sample, uptake of beads or IEs by neutrophils was quantified by measuring the percentage and (g)MFI of FITC+ or DHE+ cells within the neutrophil population. Opsonizing antibodies were expressed as the percentage of neutrophils that were FITC or DHE positive, and the data were analyzed using FlowJo. Figure 4.2 illustrates the sample gating strategy used for ADNP assay (bead-based), and DHE+ neutrophils were gated in a similar way.

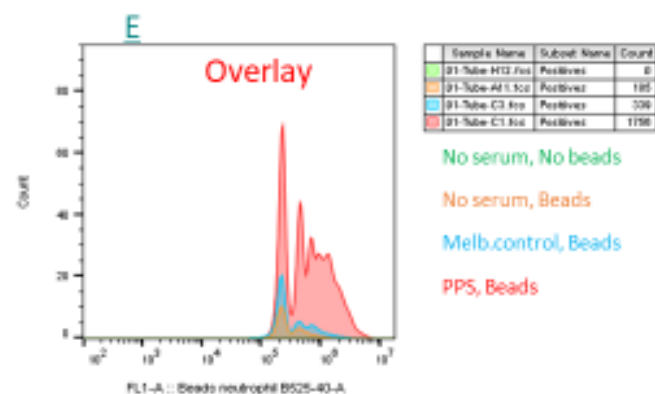
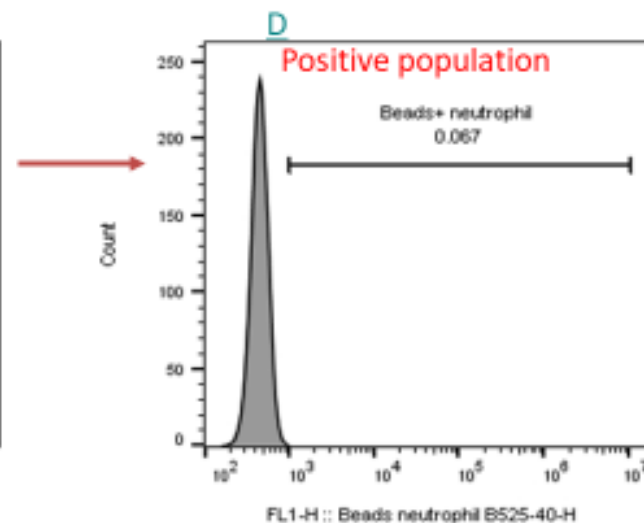
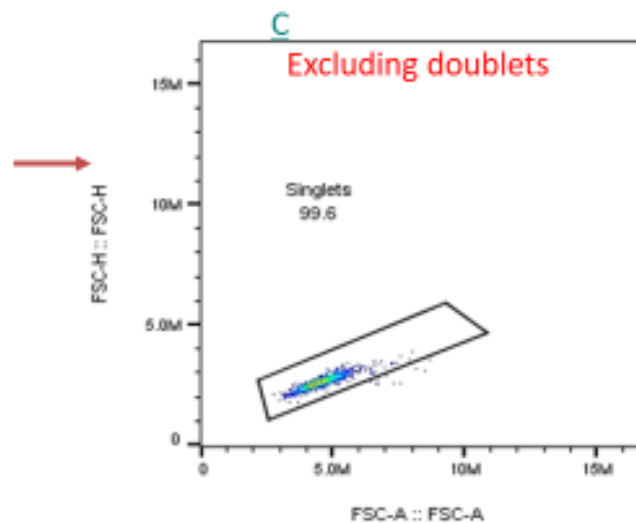
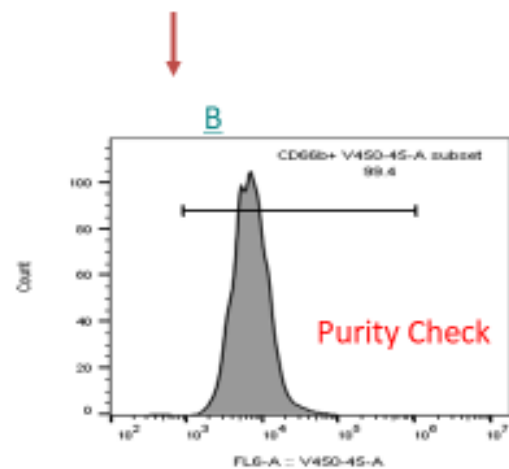
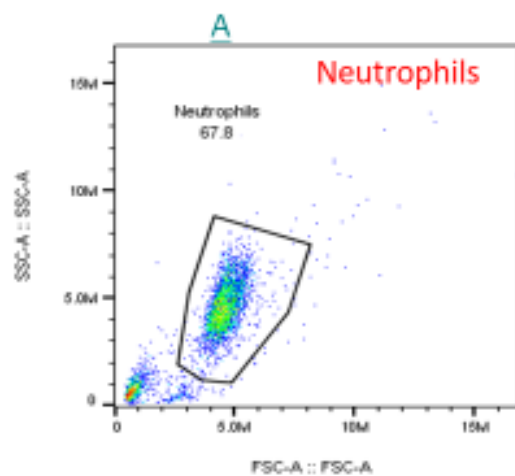


Figure 4 2 Neutrophils gating strategy. Schematic representation and gating strategy used for antigen-coated beads in the antibody-dependent neutrophil phagocytosis assay. Beads coated with recombinant VAR2CSA protein (DBL3 in this example) were opsonized with pooled plasma. Neutrophils were cocultured with opsonized beads and were fixed, and stained for CD66b marker. Cells were analyzed by flow cytometry and data were analyzed using FlowJo. Neutrophils were gated based on SSC-A and FSC-A (A) and the neutrophil purity was determined using a neutrophil-specific marker, CD66b-BV421 (B). To identify a single population, doublets were excluded by SSC-H and FSC-A (C). Uptake of beads by neutrophils was quantified by measuring the percentage and gMFI (geometric mean fluorescence intensity) of AlexaFluor 488-positive cells within the neutrophil population (D), and expressed in percent phagocytosis and phagocytic score. Phagocytic score was calculated by the percent neutrophils that had taken up beads multiplied by the fluorescent signal of beads taken up by the neutrophils (gMFI) of bead-positive neutrophils. In the overlay histogram (E), neutrophils alone are depicted (green), neutrophils incubated with unopsonized beads (orange), neutrophils incubated with beads opsonized with Melbourne control (blue) and neutrophils incubated with beads opsonized with the positive control (red).

4.3.6 Statistical analysis

In GraphPad Prism 5, A Mann-Whitney test was used to compare percent phagocytosis and phagocytic score between the groups. Donor to donor (neutrophils) variation of ADNP scores was compared by the non-parametric Spearman's rank correlation, using a two-tailed p -value. Percent phagocytosis and phagocytic score (median with interquartile range, IQR) to 3 different recombinant DBL domains (DBL2, 3, and 5) were compared using Kruskal-Wallis one-way analysis of variance (ANOVA) (Dunn's multiple comparison test) with significance considered as $p < 0.05$.

4.3.7 Optimization results:

4.3.7.1 *Human neutrophil isolation from whole blood*

In the current study, neutrophils were isolated from human healthy blood donors as described in the general thesis methods **section 3.2.7**. Two isolation methods, Ficoll and dextran gradients and EasySep direct human neutrophil isolation kit, were optimized as detailed in **section 3.2.7.1 and 3.2.7.2** respectively. The Ficoll and dextran gradients purification protocol produced $> 90\%$ cell purity with the viability of 95%. The viability of cells was assessed by the trypan blue exclusion test, and the cell purity was determined by conventional Giemsa microscopy. In addition, the purity of neutrophils was confirmed by CD66b staining (90.3% purity). In this isolation method, lymphocytes and monocytes were the identified contaminants, and some morphological changes of neutrophils like nuclear round up or releasing its nuclear contents were observed by conventional Giemsa microscopy, suggesting neutrophils may be activated by the purification (reagents or the centrifugation) steps during the isolation process, which may affect some functions of neutrophils.

Next, EasySep direct human neutrophil isolation kit was used following the manufacturer's instructions with modification as described in **section 3.2.7.2**. This isolation kit produced $\geq 98\%$ purity (microscopy, figure 4.3) and viability (trypan blue exclusion test). Furthermore, the purity of neutrophils in this method was confirmed by CD66b (conjugated with BV421) staining ($\geq 99\%$ purity). A lesser amount of nuclear round up or releasing its nuclear contents from neutrophils isolated by this isolation kit was observed compared to

the aforementioned method. Thus, neutrophils isolated by EasySep direct human neutrophil isolation kit were used for all assays done in this thesis.



Figure 4 3 Morphological appearances and purity of human neutrophils. After isolation as described in the methods section, cells were smeared onto glass slides. Slides were stained with Giemsa and photographed under visible light microscopy. Neutrophils were identified based on their segmented nucleus and granular staining.

4.3.7.2 Optimization of time points and purified IgG concentration for ADNP assay

To determine the optimum incubation time and IgG concentration for ADNP assay, freshly isolated human neutrophils were incubated in a 96 well-plate with IgG-opsonised DBL3 coated beads for 1 h and 2 h at 37°C, 5% CO₂, and ADNP was measured by flow cytometry. DBL3 coated beads were opsonized with purified IgG pooled from Malawian malaria-infected pregnant women (PPS), or non-immune Melbourne control (MC). The optimization results demonstrated that incubation of neutrophils with IgG-opsonized DBL3 coated beads for 1 h was the optimum time point (**A**) whereas when neutrophils were incubated for 2 h a decrease in % phagocytosis and an increase in background phagocytosis was observed (**B**), suggesting that prolonged exposure might lead exhaustion of neutrophils, which then stop doing their function properly (lose phagocytic function), figure 4.4.

The titration of purified pooled IgG (from 8 positive or negative control plasmas) was from 0.5 µg/ml to 0.0156 µg /ml in PBS. This optimization result showed that % phagocytosis increased with increasing concentration of purified IgG dilution for both time points (figure 4.4 **A and B**); confirming a relationship between the level of opsonizing IgG against VAR2CSA and phagocytic activity. The optimum IgG concentration was 0.125 µg/ml (**A, B**) for ADNP assay.

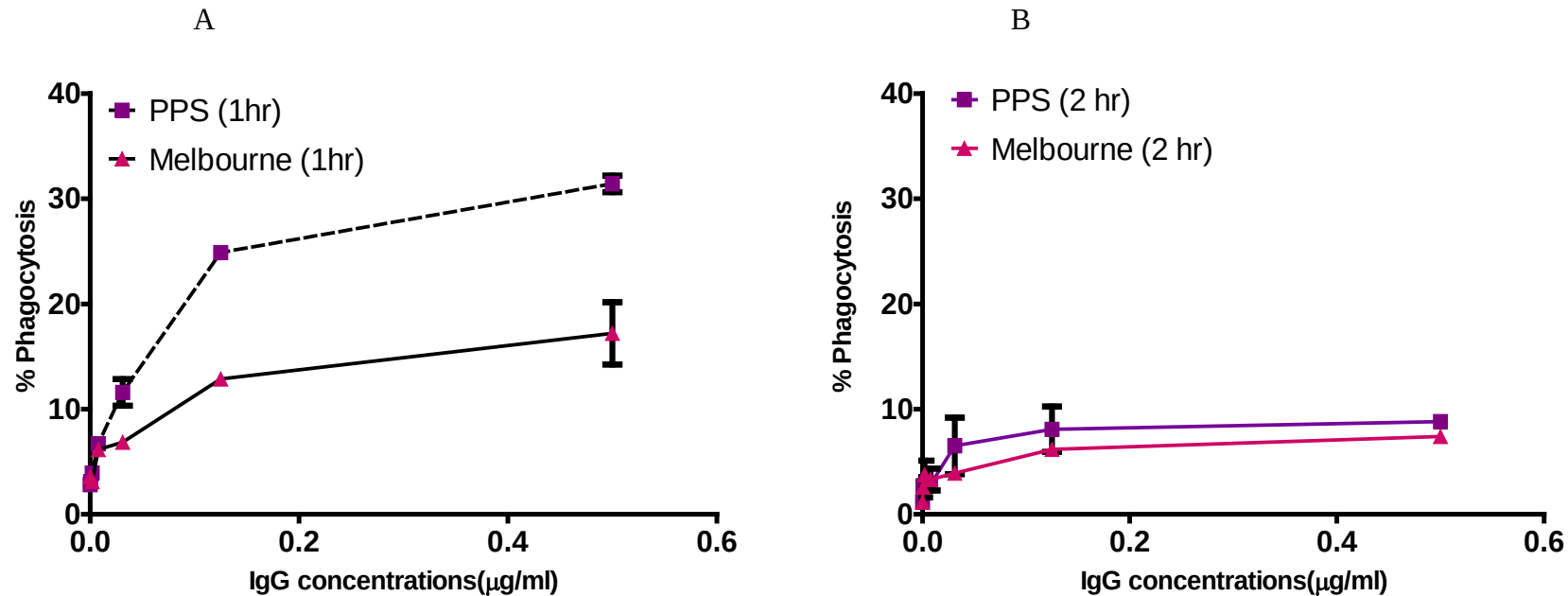


Figure 4 4 Determining time points and purified IgG concentration for ADNP assay. Levels of opsonizing IgG to DBL antigen-coated beads and incubation time points using pooled purified IgG from Malawian malaria-infected women and Melbourne healthy control. This graph is showing the percentage of phagocytosis (Y-axis) and IgG concentrations (X-axis) at different time points (A and B). Neutrophils and IgG opsonized DBL antigen-coated beads were cocultured for 1 h (A) and 2 h (B). The rectangular pink square symbol is malaria-infected pooled purified IgG (PPS) and the triangular red symbol is purified IgG from pooled Melbourne controls (MC). The subsequent concentration of purified IgG was done serially in two-fold dilution.

4.3.7.3 Optimization of the volume or number of neutrophils, beads, and purified IgG for ADNP assay

To determine the effect of reaction volume on ADNP, different volumes of neutrophils, opsonizing IgGs and VAR2CSA antigen-coated beads were used, because to process many samples in the same run, a large volume of blood (15-25 ml) from healthy neutrophil donors may be required. We then investigated whether ADNP is affected by volume or number of neutrophils, beads and opsonizing antibodies. The ratio of neutrophils (50 μ l, 5×10^4 /well) and IgG opsonized beads (10 μ l, 2×10^6 /well) was used as a reference point. In this optimization, we reduced the number of neutrophils in suspension to 30 μ l, 3×10^4 /well, from 50 μ l, 5×10^4 /well, and proportionally we reduced the volume of beads (from 10 μ l down to 6 μ l, 1.2×10^6 /well) and opsonizing antibodies (from 20 μ l, down to 12 μ l). The result of this optimization experiment showed that ADNP is not affected by the volume/number of neutrophils, beads and opsonizing antibodies if the ratio of all is proportionally adjusted (figure 4.5). In this optimization experiment, the titration of purified pooled IgG was from 0.25 μ g/ml to 0.0078 μ g /ml using PBS. Similar to previous optimization results (figure 4.4), the % phagocytosis was increased with decreasing purified IgG dilution, and the optimum IgG concentration was 0.125 μ g/ml (A, B) for ADNP assay (figure 4.5).

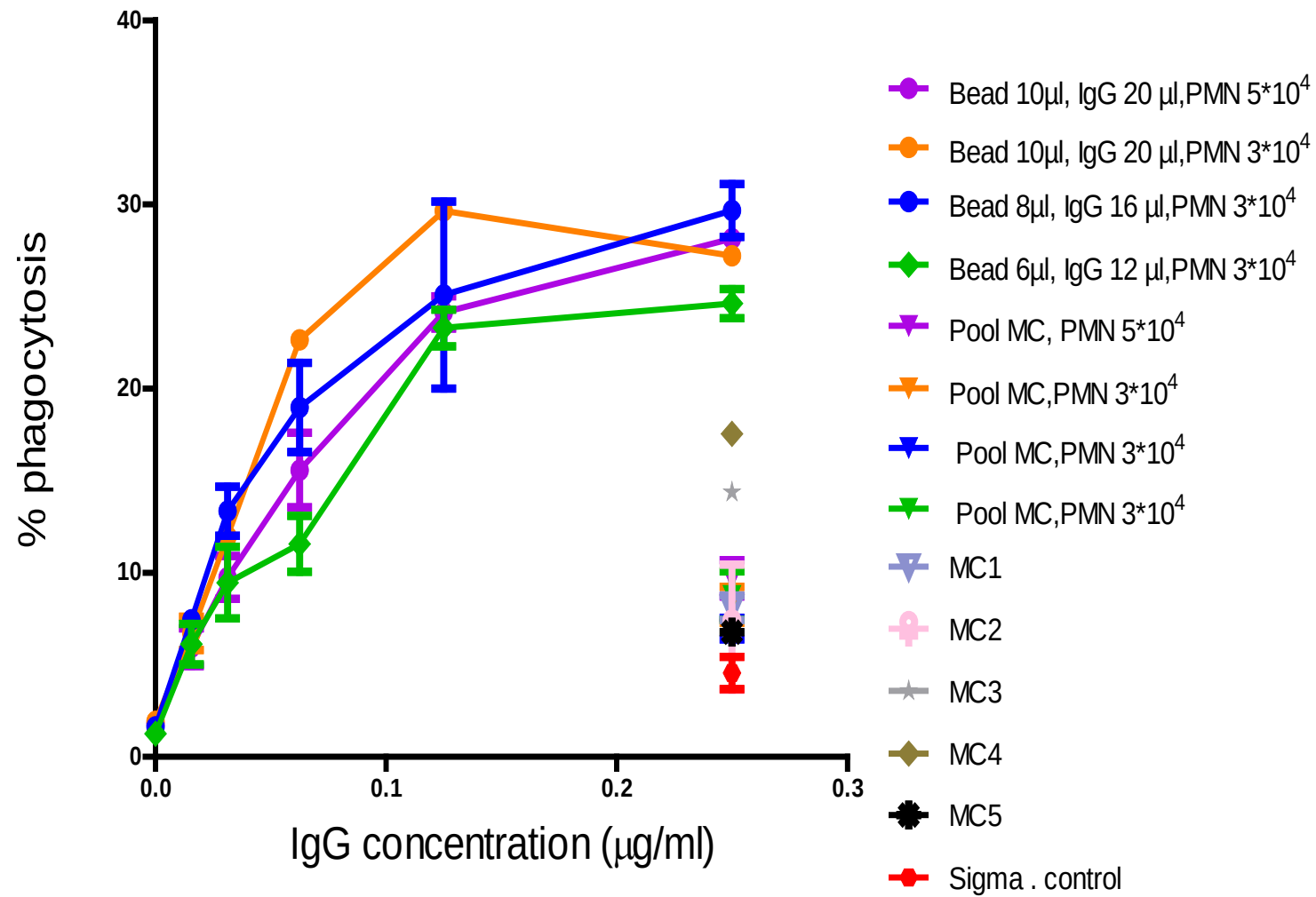


Figure 4 5 Determining the volume of neutrophils, beads and purified IgG for ADNP assay. This graph is showing the percent of neutrophils that take up one or more beads coated with DBL antigen with different volumes of neutrophils, beads, and opsonizing pooled purified IgG. DBL antigen-coated beads were opsonized with pooled IgG from malaria-infected women (PPS) or from healthy Melbourne controls (MC) or individual MC IgG (M1-M5) and commercial control from sigma. Neutrophils and IgG opsonized beads were incubated for 1 h. The Y-axis shows percent phagocytosis of beads by neutrophils and X-axis is the concentrations of purified IgG. Each point represents the mean $\pm$ SEM of duplicate wells.

4.3.7.4 Variation of ADNP between DBL antigens and neutrophil donors

The rationale for assessing variation of ADNP between neutrophil donors is important to understand the variation in phagocytic clearance of neutrophils among individuals. And also, it is important to evaluate the reproducibility of the assay test results. We sought to evaluate the ADNP of DBL antigen-coated beads among neutrophil donors. The aims of this experiment were (1) to compare the level of ADNP between domains, (2) to compare ADNP between neutrophil donors at different IgG concentrations.

Both DBL1 and DBL3 antigen-coated beads were opsonized with pooled purified IgG (PPS or MC) and cocultured with neutrophils isolated from two healthy donors, and ADNP assay was done on the same day following the same conditions. Figure 4.6 demonstrated a variation of ADNP between neutrophil donors. The result of this assay showed that the trend of ADNP for both DBL antigen-coated beads seems the same (Figure 4.6 A, B). Interestingly, we observed a donor to donor variability. ADNP was increased with increasing antibody concentration for donor 2 (**B**), but a sharp decrease was observed when the IgG concentration is beyond a certain limit (0.125 µg/ml) in donor number 1 (**A**), suggesting a 0.125 µg/ml of dilution of purified IgG is the optimal dilution.

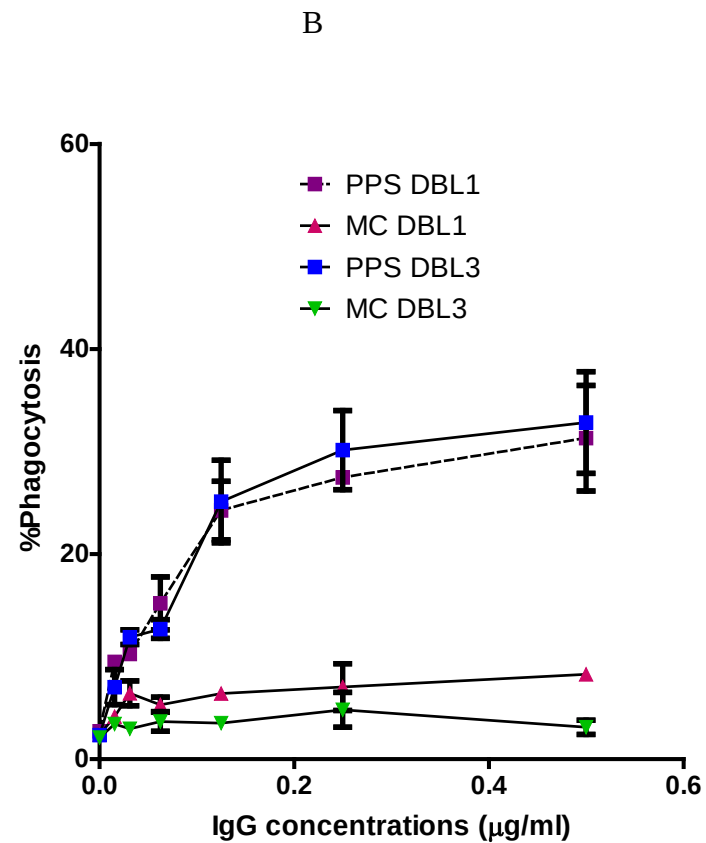
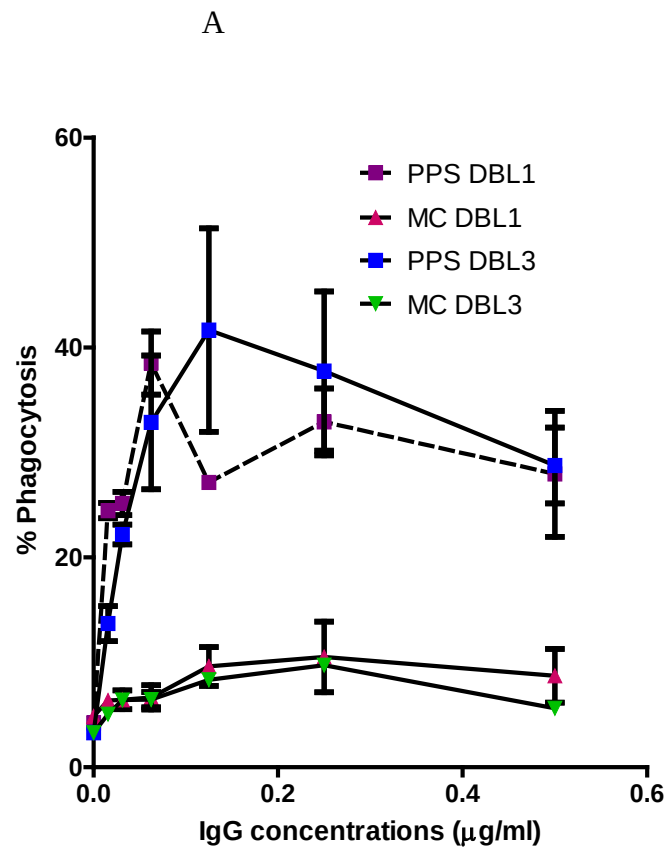


Figure 4 6 Variation of ADNP between DBL antigens and neutrophil donors. The line graph is showing the ADNP of DBL1 (PPS = pink square, MC = red triangle) and DBL3 (PPS = blue square, MC = green triangle) coated beads between neutrophil donors (A and B). Neutrophils and DBL1 or DBL3 coated beads opsonized with pooled purified IgG were incubated for 1h. The Y-axis is showing the % phagocytosis and the X-axis is the concentration of purified IgG.

4.3.7.5 Comparison of ADNP between plasma and purified IgG samples

We next sought to compare the opsonizing ability of plasma antibody and IgG purified from the same plasma samples. When the plasma sample was used, a clear difference was observed between ADNP of PPS and MC, however, beads opsonized with MC purified IgG were phagocytosed as high as the PPS (Figure 4.7 **A**). In addition, ADNP of protein coated beads was also compared between 10 randomly selected plasma antibodies and IgGs purified from the same samples, and the same finding was observed (Figure 4.7 **B**).

Although our aim was to optimize ADNP using purified IgG, because of inconsistent recognition of beads by purified IgG from MC we decided to optimize the assay protocol using plasma samples. Further studies may be needed to investigate the reason why a high level of phagocytosis was observed from MC covered beads. These inconsistent results were also observed in other antibody-dependent phagocytosis assays performed using primary monocytes and THP-1 cell lines (unpublished data in Rogerson's lab).

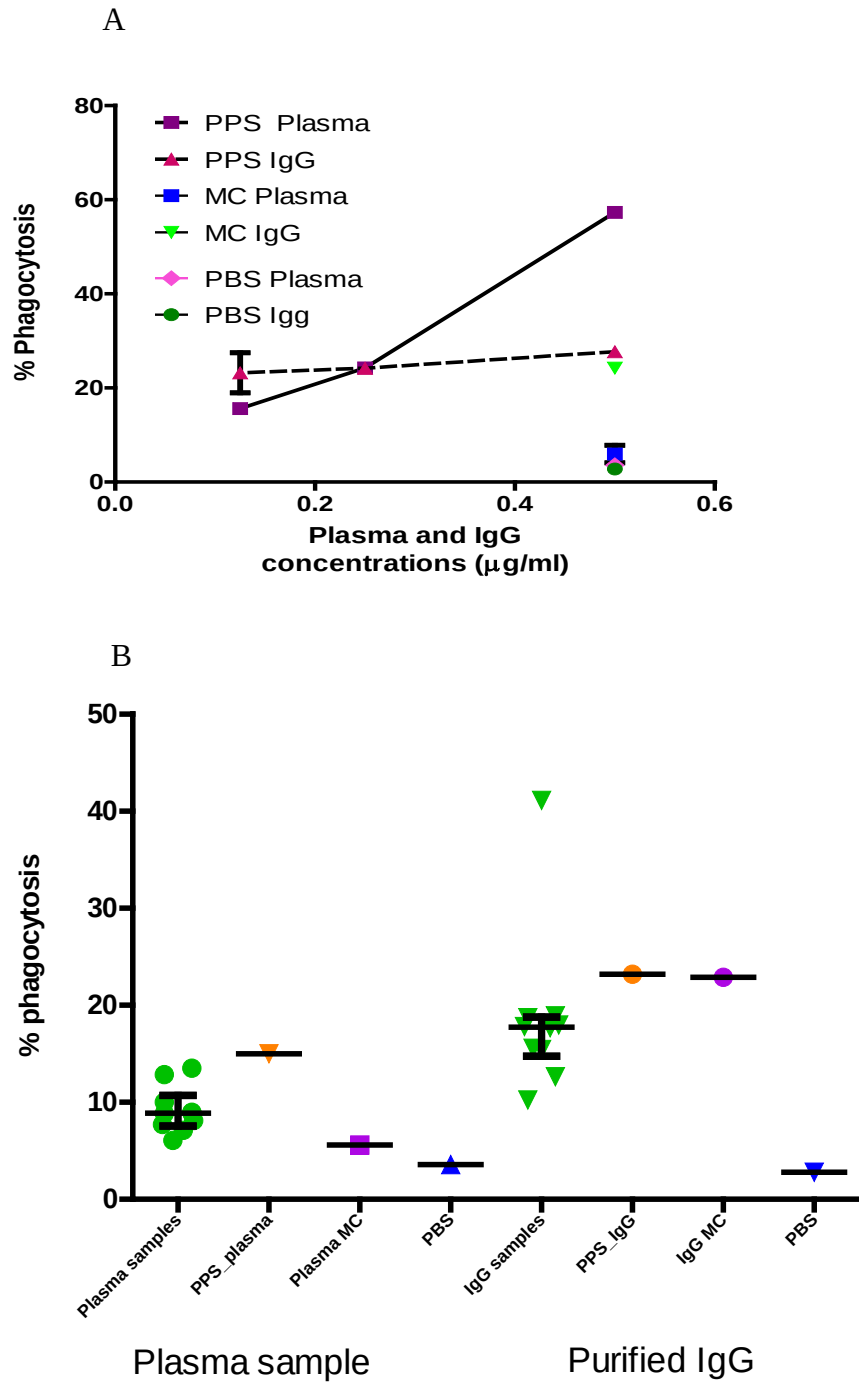
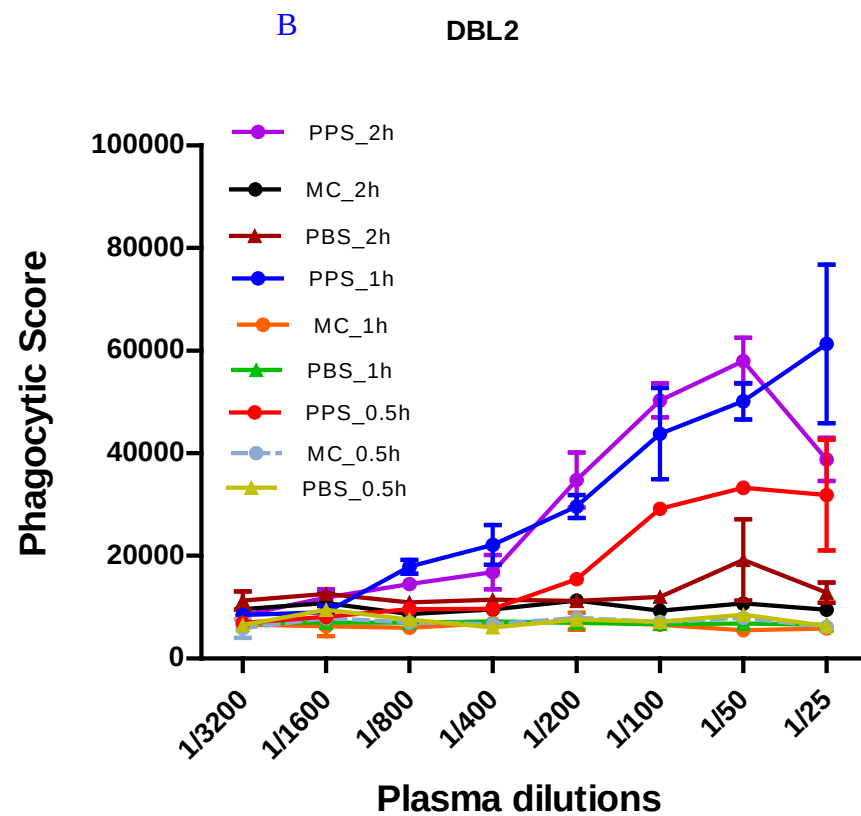
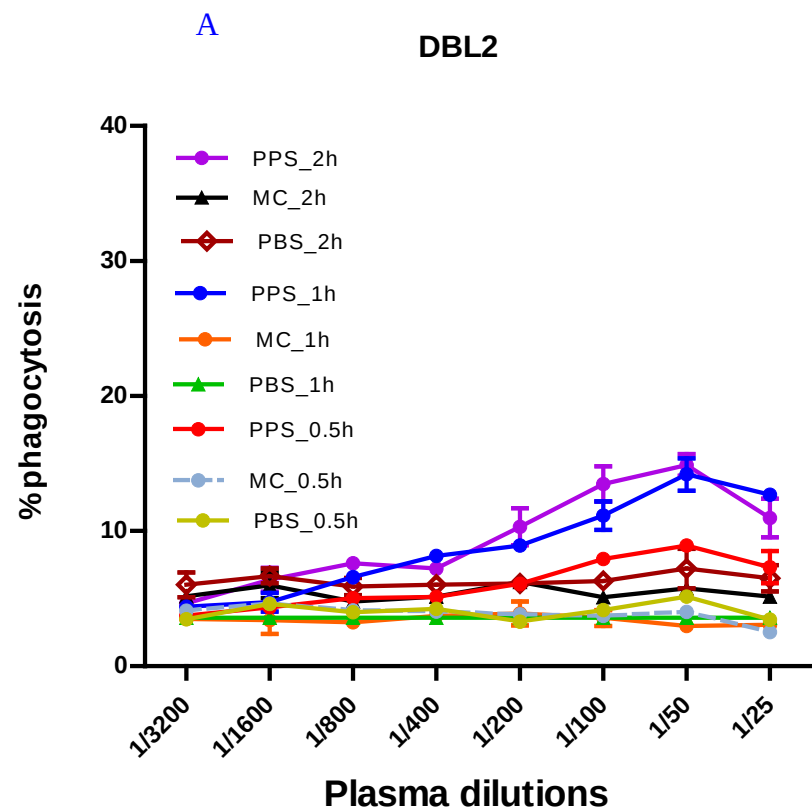


Figure 4 7 Comparison of ADNP between plasma and purified IgG opsonized beads. Representative graphs show comparisons of percent phagocytosis of beads coated to DBL antigen opsonized with pooled plasma or purified IgG samples from the same sample (figure 4.5A) and randomly selected plasma samples or purified IgG from the same sample (N =10 each) (figure 4.5B). Positive (PPS) and melbourne controls (MC) were pooled from each group of samples. Neutrophils and opsonized beads were incubated for 1 h. Percent of phagocytosis was measured by flow cytometry. The Y-axis is showing the % phagocytosis and the X-axis is the concentration of plasma or purified IgG.

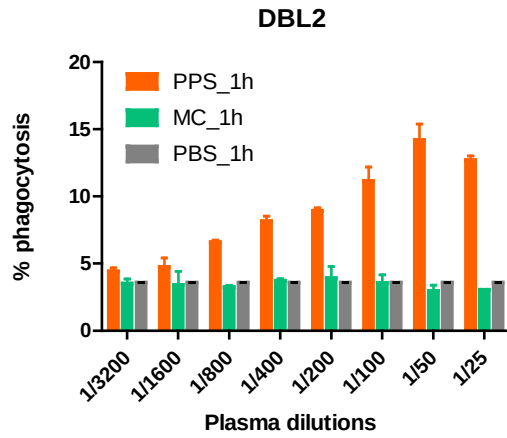
4.3.7.6 Optimization of plasma dilution and incubation time points for ADNP assay using antigen-coated beads

The initial step in the ADNP assay optimization was to determine the optimal time points and dilution of plasma to use. To determine the optimal amount of time for efficient bead uptake by neutrophils in the ADNP assays, DBL2 coated beads were opsonized with PPS or MC. To do this, neutrophils (5×10^4 /well, 50 μ l) and opsonized beads coated with antigen (2×10^6 , 10 μ l) were cocultured at different time points (0.5, 1 and 2 h) at 37°C, 5% CO₂, and ADNP was measured by flow cytometry. Both % phagocytosis and phagocytic score were calculated in this work. Beads uptake was observed as early as 0.5 h incubation and increased with increasing incubation times. The highest % phagocytosis and phagocytic score were observed at 1 and 2 h incubation, however, the background signal was higher at 2 h compared to 1 h incubation time point (figure 4.8A and B), suggesting that incubation of neutrophils and opsonized beads for 1h is optimal for ADNP assay. Thus, we selected a 1 h incubation time point for all further assays.

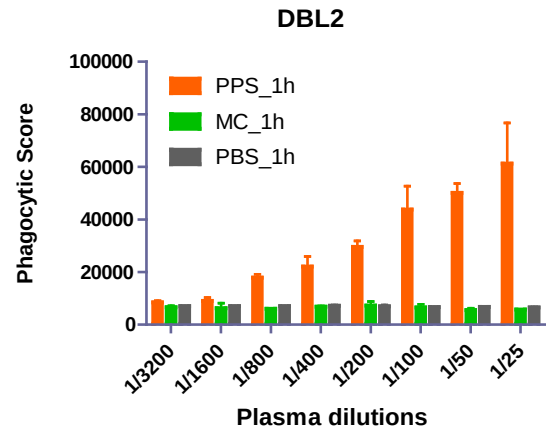
Next, we determined the influence of plasma sample dilutions to use, at 1 h incubation time points for both DBL antigens coated beads. The titration of PPS or MC was from 1:25 to 1:3,200 using high salt PBS (1% BSA /0.5 M NaCl in PBS). The result of this study showed that the ADNP of VAR2CSA-coupled beads declined with increasing plasma dilution for both domains (figure 4.8 C-F); confirming a relationship between the level of antibodies against VAR2CSA in plasma and phagocytic activity. Then, EC50 was determined. In this study, EC50 is defined as the concentration of the antibody that gives a half-maximal response. Although the highest percent phagocytosis and phagocytic score were observed at 1:25 and 1:50 dilutions, the EC50 for both DBL2 and DBL3 (figure 4.8 G-J) were at approximately a 1:200 dilution of plasma (between -2.2 to -2.4). However, to ensure robust results, throughout all experiments a slightly higher dilution of plasma (1:100) was selected for all further experiments in the current study. In summary, the optimum time points and the best plasma dilution for ADNP assay in the current study were 60 minutes and 1:100, respectively.



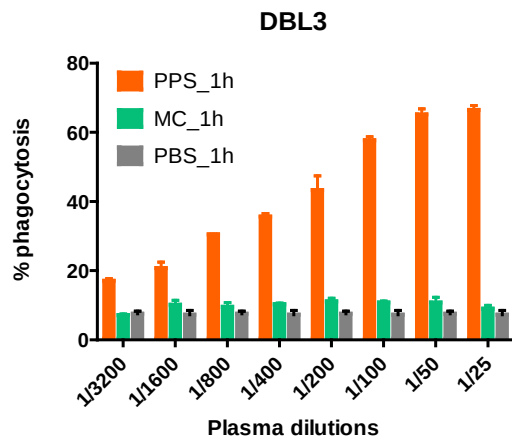
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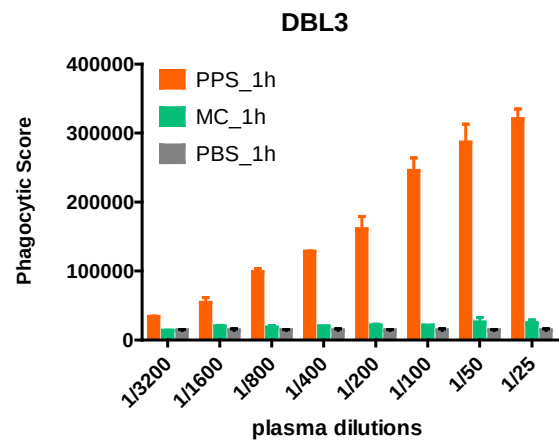
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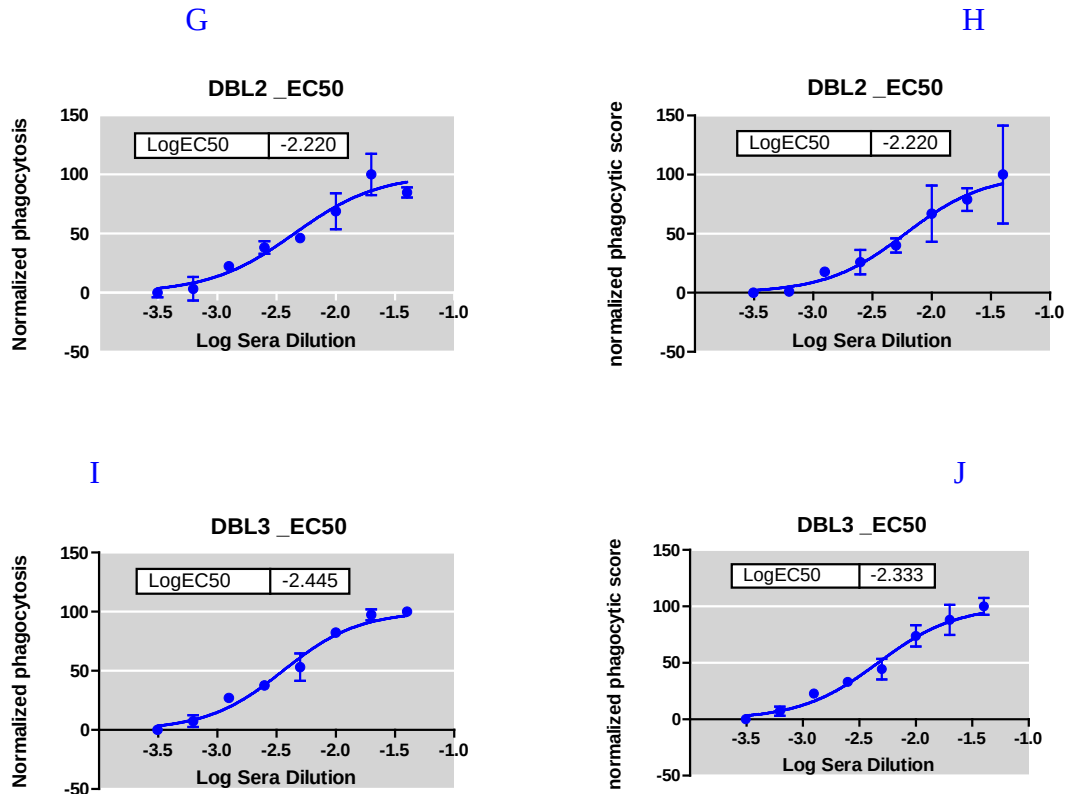


Figure 4 8 Optimization of plasma dilution and incubation time points for ADNP assay. DBL2 and DBL3 coated beads were opsonized with plasma antibody pooled from malaria-infected Malawian women (positive for each domain, PPS), or non-immune Melbourne control (MC). The pooled plasma samples were 2-fold serially diluted. Neutrophils and IgG opsonized DBL2 or DBL3 coated beads were cocultured, and ADNP was measured by flow cytometry. (A, B) ADNP at time points (0.5, 1 and 2 h) utilizing different concentrations of PPS or MC and no serum (PBS). (C-F) plasma dilution utilizing different concentrations PPS or MC and PBS at 1 h incubation. (G-J) The EC50 calculation was based on phagocytic score and % phagocytosis for DBL2 and DBL3 at 1 h incubation. The Y-axis is showing the % phagocytosis and phagocytic score whereas the X-axis is plasma dilutions.

4.4 Results

Previously, studies demonstrated that over consecutive pregnancies women acquire antibodies to VAR2CSA (273), and antibodies against CSA binding IE have been associated with protection (274). The aim of the current study was to identify protective antibodies against PM. We hypothesized that high levels of opsonizing antibodies promoted ADNP and were associated with protection from PM. To investigate the potential role of antibodies in directing neutrophil activity against VAR2CSA antigens, ADNP assay was done using freshly isolated neutrophils.

4.4.1 ADNP towards recombinant VAR2CSA antigen-coated beads

During malaria in pregnancy, VAR2CSA is the target of protective immunity. It has been reported that antibodies generated by ID1-DBL2X (275) and DBL3 and DBL5 (90) domains of VAR2CSA efficiently block IE adhesion to CSA in a similar manner to antibodies induced by the full-length VAR2CSA (275), or efficiently prompted opsonic phagocytosis of IEs by human monocytes (90), suggesting DBL2, DBL3 and DBL5 are the shortest fragments of VAR2CSA carrying major protective epitopes able to elicit inhibitory and opsonizing antibodies (90, 275). The use of full-length VAR2CSA to identify antibodies associated with protection from PM is challenging because of its large size, thus, it is important to test one or more individual recombinant DBL antigens.

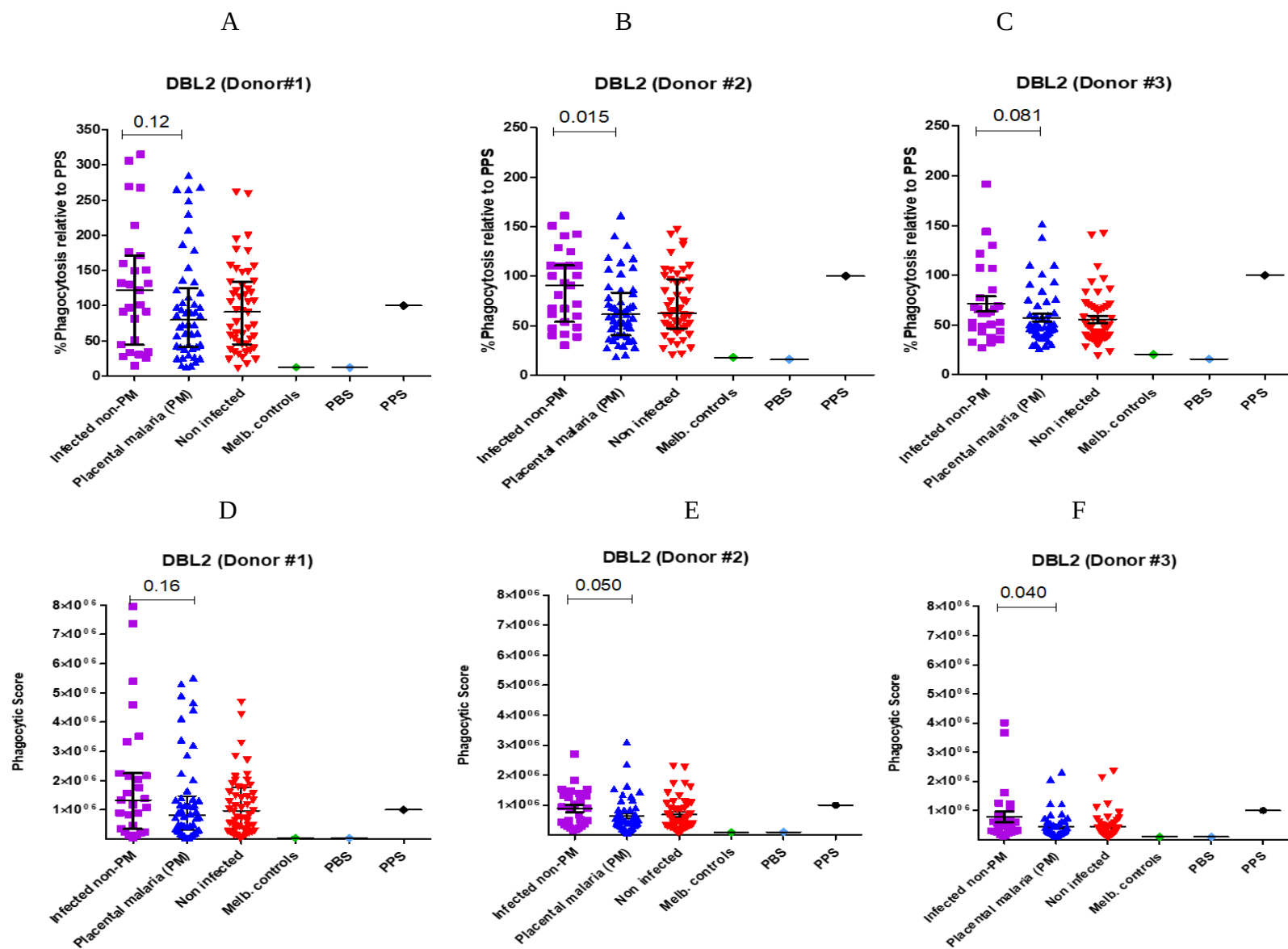
In the present study, ADNP was tested for three recombinant domains of VAR2CSA, namely DBL2, DBL3, and DBL5. These three DBL domains were selected based on the preliminary assessment of recognition of DBL domains (DBL1- 6) by FcRs (done by Timon Damelang, in Rogerson Lab, unpublished data), suggesting DBL2, DBL3, and DBL5 are the main targets of antibodies. In addition, it has been demonstrated previously that DBL2, DBL3, and DBL5 were the main targets of opsonizing antibodies from multigravida women (100). Recombinant DBL antigen-coated beads were opsonized with plasma samples from malaria-infected and non-infected pregnant women (N =127: PM =50, NPM = 27 and uninfected =50). All plasma samples were diluted (1:100) with high salt PBS. Each recombinant VAR2CSA domain was tested three times using different neutrophil donors (n = 3). The same neutrophil donor was used to test the 3 DBL antigens in the same set of the experiment in one day to limit variations due to donor variability. The

percent phagocytosis and phagocytic score were measured for each donor and compared between groups.

4.4.1.1 ADNP towards DBL2 recombinant antigen-coated beads

DBL2 protein-coated, antibody opsonized beads were incubated with neutrophils for 1 h. As demonstrated in figure 4.9, the % phagocytosis and phagocytic score didn't differ between the NPM and PM groups for neutrophil donor 1 (**A and D**). For neutrophil donor 2, % phagocytosis (**B**) ($p = 0.015$) was significantly higher in NPM compared to PM, and phagocytic score (**E**) was still within the 95% confidence interval. For the third neutrophil donor, we observed that phagocytic score (**F**) was significantly associated with protection from PM ($p = 0.040$) and % phagocytosis (**C**) was still within the 95% confidence interval. Considering the average of the three neutrophil donors, ADNP was significantly higher in NPM than PM ($p = 0.033$, for % phagocytosis and $p = 0.045$, for phagocytic score) (figure 4.9 **G and H**). Both % phagocytosis and the phagocytic score were correlated between donors ($n = 3$) (Figure 4.10**A-F**). The correlation of ADNP of DBLs between neutrophil donors indicates that neutrophils phagocytose DBL antigens with minimal inter-individual variation and outstanding reproducibility, suggesting that the assay effectively measured differences in neutrophil phagocytosis DBLs among individuals. Thus, researchers can use a single neutrophil donor to measure ADNP of DBL antigens.

Another interesting finding in this study was that the opsonizing activities of most plasma samples in this cohort from PNG were higher than the Malawian PPS (figure 4.9).



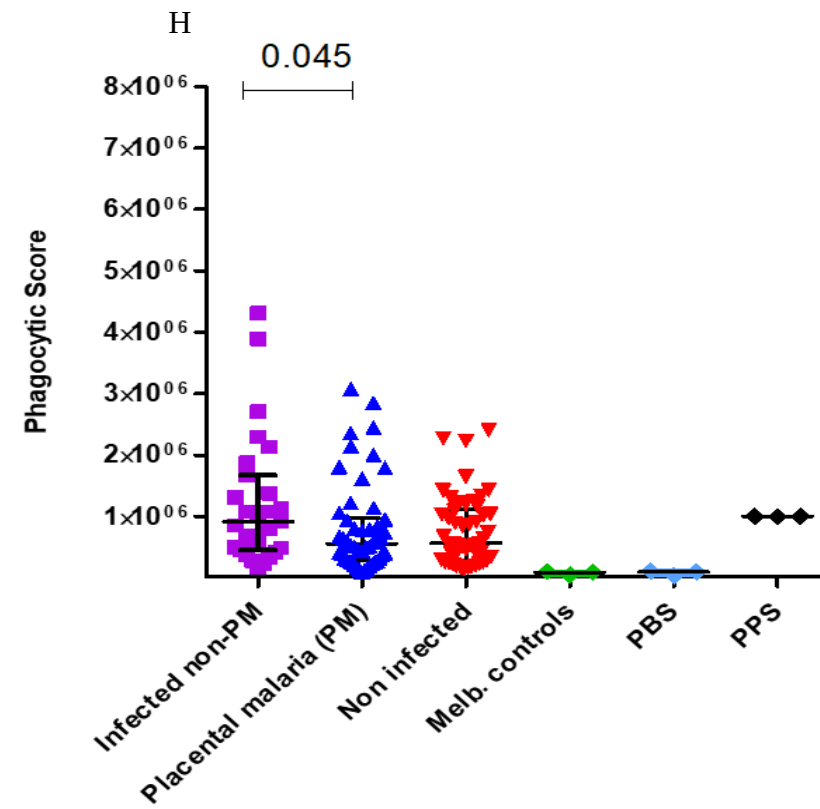
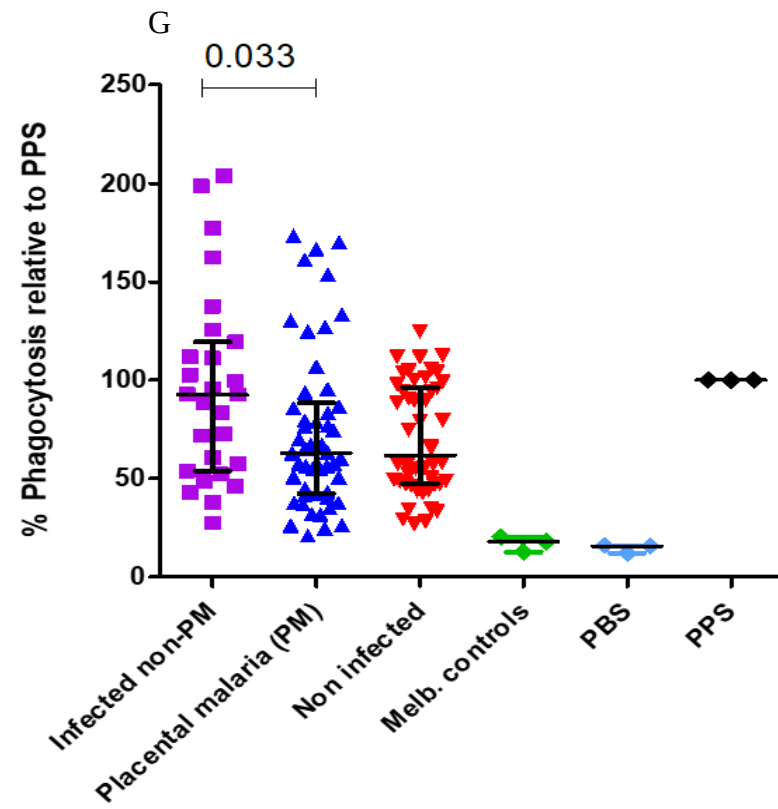


Figure 4 9 Antibody-dependent neutrophil phagocytosis using VAR2CSA DBL2-coated neutravidin beads. Neutrophils were purified from 3 donors by negative selection. Purity was >98% using light microscopy of giemsa stained slides and CD66b staining. DBL2-coated neutravidin beads were opsonised with individual plasma from pregnant women infected with malaria but not placental malaria (NPM, n = 27), pregnant women with placental malaria (PM, n = 50) and non-infected pregnant women (n = 50) or controls and incubated with neutrophils for 1 h phagocytosis and phagocytic score was assessed by flow cytometry residing in PNG. To minimize plate to plate variability the mean of positive controls was taken and normalised to phagocytosis of all samples run in the same day. Phagocytosis (arbitrary units) was defined as the percentage (%) of bead-positive events while the phagocytic score (arbitrary units) was calculated as: ((% of events positive for beads) *(the geometric mean of fluorescecent intensity of the % of events positive for AF488)). DBL2 positive pooled plasma samples (PPS) was used as a positive control and pooled plasma from Melbourne healthy controls (MC) or no antibody (PBS) were used as a negative control. Three independent experiments were performed using different neutrophil donors and the median with interquartile range (IQR) percent phagocytosis and phagocytic score are displayed without subtraction of the background (PBS). Neutrophil donor 1; percent phagocytosis (A) and phagocytic score (D). Neutrophil donor 2; percent phagocytosis (B) and phagocytic score (E). Neutrophil donor 3; percent phagocytosis (C) and phagocytic score (F). The average of the 3 neutrophil donors; percent phagocytosis (G) and phagocytic score (H). Relative phagocytosis normalized to PPS = 100 and phagocytic score to PPS = 1,000,000. ADNP levels between women with PM and NPM were compared using Mann–Whitney test. P-values of < 0.05 were considered significant.

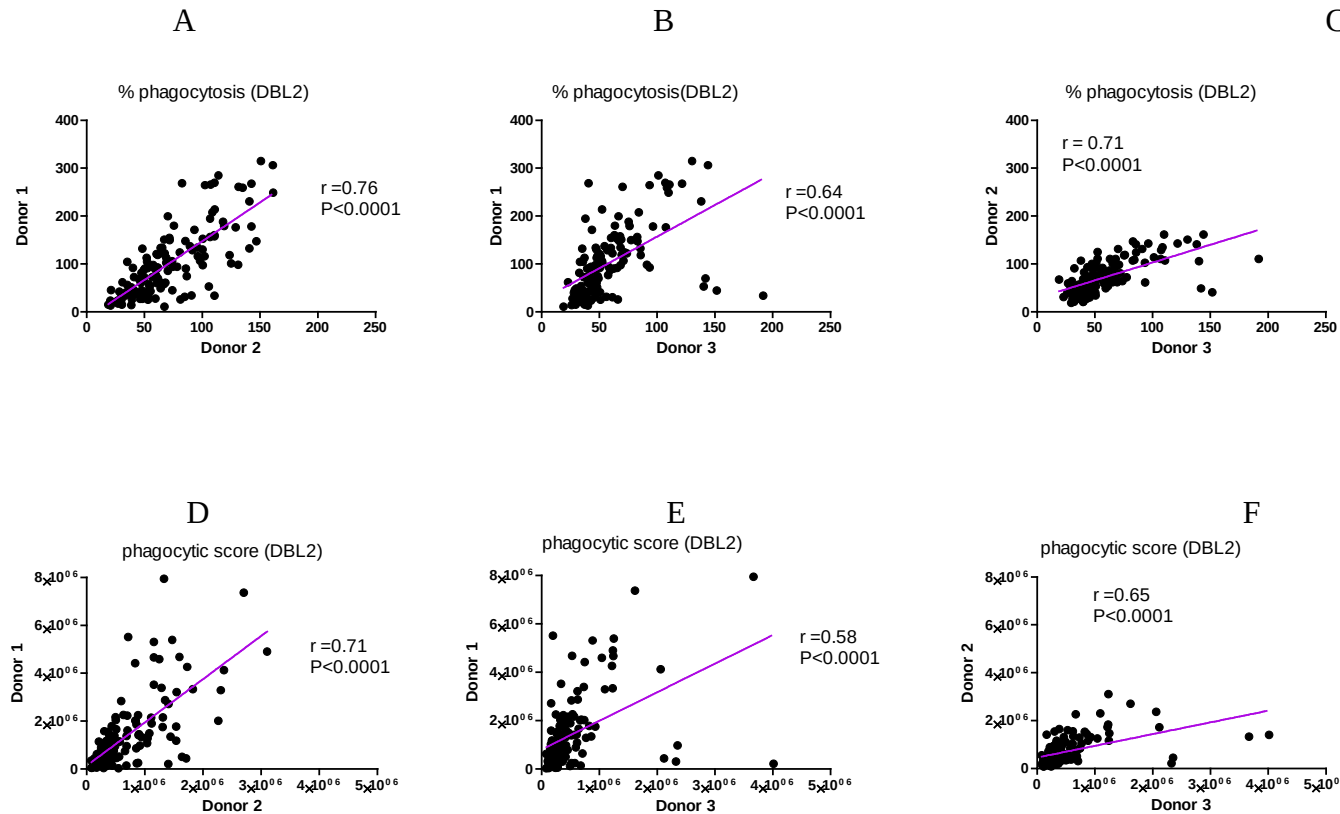
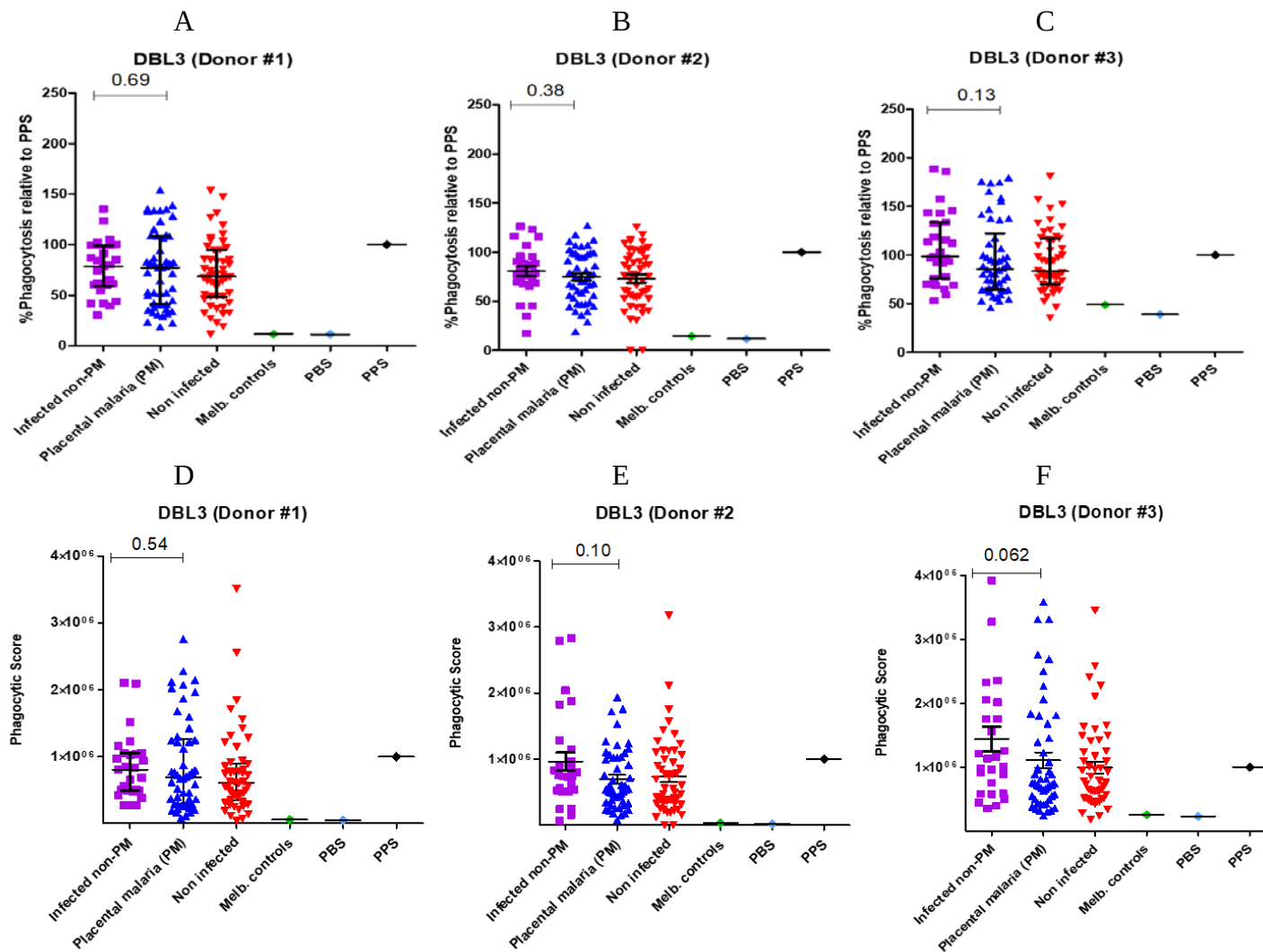


Figure 4 10 Correlation of ADNP of DBL2 protein-coated beads between neutrophil donors. This figure illustrated both percent phagocytosis and phagocytic score. Comparison of ADNP of beads opsonized with plasma from malaria-infected and non-infected PNG women (N =127) between neutrophil donors. Correlation of percent phagocytosis and phagocytic score between donor 1 (Y-axis) and donor 2 (X-axis) (A, D); donor 1 (Y-axis) and donor 3 (X-axis) (B, E); and donor 2 (Y-axis) and donor 3 (X-axis) (C, F). Correlations were determined by Spearman's correlation coefficient. $r \leq 0.2$; $r = 0.2-0.4$; $r = 0.4-0.59$ and $r \geq 0.6$ were considered as very weak, weak; moderate and strong correlation. P-values of < 0.05 were considered significant.

4.4.1.2 ADNP towards DBL3 and DBL5 recombinant antigen-coated beads

For DBL3 and 5, no evidence of a difference in ADNP was observed between NPM and PM groups for all neutrophil donors (figure 4.11 and 4.13).

To evaluate donor variability, both % phagocytosis and the phagocytic score were compared between donors ($n = 3$) and they were significantly correlated (figure 4.12 and 4.14). Like the DBL2 antigen, the opsonizing activities of most plasma samples in the cohort for beads coated with DBL3 and DBL5 antigens were higher compared to PPS (figure 4.11 and 4.13).



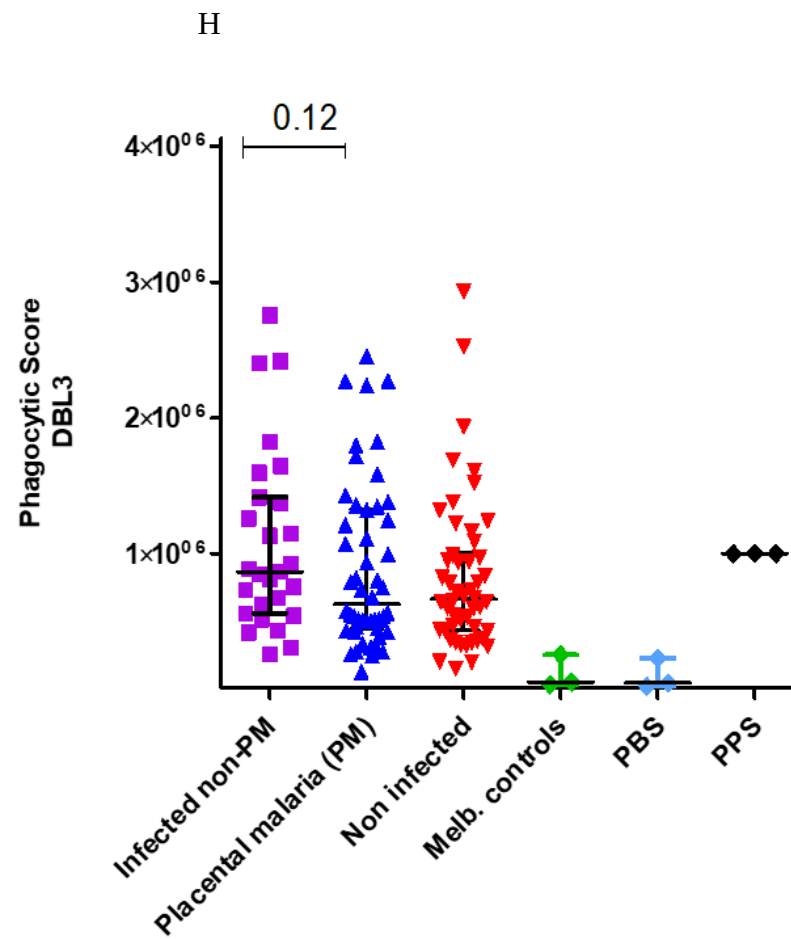
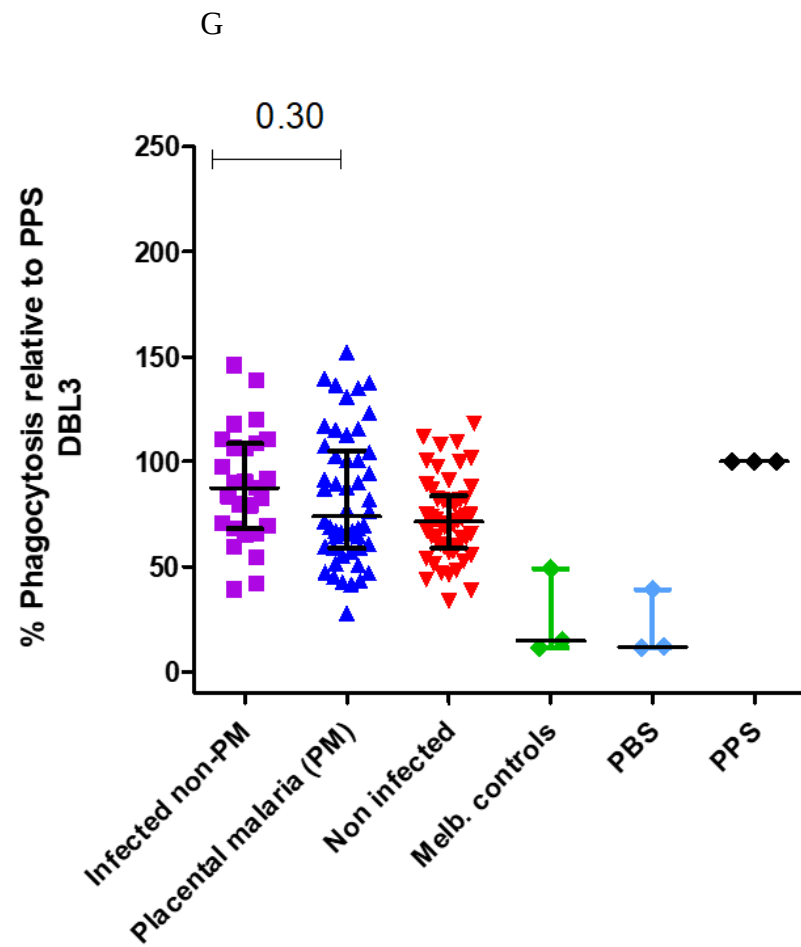


Figure 4 11 Antibody-dependent neutrophil phagocytosis using VAR2CSA DBL3-coated neutravidin beads. Neutrophils were purified from 3 donors by negative selection. Purity was >98% using light microscopy of Giemsa stained slides and CD66b staining. DBL3-coated neutravidin beads were opsonized with individual plasma from pregnant women infected with malaria but not placental malaria (NPM, n = 27), pregnant women with placental malaria (PM, n = 50) and non-infected pregnant women (n = 50) or controls and incubated with neutrophils for 1 h phagocytosis and phagocytic score was assessed by flow cytometry residing in PNG. To minimize plate to plate variability the mean of positive controls was taken and normalised to phagocytosis of all samples run in the same day. Phagocytosis (arbitrary units) was defined as the percentage (%) of beads positive events while the phagocytic score (arbitrary units) was calculated as: ((% of events positive for beads) *(the geometric mean of fluorescecent intensity of the % of events positive for AF488)). DBL3 positive pooled plasma samples (PPS) were used as a positive control and pooled plasma from Melbourne healthy donors (MC) or no antibody (PBS) were used as a negative control. Three independent experiments were performed using different neutrophil donors and the median with IQR % phagocytosis and phagocytic score are displayed without subtraction of the background (PBS). Neutrophil donor 1; percent phagocytosis (A) and phagocytic score (D). Neutrophil donor 2; percent phagocytosis (B) and phagocytic score (E). Neutrophil donor 3; percent phagocytosis (C) and phagocytic score (F). The average of the 3 neutrophil donors; percent phagocytosis (G) and phagocytic score (H). ADNP levels between women with placental malaria and non-placental malaria were compared using the Mann–Whitney test. P-values of < 0.05 were considered significant.

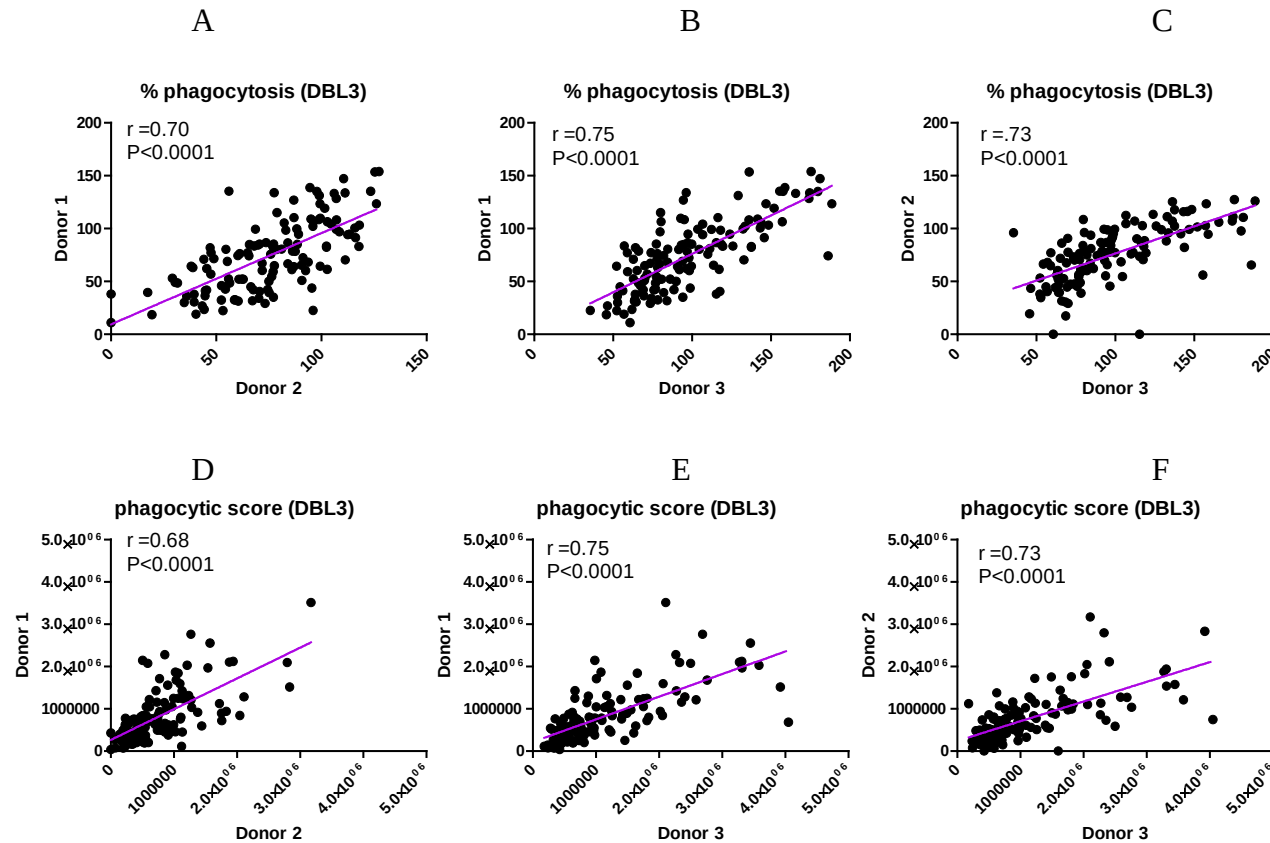
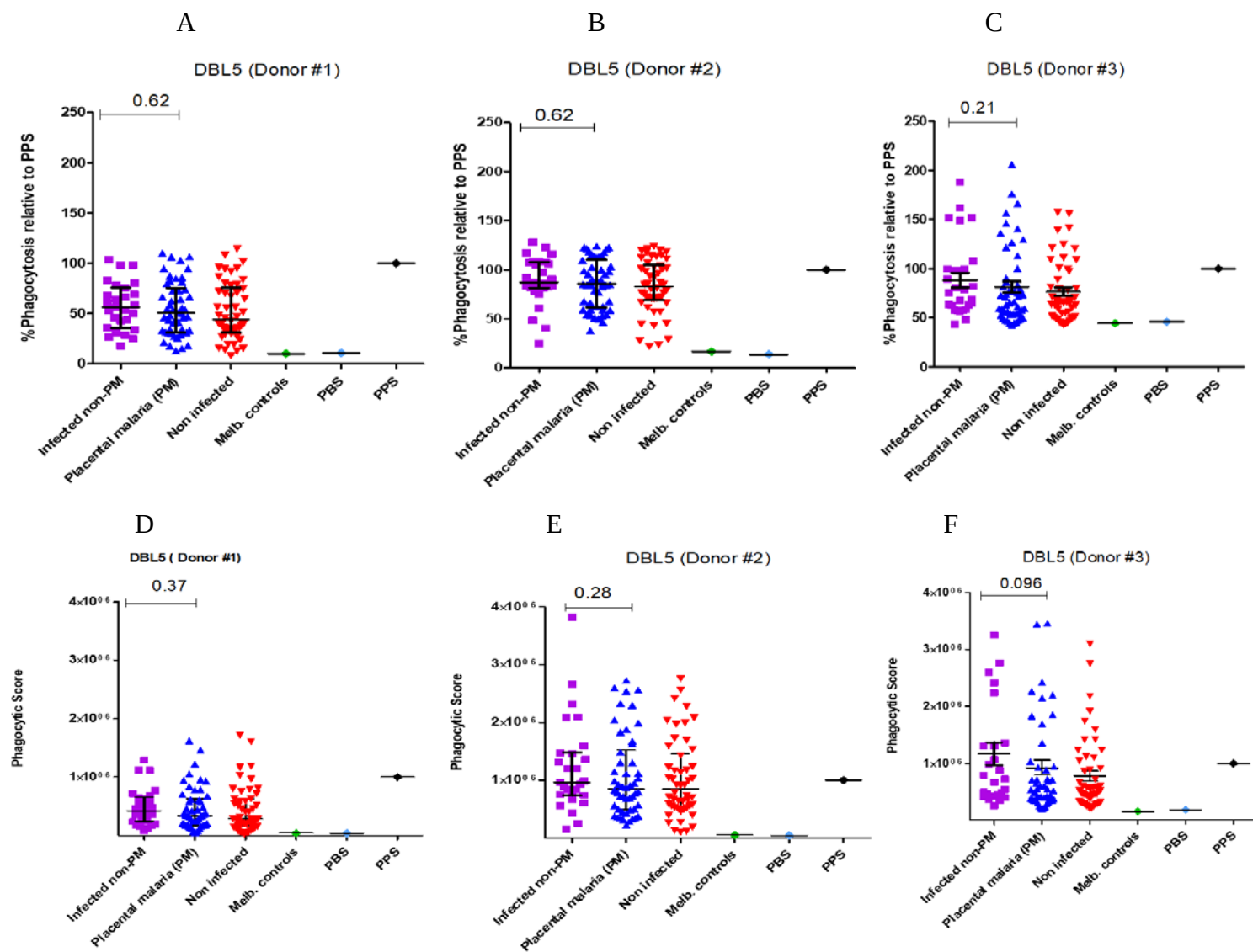


Figure 4 12 Correlation of ADNP of DBL3 protein-coated beads by neutrophil donors. This figure illustrated both percent phagocytosis and phagocytic score. Comparison of ADNP of bead opsonized with plasma from malaria-infected and non-infected PNG women (N =127) between neutrophil donors. Correlation of percent phagocytosis and phagocytic score between donor 1 (Y-axis) and donor 2 (X-axis) (A, D); donor 1 (Y-axis) and donor 3 (X-axis) (B, E); and donor 2 (Y-axis) and donor 3 (X-axis) (C, F). Correlations were determined by Spearman's correlation coefficient. $r = < 0.2$; $r = 0.2-0.4$; $r = 0.4-0.59$ and $r \geq 0.6$ were considered as very weak, weak; moderate and strong correlation. P-values of < 0.05 were considered significant.



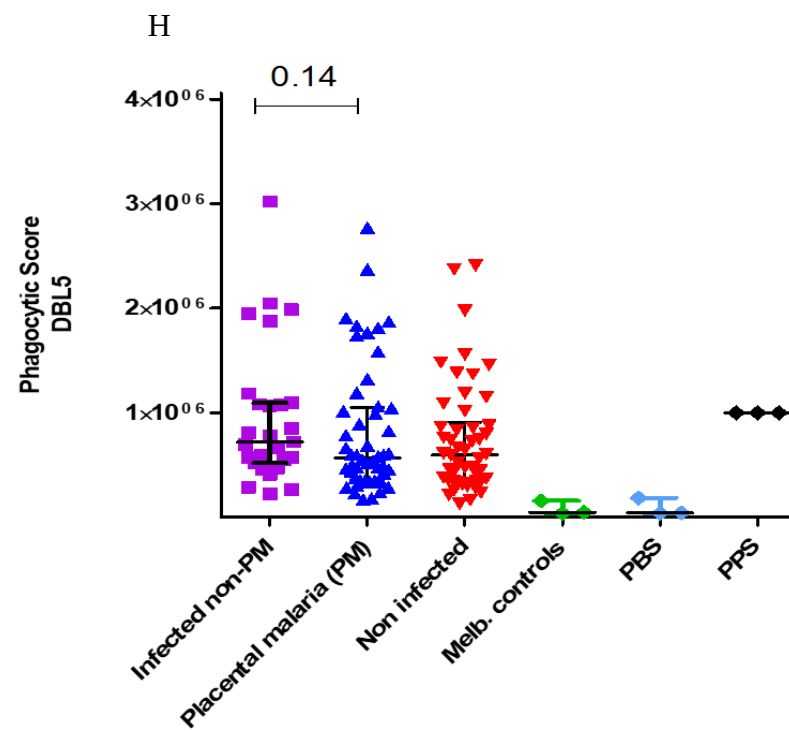
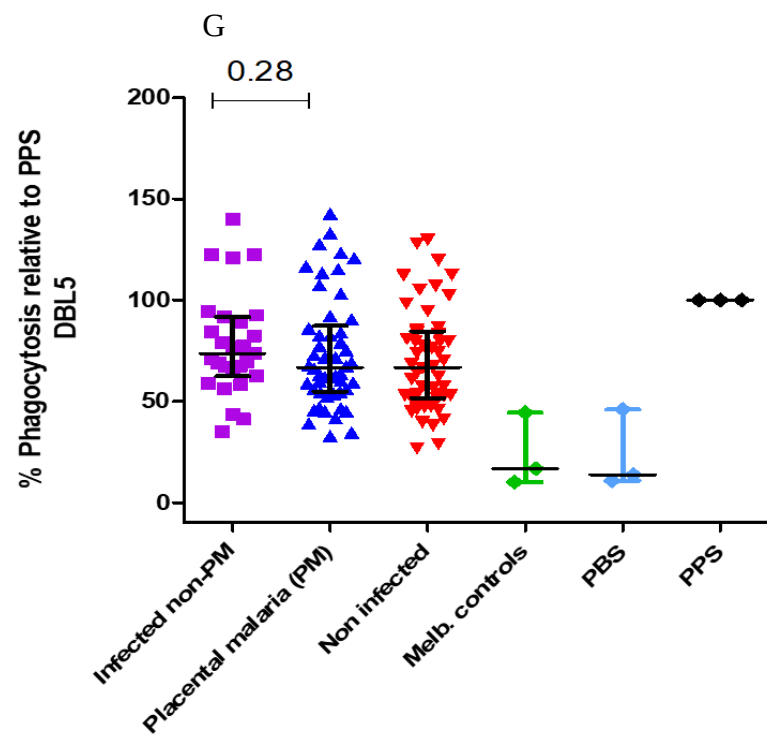


Figure 4 13 Antibody-dependent neutrophil phagocytosis using VAR2CSA DBL5-coated neutravidin beads. Neutrophils were purified from 3 donors by negative selection. Purity was >98% using light microscopy of Giemsa stained slides and CD66b staining. DBL5-coated neutravidin beads were opsonized with individual plasma from pregnant women infected with malaria but not placental malaria (NPM, n = 27), pregnant women with placental malaria (PM, n = 50) and non-infected pregnant women (n = 50) or controls and incubated with neutrophils for 1 h phagocytosis and phagocytic score was assessed by flow cytometry. To minimize plate to plate variability the mean of positive controls was taken and normalised to phagocytosis of all samples run in the same day. Phagocytosis (arbitrary units) was defined as the percentage (%) of beads positive events while the phagocytic score (arbitrary units) was calculated as: ((% of events positive for beads) *(the geometric mean of fluorescecent intensity of the % of events positive for AF488)). DBL5 positive pooled plasma samples (PPS) were used as a positive control and pooled plasma from Melbourne healthy donors (MC) or no serum (PBS) were used as a negative control. Three independent experiments were performed using different neutrophil donors and the median with IQR % phagocytosis and phagocytic score are displayed without subtraction of the background (PBS). Neutrophil donor 1; percent phagocytosis (A) and phagocytic score (D). Neutrophil donor 2; percent phagocytosis (B) and phagocytic score (E). Neutrophil donor 3; percent phagocytosis (C) and phagocytic score (F). The average of the 3 neutrophil donors; percent phagocytosis (G) and phagocytic score (H). ADNP levels between women with placental malaria and non-placental malaria were compared using the Mann–Whitney test. P-values of < 0.05 were considered significant.

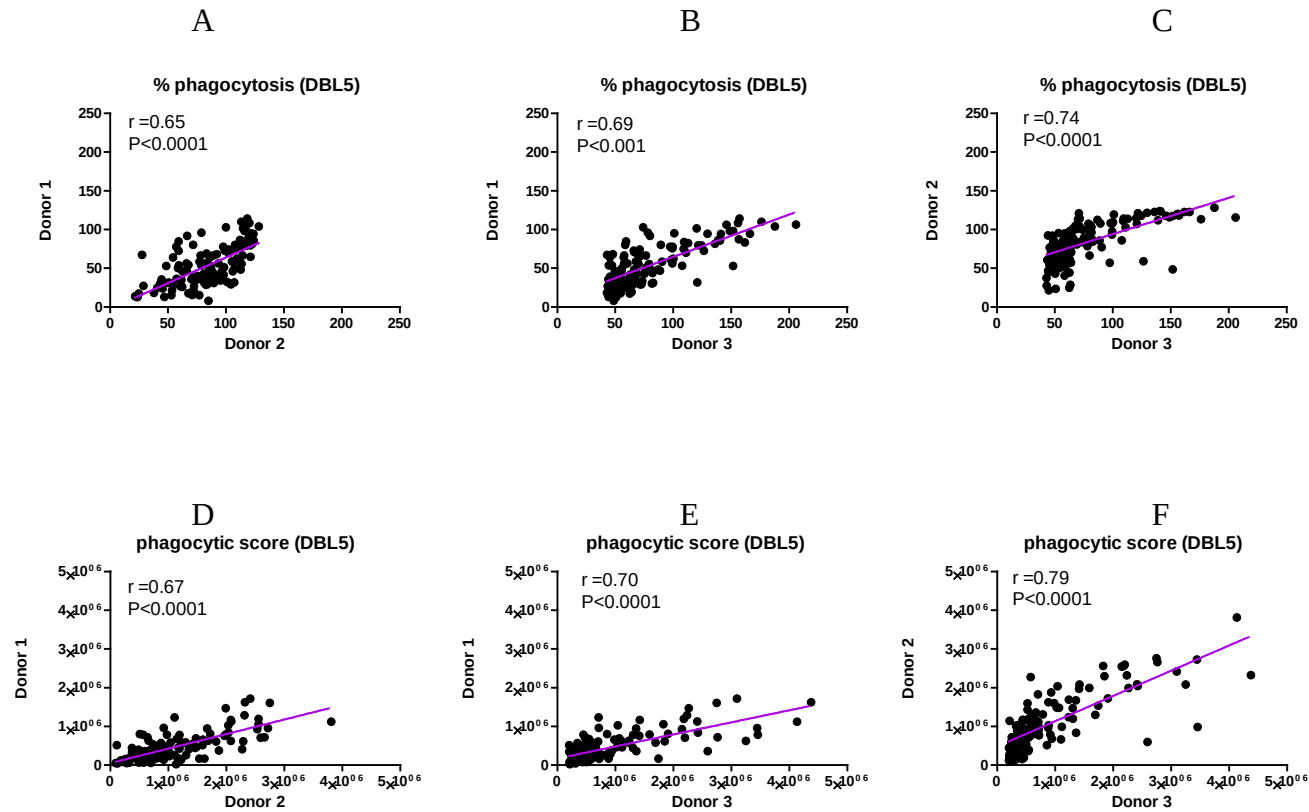


Figure 4 14 Correlation of ADNP of DBL5 protein-coated beads by neutrophil donors. This figure illustrated both percent phagocytosis and phagocytic score. Comparison of ADNP of bead opsonized with plasma from malaria-infected and non-infected PNG women (N =127) between neutrophil donors. Correlation of percent phagocytoses and phagocytic score between donor 1 (Y-axis) and donor 2 (X-axis) (A, D); donor 1 (Y-axis) and donor 3 (X-axis) (B, E); and donor 2 (Y-axis) and donor 3 (X-axis) (C, F). Correlations were determined by Spearman's correlation coefficient. $r = <0.2$; $r = 0.2-0.4$; $r = 0.4-0.59$ and $r \geq 0.6$ were considered as very weak, weak; moderate and strong correlation. P-values of <0.05 were considered significant.

ADNP of beads coated with recombinant DBL antigens between neutrophil donors (n=3) were compared using one-way ANOVA (analysis of variance) with significance considered as $p < 0.05$. In the present study, neutrophils from the 3 donors exhibited a different ADNP profile for each DBL domain. For instance, ADNP of beads coated with DBL2 antigen was significantly higher compared to DBL5 in neutrophil donor 1 (figure 4.15). Whereas, ADNP of beads coated with DBL3 and 5 antigens was significantly higher compared to DBL2 in donor 3. ADNP of beads coated with DBL3 antigen were significantly higher than DBL5 in neutrophil donor 1 and 3 (figure 4.15). Compared to DBL3 and 5, beads coated with DBL2 antigens were more highly recognized by neutrophil donor 1 and less well recognized by neutrophil donor 3 (figure 4. 15).

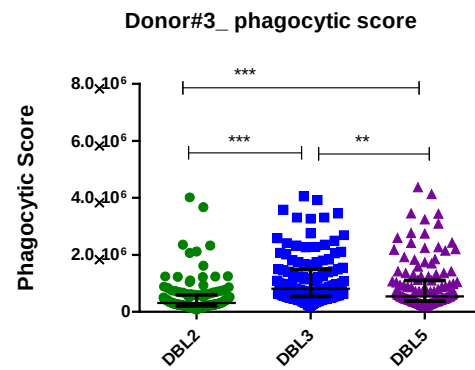
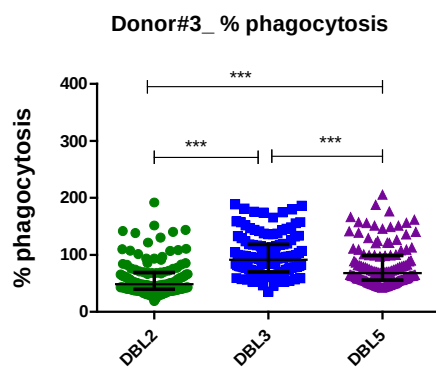
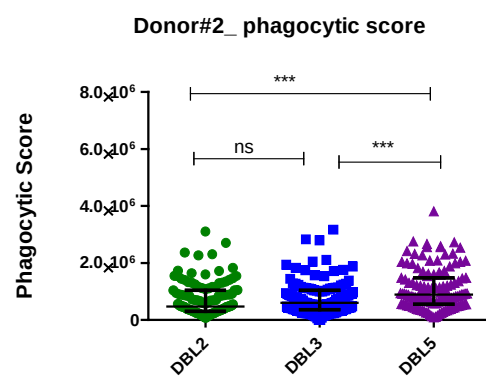
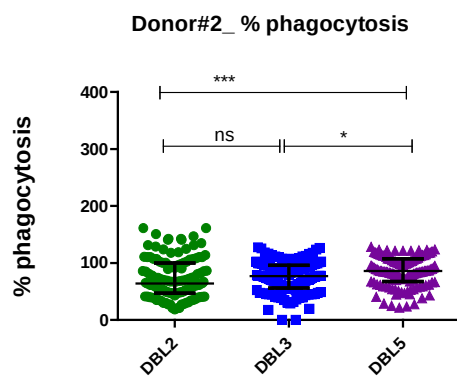
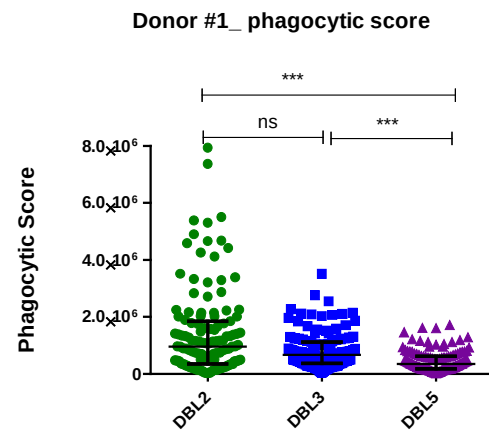
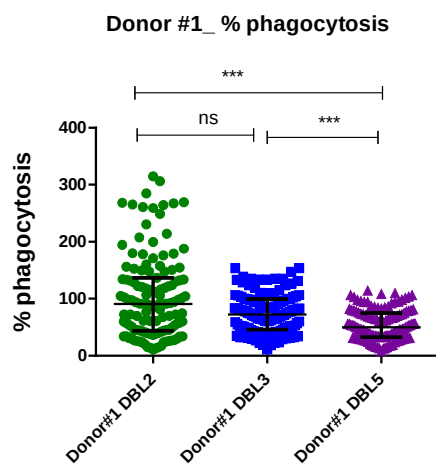


Figure 4 15 Comparison of ADNP between recombinant DBL antigens. ADNP of individual recombinant DBL domain- coated bead types were measured. Three independent experiments were performed using different neutrophil donors and the median with IQR % phagocytosis and phagocytic score are displayed for each assay. (A and B) Neutrophil donor 1. (C and D) Neutrophil donor 2. (E and F) Neutrophil donor 3. ADNP levels between individual DBL domains were compared using a one way ANOVA (Kruskal–Wallis correction test). P-values of < 0.05 were considered significant.

4.4.2 ADNP assay towards IE

Previously, it has been well-documented that human monocytic cell line THP-1 has phagocytic activity against opsonized particles (e.g., latex beads and RBCs) and apoptotic bodies (97-99, 276) as well as IE (97). In the present assay, Percoll purified IE expressing VAR2CSA were obtained from *in vitro* cultures, stained with dihydroethidium (DHE), and incubated with human neutrophils prior to analysis by flow cytometry. The assays were repeated with three different neutrophil donors in duplicate.

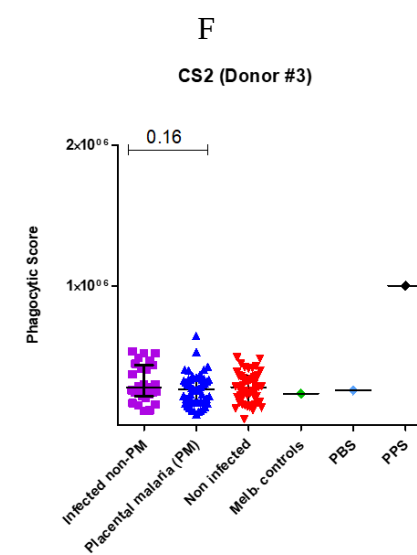
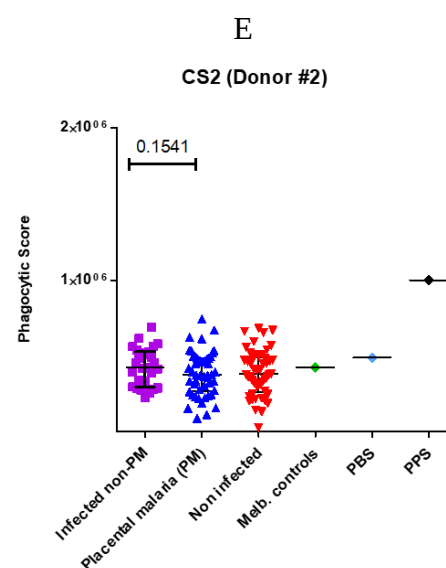
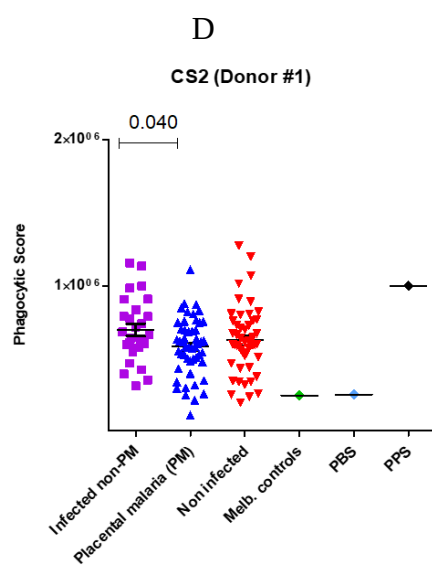
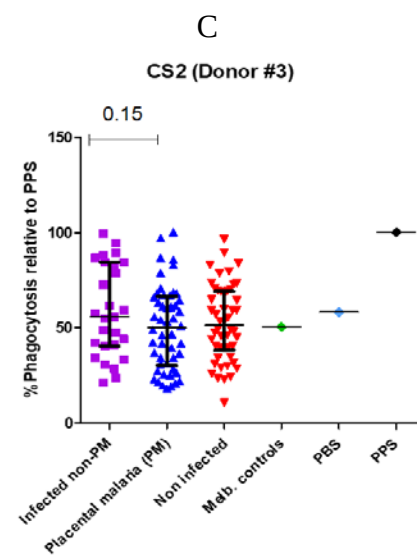
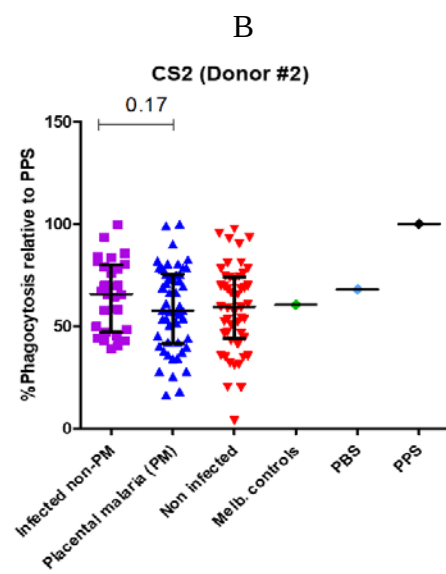
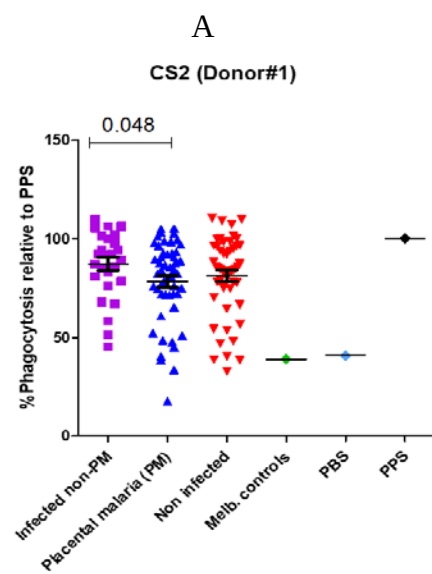
As demonstrated in figure 4.16, higher levels of ADNP were observed in NPM compared to PM (**A** and **D**; $P = 0.048$, for % phagocytosis and $P = 0.040$, for phagocytic score) for neutrophils from donor 1. Both % phagocytosis (**B**, **C**) and % phagocytic score (**E**, **F**) did not differ between NPM and PM for the neutrophil donors 2 and 3. When the average of the three neutrophil donors was considered, higher levels of ADNP to IEs was observed in NPM compared to PM (**G** and **H**; $p = 0.012$, for % phagocytosis and $p = 0.0070$, for phagocytic score), suggesting a higher level of opsonizing antibody against VAR2CSA is associated with protection against PM.

In this study, high background phagocytosis and phagocytic score were observed in two of the assays done using neutrophil donors 2 and 3.

In IE based ADNP, almost all test samples fall below positive controls in all three assays.

Neutrophils were compared for ADNP of IEs. In contrast to bead-based ADNP results, which were strongly correlated, a weak correlation was observed between neutrophil donors 1 and 2 (figure 4.17**A** and **D**) and donors 2 and 3 (figure 4.17**F**).

Next, the bead-based ADNP assay was compared with IE based ADNP assays (the first experiment from each assay, as the same neutrophil donor was used). Results from the two assays didn't correlate each other (figure 4.18)



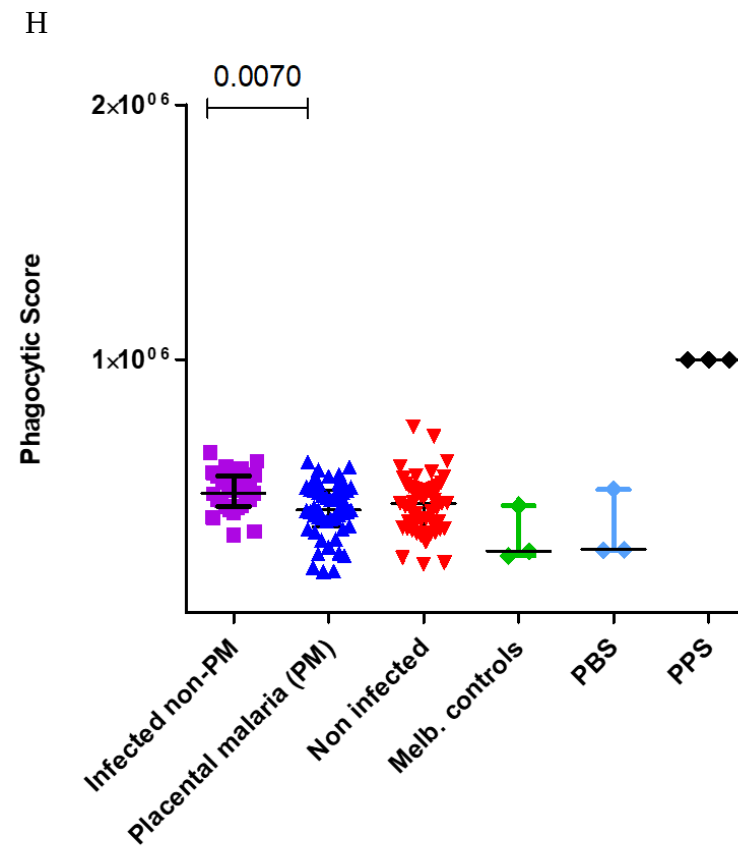
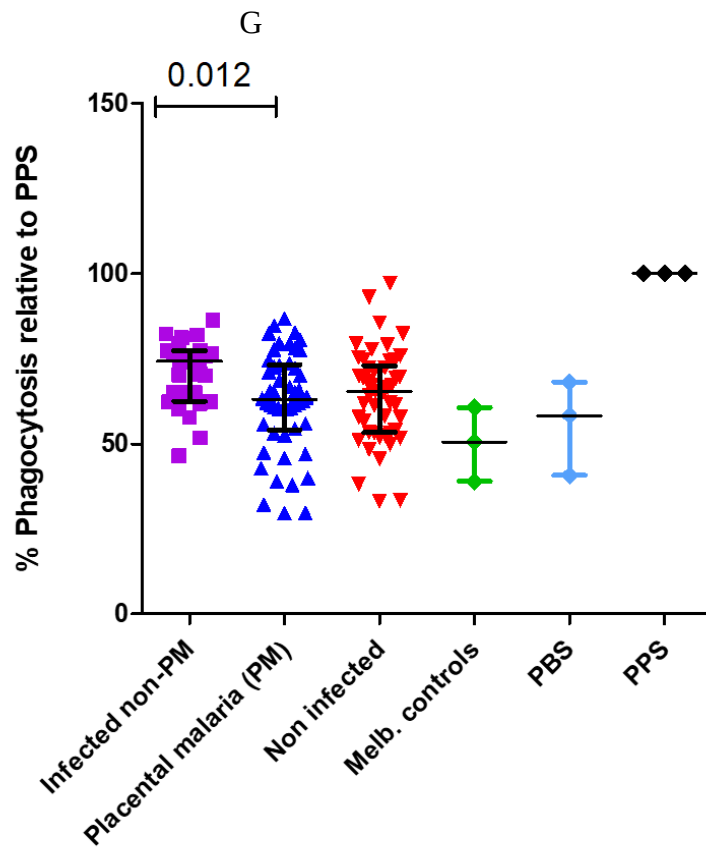


Figure 4 16 Antibody-dependent neutrophil phagocytosis using VAR2CSA IE antigen. Neutrophils were purified from 3 donors by negative selection. Purity was >98% using light microscopy of Giemsa stained slides and CD66b staining. DHE stained IEs were opsonized with individual plasma from pregnant women infected with malaria but not placental malaria (NPM, n = 27), pregnant women with placental malaria (PM, n = 50) and non-infected pregnant women (n = 50) or controls and incubated with neutrophils for 1 h phagocytosis and phagocytic score was assessed by flow cytometry. To minimize plate to plate variability the mean of positive controls was taken and normalised to phagocytosis of all samples run in the same day. Phagocytosis (arbitrary units) was defined as the percentage (%) of IEs positive events while the phagocytic score (arbitrary units) was calculated as: ((% of events positive for IE) *(the median of fluorescent intensity of the % of events positive for DHE)). Pooled plasma samples (PPS) from malaria-infected Malawian pregnant women were used as a positive control and pooled plasma from Melbourne healthy donors (Melb. control) and no antibody (PBS) was used as a negative control. Three independent experiments were performed using different neutrophil donors and the median with IQR % phagocytosis and phagocytic score are displayed without subtraction of the background (no antibody, PBS). Neutrophil donor 1; percent phagocytosis (A) and phagocytic score (D). Neutrophil donor 2; percent phagocytosis (B) and phagocytic score (E). Neutrophil donor 3; percent phagocytosis (C) and phagocytic score (F). The average of the 3 neutrophil donors; percent phagocytosis (G) and phagocytic score (H). ADNP levels between women with placental malaria and non-placental malaria were compared using the Mann–Whitney test. P-values of < 0.05 were considered significant.

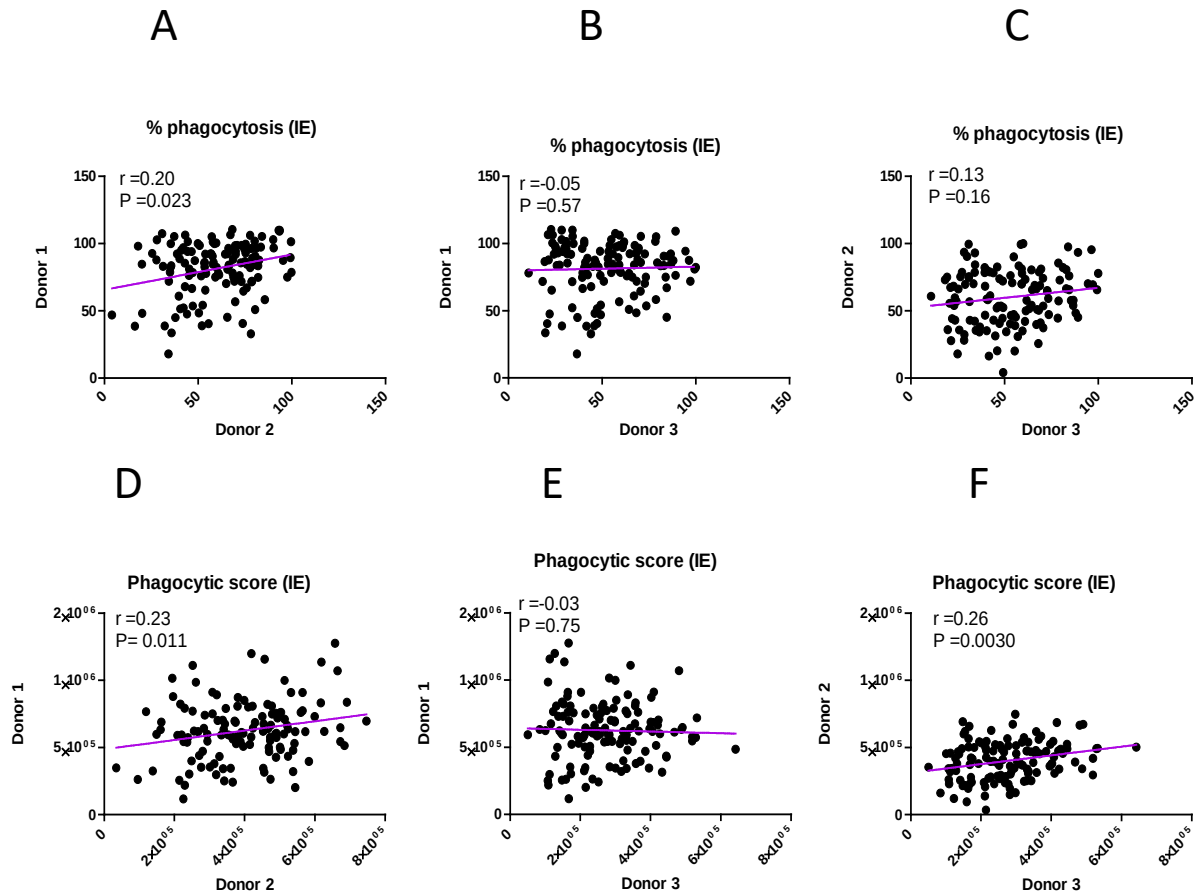


Figure 4 17 Correlation of ADNP to native VAR2CSA- expressing IE by neutrophil donors. This figure demonstrated both percent phagocytosis and phagocytic score. Comparison of ADNP of IE opsonized with plasma from malaria-infected and non-infected PNG pregnant women (N =127) by neutrophil donors. Correlation of percent phagocytosis and phagocytic score between donor 1 (Y-axis) and donor 2 (X-axis) (A, D); donor 1 (Y-axis) and donor 3 (X-axis) (B, E); and donor 2 (Y-axis) and donor 3 (X-axis) (C, F). Correlations were determined by Spearman's correlation coefficient. $r < 0.2$; $r = 0.2-0.4$; $r = 0.4-0.59$ and $r \geq 0.6$ were considered as very weak, weak; moderate and strong correlation. P -values of < 0.05 were considered significant.

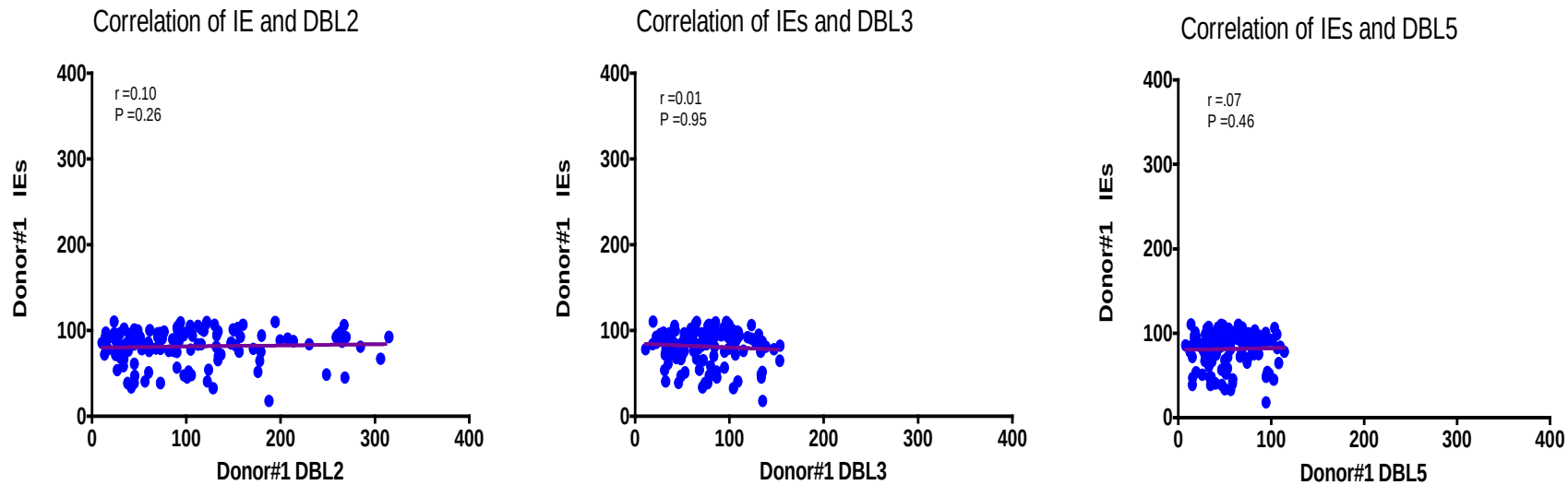


Figure 4 18 Correlation of percent phagocytosis of IE and recombinant DBL antigen-coupled beads. Comparison of ADNP of IE and DBL antigen coated beads opsonized with plasma from malaria-infected and non-infected PNG pregnant women (N =127) by neutrophil donors. Correlation of percent phagocytoses of IE (Y-axis) and percent phagocytosis of DBL antigens (X-axis) by neutrophil donor 1. Correlations were determined by Spearman's correlation coefficient. $r \leq 0.2$; $r = 0.2-0.4$; $r = 0.4-0.59$ and $r \geq 0.6$ were considered as very weak, weak; moderate and strong correlation. P-values of < 0.05 were considered significant

4.5 Discussion, conclusion and future directions

There is an increasing interest in the role of Fc-mediated effector functions like antibody-dependent phagocytosis (ADP) in protective immunity in malaria. However, there has been a lack of studies into the role of ADNP in the context of *P. falciparum* infections. This is the first study to investigate ADNP towards placental binding parasites expressing VAR2CSA antigens. The current study aimed to evaluate whether antibody responses to VAR2CSA are associated with protection against placental malaria in PNG pregnant women. To test our hypothesis, neutrophils isolated from healthy donors were cocultured with beads coated with recombinant DBL antigens or IEs expressing VAR2CSA on their surface opsonized with plasma from malaria-infected or noninfected pregnant women, and ADNP was measured using flow cytometry.

The main findings in this study demonstrated that: (1) ADNP of DBL2 coated beads, but not of DBL3 and DBL5 coated beads, differed between PM and NPM; (2) ADNP to recombinant DBL antigens was significantly correlated between donors, and (3) ADNP of IE differed between PM and NPM.

4.5.1 Assay optimization

Using human neutrophils and opsonized latex beads coated with VAR2CSA antigens we have optimized a phagocytosis assay; thus, researchers can adapt the assay method to other antigens. Previous evidence showed that, in addition to blocking activities, antibodies are able to facilitate innate immune cells to eradicate opsonized pathogens like promoting phagocytosis, which mediates clearance of invading pathogens (268), suggesting that identifying the target antigens that trigger specific-antibody mediated phagocytosis is important to study protective immunity and develop a vaccine. Neutrophils, the predominant innate immune cells in the blood, express Fc receptors such as FcRII (CD32), FcRIII (CD16) and (when activated) FcRI (CD64), which mediate phagocytic uptake of antigen opsonized with antibodies, reviewed in (121).

For bead-based ADNP assay optimization, yellow fluorescent neutravidin beads were coupled to recombinant DBL antigens and used to assay the phagocytic activity of antibodies in clinical samples (97, 268). Plasma samples collected from PNG pregnant women participating in an IPTP study (249, 277) were used as the source of antibody. To determine the optimal amount of time for efficient uptake of beads by neutrophils in the ADNP assay, beads were opsonized with positive and negative control plasma and cocultured with neutrophils for different time points. In this study, higher % phagocytosis and phagocytic score were observed at 1 and 2 h incubation. However, background phagocytosis was increased at 2 h incubation time point (figure 4.10A and B), suggesting that incubation of neutrophil with beads for a prolonged time may cause non-specific uptake, which could be because of overactivation or exhaustion. Thus, because of the low levels of ADNP at 0.5 h and non-specific uptake of beads at 2 h incubation time, we chose a 1 h incubation time point for our ADNP assay. In agreement with our finding, a similar incubation time point was reported using beads coated with gp120 antigen, (278, 279), and another study preferred 2 h incubation time for their ADNP assay using similar antigen (280). Using THP-1 cell lines, antibody-dependent phagocytosis of IE was done at 40 minutes incubation time points (97, 99, 101).

We optimized the plasma dilutions to use and the EC₅₀ (dilution of the plasma at which neutrophil phagocytosis is half its maximal) was calculated. Pooled plasma samples were titrated from 1:25 to 1:3,200, and ADNP of beads declined with increasing plasma dilution (figure 4.6 C-F); confirming a relationship between the level of antibodies against VAR2CSA in plasma and phagocytic activity (97). Although the highest percent phagocytosis and phagocytic score were observed at 1:25 and 1:50 dilutions, the EC₅₀ (figure 4.8G- J) were at approximately a 1:200 dilution of plasma. Since this optimization assay was done using pooled plasma samples which might contain high levels of antibody, to ensure robust results and maximize differences in % phagocytosis and phagocytic score between positive and negative pool plasma controls, throughout all experiments a slightly higher dilution of plasma (1:100) was selected for the current study. Our plasma dilutions are supported by ADNP optimization findings by Karsten *et al* (278) and Butler *et al* (279), using viral antigens. However, no studies have been done in the context of malaria antigens using neutrophils. In this study, we used primary human neutrophils, to explore the extent to which donor to donor variability and natural neutrophil reactivity to immune complexes

may affect ADNP (278). The intra- and inter-individual variation in the number of neutrophils is characterised and the intra-individual variation is 16.1% and the inter-individual variation is 32.8% (281). A research has shown that the expression of FcγR mRNA and granulocyte release of ROS are both affected by variations in the allele and copy number of FcγR genes. FcγR encoding genes show regular variations in the number of sequences and gene copies that affect their functional properties (282).

Here, donor to donor variability of ADNP towards recombinant DBL antigen-coated beads was evaluated and the result of our study demonstrated that neutrophil function (ADNP) is variable between individuals. Beads were opsonized with pooled purified IgG (PPS or MC) and cocultured with neutrophils from two healthy donors. The ADNP assay was done on the same day following the same conditions. We have observed similar trends of ADNP towards beads coated with both DBL antigens (figure 4.6 **A** and **B**), however, donor to donor variation was observed in an IgG concentration-dependent manner, demonstrating an increase in ADNP with increasing IgG concentration in one neutrophil donor and a sharp decrease in a second neutrophil donor after a certain IgG concentration. Although the level of phagocytic reactivity of PPS against DBL antigens was variable between neutrophil donors, the levels of phagocytosis using MC remained stable. The possible hypothesis for this variability could be the genetic variability of human neutrophil FcRs and other proteins involved in phagocytosis (283). Data from recent studies demonstrated that phagocytic activities of neutrophils were different between neutrophil donors (278) and the ability of neutrophils from different donors to respond to *Mycobacterium tuberculosis* lipoprotein was variable (284). In other studies, it has been reported that most FcR encoding genes display genetic variation either in the form of single nucleotide polymorphisms (SNPs) or variation in their copy number which impacts the expression level, affinity, and functionality of FcRs (278, 285). These genetic variants display heterogeneity among ethnic groups (286). In a recent study, ADNP was measured using beads coated with gp120 and opsonized with HIV specific antibodies, and neutrophils from each of the 5 donors exhibited a different ADCP profile (283), suggesting that antibody-mediated neutrophil-based assays should prove useful in dissecting target-specific immunity (280). It is interesting to speculate about the impact of variation in ADNP between different donors. Cells from different individuals may have different sensitivities for responding to the target antigen.

In this thesis, ADNP was expressed in % phagocytosis and phagocytic score. I decided to put both % phagocytosis and phagocytic score because, although previous researchers reported their data in % phagocytosis (which shows the percent of cells which take up one or more IEs or beads coated with the target antigen), more recent studies expressed their findings in phagocytic score, which is a score of both % phagocytosis and the fluorescent signal of beads taken up by the cells, and indicates the cells' efficiency.

4.5.2 ADNP towards IEs and recombinant DBL antigens

In malaria-endemic areas, pregnant women produce antibodies to VAR2CSA, which increase over consecutive pregnancies (parity-dependent) (287), such that the responses are robust in multigravida women (288). Earlier studies demonstrated that pregnant women who had previous exposure to malaria produce antibodies to VAR2CSA that inhibit the attachment of IE to CSA, and these adhesion-blocking antibodies have been associated with protection (95, 289). In other studies, opsonizing antibody decreased malaria-associated complications (71, 84, 99, 264), suggesting antibodies to VAR2CSA play a significant role in protecting pregnant women from PM, however, the principal mechanisms by which anti-VAR2CSA antibody protects against PM and its clinical outcomes is not clear.

In the current study, the uptake of beads coated with recombinant DBL antigens and VAR2CSA-expressing IEs by human neutrophils was assessed. The results of this study showed that ADNP of beads coated with DBL2 and IEs, but not beads coated with DBL3 and DBL5, were associated with protection against PM.

Antibodies against VAR2CSA could increase with infection, so we chose to compare within women with infection between those with and without placental infection. Previous studies have shown the association of high levels of antibodies measured by ELISA and protection from PM (89, 95, 289). However, the levels of antibodies against CSA binding parasites have been controversial as different studies have presented conflicting results. For example, some authors reported that high levels of antibodies in plasma samples from pregnant women reflect exposure to malaria parasites or active placental infection (290, 291) while others reported high levels of antibodies are associated with protection from PM (89).

Since the study subjects were categorized into three groups at delivery and there is no definite evidence of infection or not before delivery for the uninfected group, comparing infected and uninfected groups was challenging in this study. In addition, it is not clear whether this group is uninfected because of protection from PM or they were not exposed to malaria at all.

Antibody-dependent phagocytosis has been reported previously using beads coated with HIV envelope protein, gp120 (102, 272, 280, 283), purified protein derivative (PPD) from *Mycobacterium tuberculosis* (103), full-length recombinant VAR2CSA antigen (97), and using IE (71, 97, 99-101, 292) as a target and neutrophils (280, 283), monocytes (100, 283, 292) and THP-1 cell lines (71, 97, 99-103, 272, 283) as phagocytes to determine the opsonizing activities of antibodies to mediate phagocytosis of the target antigen. Using THP-1 cell lines, studies demonstrated the association of opsonizing antibodies and percent phagocytosis (97, 101). However, the interaction of antibodies, infected erythrocytes and neutrophils is not investigated in the context of malaria before this study. Here, we developed novel assays of ADNP using primary human neutrophils, with IE and DBL-coated beads as their targets. The percent phagocytosis of beads and IE were significantly correlated and day to day variation of percent phagocytosis was not observed (97). Other studies have also demonstrated that PM-specific plasma antibodies from malaria exposed multigravida Kenyan women (292) and Malawian women (99) promoted the opsonic phagocytosis of CSA-binding IEs compared to women without malaria infection, suggesting the association of antibodies and protection from placental infection. However, the interaction of antibodies and neutrophils is not investigated in the context of malaria. The antibody mediated Fc γ R function could be different depending on the types of Fc γ Rs present on the surface of certain cell types, which suggests that ADNP may differ from ADP using the THP-1 cell line (283). Here, we evaluated anti-VAR2CSA antibody activities in promoting phagocytosis by primary human neutrophils.

4.5.3 ADNP of DBL2 coated beads, but not DBL3 and DBL5, did differ between NPM and PM

The results of the present study showed that the levels of ADNP towards DBL2 coated beads, expressed as the average of 3 neutrophil donors, differed between NPM and PM. For one of the three neutrophil donors, we saw no evidence of a difference for both %

phagocytosis and phagocytic score in NPM compared to PM. Overall there were significantly higher levels of % phagocytosis and phagocytic score in NPM compared to PM as presented in figures 4. 9.

For beads coated with DBL3 and DBL5 antigens, we observed no evidence of a difference between NPM and PM groups for all neutrophil donors (% phagocytosis and phagocytic score). A previous study reported that higher levels of antibodies were associated with active placental malaria infections (130, 293). Data from previous studies demonstrated that higher levels of antibody to DBL2 and DBL3 domains were reported in women with histological evidence of infection than women without evidence of infection (100, 291), and phagocytic antibodies were higher in PM+ women than in PM- women (98). On the other hand, studies have shown that serum IgGs of malaria-exposed multigravida women predominantly recognized the DBL2, DBL3 and DBL5 domains of VAR2CSA (100, 294) and high levels of antibody to multiple VAR2CSA domains during pregnancy were associated with protection from PM (PM- women had high antibody than PM+).

In the current study, ADNP of beads coated with individual recombinant DBL antigens was variable between neutrophils donors (figure 4.15). For instance, although beads were opsonized with the same plasma antibody neutrophils from donor 1 phagocytosed significantly more beads coated with DBL2 and DBL3 while neutrophils from donor 3 phagocytosed more beads coated with DBL 3 and DBL5 as presented in figure 4. 15. It might be important to note that recognition of the individual recombinant DBL antigens by human neutrophils may be different. The possible hypothesis for this difference could be (a): the opsonizing ability of antibodies against the targeted antigens, (b): the genetic variability of human neutrophil FcRs and other proteins involved in phagocytosis. Most FcR encoding genes display genetic variation either in the form of single nucleotide polymorphisms (SNPs) or variation in their copy number which impact the expression level, affinity, and functionality of FcRs (278, 285).

Another interesting finding in this bead based ADNP study was the % phagocytosis and phagocytic score of most plasma samples in the cohort (PNG pregnant women) were higher than PPS. The plausible hypothesis for this difference might be due to the difference in the source of plasma samples. In this study, the positive control was pooled from plasma samples from malaria-infected Malawian pregnant women, where the *plasmodium* parasite

species circulating are *P. falciparum*, *P. malariae*, and *P. ovale*, but not *P. vivax* (295), while both *P. falciparum* and *P. vivax* are found in PNG (296). Double infection by *P. falciparum* and *P. vivax* may be possible in PNG that might result in the production of antibodies against both species, which may lead to cross-reactivity of antibodies (297, 298). Thus, further investigation is recommended to confirm the hypothesis of cross-reactivity of antigens between *P. falciparum* and *P. vivax*. For future assays in the lab, it might be important to use positive controls pooled from the same cohort.

4.5.4 ADNP towards IE differed between NPM and PM.

A study reported that neutrophils likely play a role in the clearance of the malaria parasites by enhanced phagocytic activity as well as superoxide production (50). Neutrophils phagocytose both IEs and merozoites (130), and *in vitro* studies have also shown that neutrophils from healthy blood donors phagocytosed IE than normal RBC (124). The phagocytic activity of neutrophils is highly increased by the addition of immune sera, suggesting that opsonization of IE by purified IgG enhances *P. falciparum* clearance by human neutrophils (124), the increased phagocytic activity of IE after opsonization by sera from immune individuals was confirmed (299), indicating complement heat inactivation of serum does not alter opsonic activity directed against IE (292, 299). Neutrophils also phagocytose merozoites both *in vitro* and *in vivo* (134). Merozoites are highly exposed to the immune system and a strong antibody response is mounted against the merozoite surface coat. Immune sera from individuals in the Solomon Islands and PNG (134), and Ghana (132) increased phagocytosis of merozoites by neutrophils compared to nonimmune sera.

In the present study, the ADNP of IEs was only different for 1 of the 3 neutrophil donors between study groups. Overall (average of three donors), a significantly higher level of ADNP of IEs was observed in pregnant women with NPM compared to PM (figure 4.16 **G** and **H**); suggesting a higher level of opsonizing antibody against VAR2CSA in NPM is associated with protection against PM.

In the current IE based ADNP assay, high background phagocytosis (nonopsonic phagocytosis) was observed for neutrophil donors 2 and 3 (figure 4.16). That is IE

opsonized with plasma from Melbourne control and the nonopsonic phagocytosis were equal or greater than the median ADNP of IEs opsonized with plasma samples from malaria exposed women, which was not observed in bead-based ADNP assay. The negative control used for both the IE and bead-based ADNP assay was the same pooled plasma sample from Melbourne healthy donors. One plausible hypothesis for this could be the adhesion of IE on neutrophils. In support of this hypothesis, Beeson and Brown in 2002, reviewed that *P. falciparum* IE adhere to receptors expressed on host cells such as a cluster of differentiation (CD36), intercellular adhesion molecule 1 (ICAM-1), platelet endothelial cell adhesion molecule 1 (PECAM-1), complement receptor 1 (CR1), chondroitin sulphate A (CSA), hyaluronic acid (HA) (300), and these receptors may be expressed by neutrophils (301). In addition, one unpublished data (an honors student project in Rogerson's lab) has shown that IE binds to neutrophils at varying levels in serum-free assays. The adhesion of IE to neutrophils was CSA dependent and the binding was varied with the neutrophil donor (unpublished data). It has been also demonstrated that glycosaminoglycans (GAGs) are expressed by neutrophils, and CSA constitutes the major parts of GAGs (85-90%) (302). Thus, the CSA expressed by neutrophils may bind to placental binding IE. Furthermore, CSA functions as the physiologically binding site for Platelet factor 4 on neutrophils. Platelets modulate multiple neutrophil functions including phagocytosis, and isolated neutrophils have been shown to contain remnants of platelets which produced platelet factor 4, which is a platelet-derived alpha-chemokine that binds to and activates human neutrophils to undergo specific functions like adhesion and CSA present on the surface of neutrophils supports the binding of platelet factor 4 (301).

Another possible explanation for the assay to assay variation could be the age of red blood cells used for parasite culture. We have been receiving blood for parasite culture from Australia Red Cross Blood Service every 3 weeks, thus when red blood cells used in their last week of reception for parasite culture their morphology may be changed, thus, neutrophils may consider them as foreign and phagocytose them.

Our finding is suggesting further assays to investigate whether IE binds to neutrophils. I was keen to evaluate the adhesion ability of IE to neutrophil if any, but due to the scarcity of time, this will need to be done in future studies.

4.5.5 ADNP to VAR2CSA antigens correlated between donors.

In the current study, ADNP to recombinant DBL antigens was significantly correlated between neutrophil donors but weakly correlated to IE-based assays, but it is not clearly understood why this happened. The bead-based and IE-based ADNP assay were compared and the results from the two assays didn't correlate with each other. This finding is suggesting further assays to investigate this variation. Although it is labor-intensive, it is possible that testing both assays in parallel using the same neutrophil donor may be helpful to minimize assay to assay variation as well as a donor to donor variation. In addition, testing a larger set of neutrophil donors for both IE and bead based assays may be important to see the trends of phagocytic activity in each donor.

4.5.6 Strength and limitations of the study

One strength of this work is the study design which controls for infection (which identified as a major confounding factor). Another strength is the use of a direct neutrophil isolation kit which allowed to get pure and unstimulated neutrophils. The use of primary human neutrophils may help to explore donor to donor variability and natural neutrophil reactivity to immune complexes. The other strength of this study is the use of both IE expressing VAR2CSA antigen and individual recombinant DBL antigens to testing ADNP.

Although neutrophils are the major innate immune cells, exhibit a unique FcR profile and active at the sites of infection, they are quite a difficult cell to work with due to their short half-life, the lack of ability to freeze isolated cells and the likelihood of overactivation and exhaustion. Donor to donor variation of neutrophil response was observed in the current study, which could be associated with priming cytokines, sex of neutrophil donors or other factors possibly due to the genetic variability of human neutrophil FcRs. The other limitation could be a small sample size as a small sample size decreases statistical power. We used a small sample size in this study because the study groups were frequency matched for age, gravidity, IPTp regime, rurality and bed net use, which makes it hard to get the large sample size.

Another challenge of using primary human neutrophils was to get a sufficient number of cells for the designed assay because the number of neutrophils isolated from different neutrophil donors or the same donor on different days per ml of blood is unpredictable. In the present study, to evaluate donor to donor or day to day variability of neutrophil numbers, different volumes of whole blood (5-15 ml) were collected from different/same

healthy volunteers. Donor to donor variations of neutrophil numbers from the same volume of blood was observed, and the number of neutrophils has also been variable between days of the same donor. For instance, 5×10^7 cells (neutrophil donor A), 2×10^7 cells (neutrophil donor B) and 2.5×10^7 cells (neutrophil donor C) were isolated from 15 ml of whole blood. In addition, 2×10^7 cells (day 1), 1.25×10^7 cells (day 2), and 3.5×10^7 cells (day 3) from 5 ml of whole blood from the same neutrophil donor. Though the number of neutrophils was variable between donors or days, the purity of the isolated neutrophils was >98%.

4.6 Future directions

From all discussion points in this chapter, the ADNP assays were done against placental binding IE (CS2-IE) and VAR2CSA DBL2, DBL3 and DBL5 antigens. For further investigations, all the 6 recombinant DBL domains as well as other types of parasite lines, should be tested for a complete picture.

4.7 In conclusion

There were differences of ADNP of IE and DBL2 as demonstrated by comparing the two infected groups (no PM and PM), which is illustrated in figure 4.9 and 4.16. ADNP of DBL2 coated beads and IEs was able to separate our groups. ADNP to recombinant DBL antigens was significantly correlated between neutrophil donors but ADNP to whole parasite antigens was weakly correlated. The current high throughput ADNP assay may be an important tool to measure acquired immunity to malaria.

5 Chapter 5: Neutrophil extracellular traps in response to *Plasmodium falciparum* parasites

5.1 Background

Previous studies have shown that microbial pathogens (116, 169, 170), mitogen phorbol myristate acetate (PMA), lipopolysaccharide (LPS) (161, 163, 164), calcium ionophore (181) and inflammatory cytokines and chemokines (163, 164) can promote the formation and release of neutrophil extracellular traps (NETs). NETs are extracellular structures composed of decondensed chromatin and histones decorated with different antimicrobial granular proteins (148-150). NETs have been shown to be important in the human immune response to pathogens such as bacterial, fungal, viral, and protozoan infections (116, 169, 170). The role of NETs in the innate immune response to malaria has not been characterized in detail, and mechanisms by which malaria-infected erythrocytes (IEs) alter NET formation are largely unknown. Knowing the mechanism of NET formation is crucial to understand the role of NETs in malaria pathogenesis.

Previous studies demonstrated an *in vivo* formation of NETs in response to *Plasmodium* parasites to trap circulating parasites. This trap structure has been reported to be detectable in the blood of children with uncomplicated malaria (120), adults with symptomatic malaria (176), and in the mouse malaria species *P. berghei* (174) and *P. yoelii* NL (303). A recent study showed that *P. falciparum* infection induces accumulation of NETs in circulation (304), in their study neutrophils were exposed to IEs, free merozoites, and parasite digestive vacuoles (DVs), as well as heme, and only heme forms NETs in neutrophils primed by TNF- α (304). However, the *in vitro* capacity of *P. falciparum* to induce NETs formation was not evaluated. Furthermore, *Plasmodium* parasites have been reported to induce *in vivo* NET formation, while an inhibitory or degradative effect on NET formation is not reported. A previous study reported that *P. falciparum* releases deoxyribonuclease (DNase) to counteract NETs (183), suggesting *P. falciparum* may possess escape mechanisms enabling it to evade several immune responses. Thus, the aim of the current study is to investigate whether *P. falciparum* IEs induce NET formation and how IEs could influence NET release by human neutrophils. A stimulatory or an inhibitory effect of *P. falciparum* on NET formation was investigated.

5.2 Materials and Methods

5.2.1 Primary Human Neutrophils and ethical considerations

Ethical approval was obtained from the Human Research Ethics Committee of Melbourne Health. Each participant was recruited and interviewed in accordance with Research Ethics Committee approval. Once signed informed consent has obtained a sample of peripheral whole blood (5-10ml) was collected. All peripheral blood samples were collected by a trained clinician or phlebotomist into plastic heparin blood collection tubes. Neutrophils were purified ($\geq 98\%$ purity) using EasySep direct human neutrophil isolation kit according to the manufacturer's instructions, as described in detail in the general thesis method **section 3.2.7.2**.

5.2.2 Parasite culture and synchronization

The laboratory adapted *P. falciparum* parasite lines CS2 (placental-binding), Skeleton-binding protein 1 knockout parasites (CS2 SBP1KO) and green fluorescent protein expressing *P. falciparum* parasite line (CS2-GFP) were cultured in 25 ml petri dishes in RPMI-HEPES medium supplemented with Albumax and heat-inactivated non-immune human sera, as described in **section 3.2.6.1**. Sorbitol and gelatin treatment was performed every 2 or 3 weeks as needed to select the ring and trophozoite stages of IEs respectively. The detailed procedures are described in **section 3.2.7.3 and 3.2.7.2** respectively

5.2.3 Merozoite purification

Merozoite purification was performed as described elsewhere (132, 250), with some modifications. The detailed procedures are described in **section 3.2.6.5** of the general methods of this thesis.

5.2.4 Opsonization of *P. falciparum* infected erythrocytes

P. falciparum IEs (1×10^6 cells/well) were opsonized with antibodies (pooled positive sample, PPS 3.3 μ l / well (purified antibodies from malaria-infected PNG pregnant women) for 30 min at room temperature. Afterward, the IEs were washed with RPMI-HEPES at room temperature for 5 min at 252 x g to remove any unbound antibody. Subsequently, unopsonized and opsonized IEs were used in the experiments of interaction with neutrophils.

5.2.5 Induction and quantification of NETs

NET induction assay was performed in Costar 24-well cell culture plates containing 13 mm round glass coverslips. Neutrophils were incubated with varying ratios of IEs, as described in detail in the general method **section 3.3.1.1**. NETs were observed by confocal microscopy. NET formation was also quantified fluorometrically using the membrane-impermeable fluorescent dye, SYTOX green, as described previously (116, 255-257), as described in **section 3.3.1.3**. NET inhibition effects of IEs and merozoites (NET inhibition assay) were done as described in **section 3.3.1.4**.

5.2.6 ELISA to quantify myeloperoxidase release

ELISA was used to quantify myeloperoxidase (MPO) release as a measure of neutrophil degranulation from cell-free supernatant using DuoSet ELISA kits, details in **section 3.3.4**.

5.2.7 HL-60 cell lines differentiation and NET induction

DMSO differentiated human leukemia cell lines (HL-60 cells) were evaluated for NET production. The HL-60 cell lines are considered an alternative cell culture model to study neutrophil functions (305). The myeloid leukemia cell line HL-60 cells were differentiated into neutrophil-like cells using 1.3% dimethyl sulfoxide (DMSO). HL-60 cells were passaged on the previous day before stimulation and resuspended so that the cell density is 1×10^5 cells/ml in fresh Iscove's Modified Dulbecco's Media (IMDM) (500 ml IMDM, 20% FBS, 1% P/S and G). Then, sterile DMSO was added to a final concentration of 1.3% and cells were cultured in a 75 cm² TC flask at 37°C, 5% CO₂ for 5 days.

For experiments, differentiated cells were harvested by centrifugation at 500 X *g* for 10 minutes at room temperature and washed once with the medium used for NET experiments. For NET induction assay, 1×10^5 stimulated HL-60 cells in HL-60 medium (IMDM, 20% FBS, 1% PSG), 100 µl/well, was added into a 96 well black plate. NET formation or inhibition was quantified fluorometrically using the membrane-impermeable fluorescent dye, SYTOX green (a final concentration of 10 µM), described in detail in **section 3.3.1.3**.

5.2.8 Statistical analysis

Data were analysed by GraphPad Prism software (Version 5), and are presented as mean ± SEM. Paired t-test was used to compare the mean of two groups (when combining results

of different experiments) and the Mann-Whitney test was used to compare two independent groups. Results were considered significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

5.3 Results

5.3.1 Observation and quantification of NETs with *P. falciparum* stimulation

To evaluate whether *P. falciparum* is capable of inducing NETs, isolated human neutrophils were exposed to *P. falciparum* IEs and evaluated by microscopy and fluorometric measurements.

5.3.1.1 Microscopic observation of NETs formation *in vitro*

To evaluate the ability of IEs to induce NETs by human neutrophils microscopically, neutrophils (1×10^5 cells/well in 100 μ l) were placed on a cover glass for 1 h to allow cell attachment. Then, *P. falciparum* IE constitutively expressing the Green Fluorescent Protein (GFP) throughout the life cycle were added and incubated for 3 h. Glass coverslips containing IE-stimulated neutrophils were stained with DAPI and placed on glass microscopic slides (face down), and NETs formation was evaluated by confocal microscopy.

The result of the current study shows only very limited NET formation when neutrophils were cocultured with IEs (figure 5.1D) compared with PMA-induced NET formation (figure 5.1C). Interestingly, we also observed that the nucleus of neutrophils stimulated with IE was observed as clearly as the nucleus of unstimulated cells (figure 5. 1 A and B). When viewed on higher magnification, some GFP-expressing *P. falciparum* IEs were observed to be adherent around neutrophils, and a few *P. falciparum* IEs were phagocytosed by neutrophils (figure 5.1D). Given these findings, next we measured extracellular DNA fluorometrically as measure of NETs in the supernatant

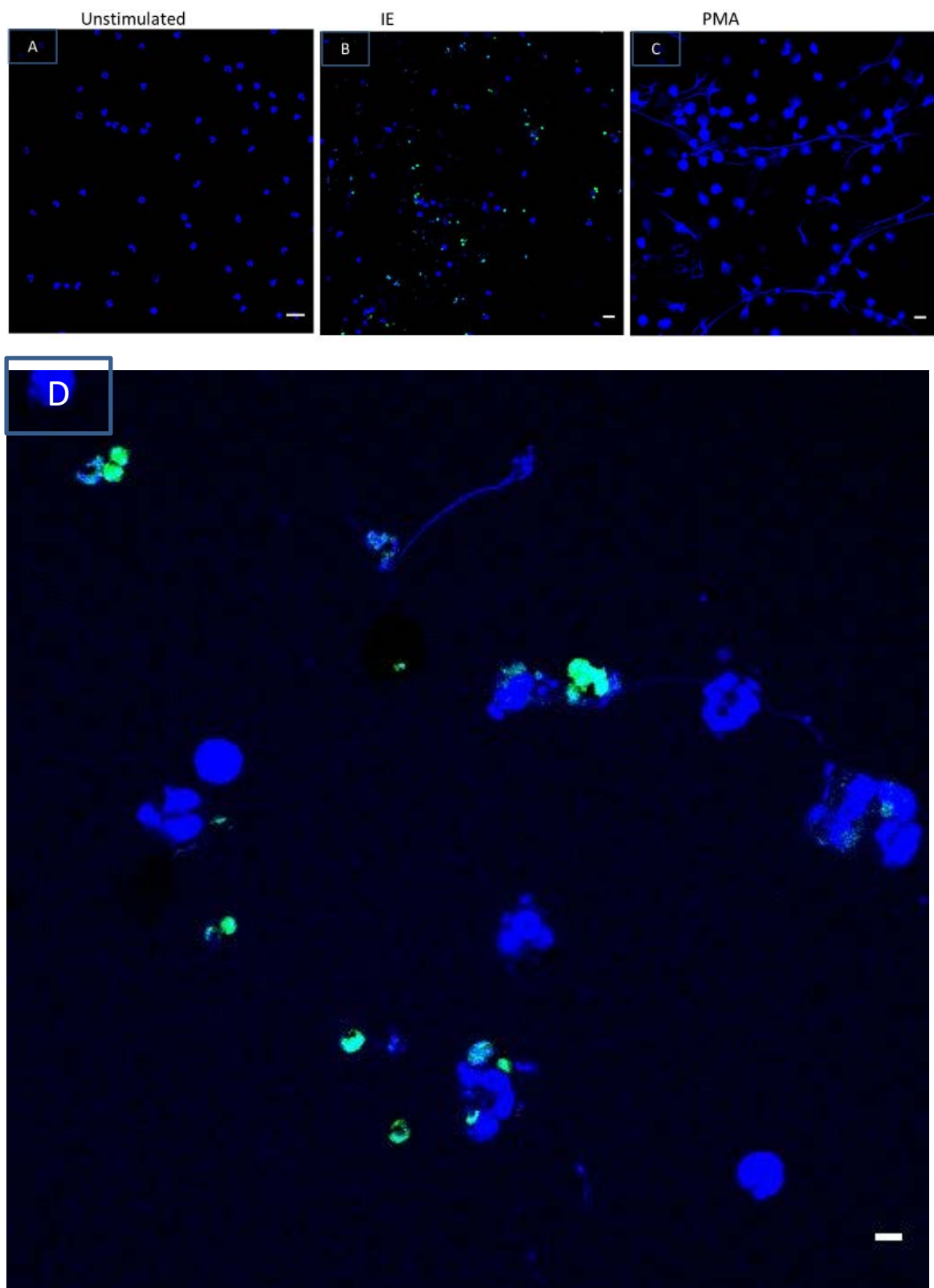


Figure 5 1 *P. falciparum*-infected erythrocytes induce very limited NETs formation by human neutrophils. Human neutrophils isolated by negative selection were cocultured with GFP-expressing *P. falciparum*-infected erythrocytes (green) for 3 h at 37°C, 5% CO₂. NETs formation was evaluated with DAPI staining (blue). Representative images, unstimulated (A), treated with IEs (B) treated with PMA (C) and enlarged images of B (D). Very limited NETs formation (red arrow), some GFP-expressing *P. falciparum* IEs adherent around neutrophils, and few *P. falciparum* IEs phagocytosed by neutrophils (white arrow) (E). Images are representative of 2 individual experiments. (A-C) 20x magnification. (D) 63x magnification of B. Scale bars 20 µm.

5.3.1.2 Fluorometric quantification of IE induced NETs formation

We sought to apply the fluorometric technique to detect NET formation in human neutrophils. A previous study has been reported that washing steps could potentially disrupt NETs formation (303). This suggests the fluorometric quantification of extracellular DNA as a measure of NETs formation from the supernatant solution. As stimulated neutrophils release their content to the extracellular environment, extracellular DNA release was quantified from the supernatant solutions fluorometrically.

The result of the current study showed that neutrophils stimulated with IEs and unstimulated neutrophils released similar levels of extracellular DNA into the solution suggesting no NETs formation ($P > 0.05$), whereas an increased amount of extracellular DNA was released after PMA stimulation as expected (185) (Figure 5.2).

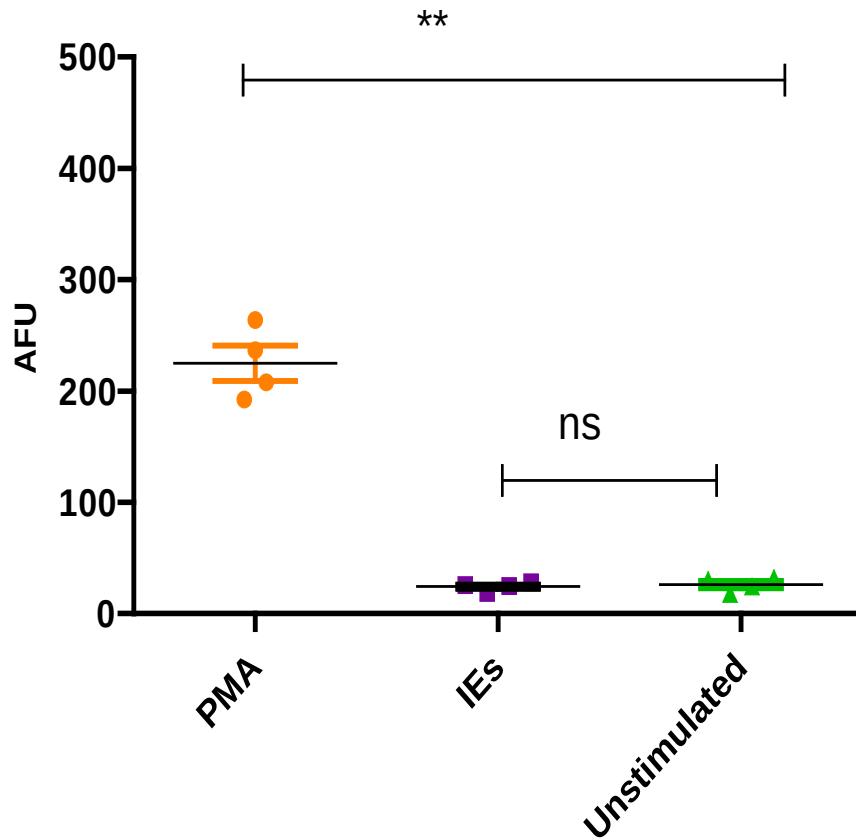


Figure 5 2 *P. falciparum*-infected erythrocytes (IEs) did not induce NET formation. Neutrophils were stimulated with IEs for 5 h and the signal of SYTOX green, a final concentration of 10 Mm, stained extracellular DNA in the solution was measured. The amount of fluorescence corresponds to the amount of extracellular DNA present as a result of NET formation. Four separate experiments were performed using neutrophils from 4 different donors, with triplicate assays performed within each experiment. The results are expressed as fluorescence relative to unstimulated neutrophils and are presented as arbitrary fluorescence units (AFU).; mean $\pm$ SEM; ** significant difference ($p < 0.01$), ns= no significant difference ($P \geq 0.05$), paired T-test.

5.3.1.3 *Parasite density and opsonization of IE with human IgG does not change neutrophil response towards NETs formation*

Previously, it has been reported that NETs are induced by the recognition of stimulants by multiple receptors on the neutrophil or following opsonic phagocytosis (306). We aimed to investigate whether opsonization of IEs with human IgG change neutrophil response to IE for releasing NETs compared with unopsonized IE. To do this, human neutrophils were incubated with IEs with or without opsonizing antibody (purified antibody from malaria infected women), the signal of SYTOX green-stained extracellular DNA in the solution was measured. In this study, NETs formation was not observed in the presence of both opsonized and unopsonized IE compared to PMA stimulation (Figure 5.3).

To evaluate the influence of parasite density on NETs formation increasing ratios of IEs (1:5, 1:10 and 1:20) were cocultured with neutrophils for 5 h. In the present study, all varying ratios of IEs failed to induce NETs formation compared to PMA stimulated neutrophils (Figure 5.4). Given these findings, we wanted to know if the parasite has same inhibitory effect on NET formation.

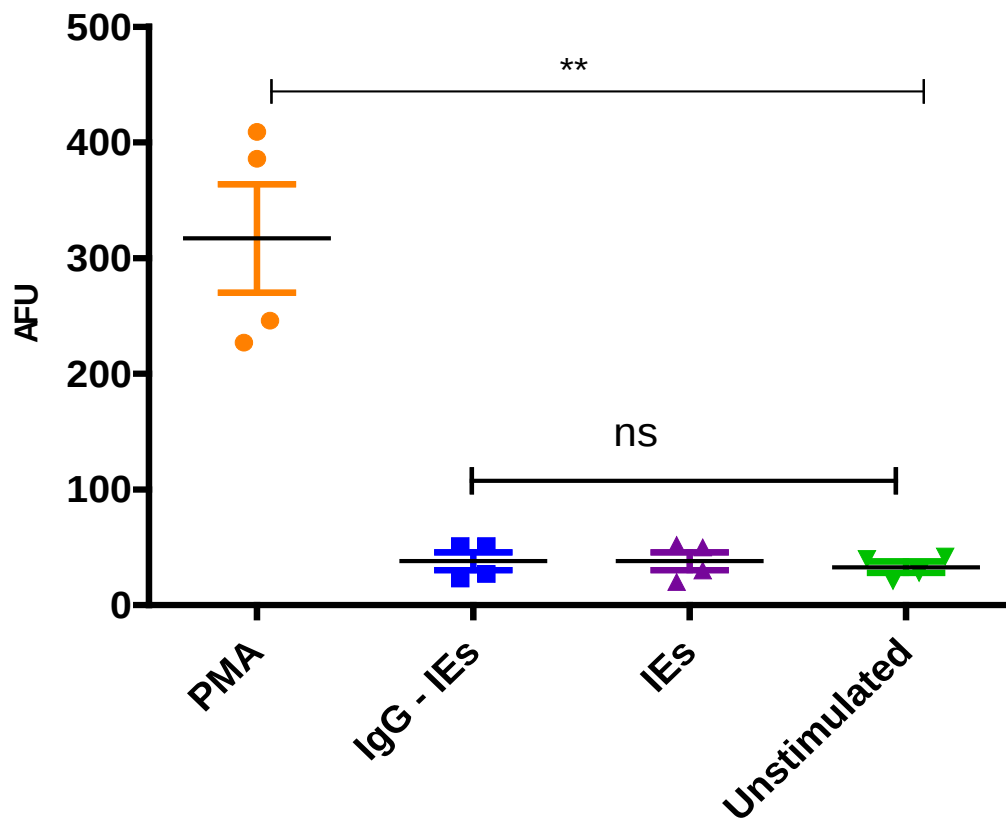


Figure 5 3 Opsonization did not enhance IE-induced NET formation. Neutrophils were stimulated with opsonized or unopsonized IEs for 5 h. The signal of SYTOX green-stained extracellular DNA in the solution was measured by plate reader. The amount of fluorescence corresponds to the amount of extracellular DNA present as a result of NET formation. Four separate experiments were performed using neutrophils from 4 different donors, with triplicate assays performed within each experiment. The results are expressed as fluorescence (AFU) relative to unstimulated neutrophils; mean $\pm$ SEM: ns=no significant difference (significant difference = $P \geq 0.05$), paired T-test.

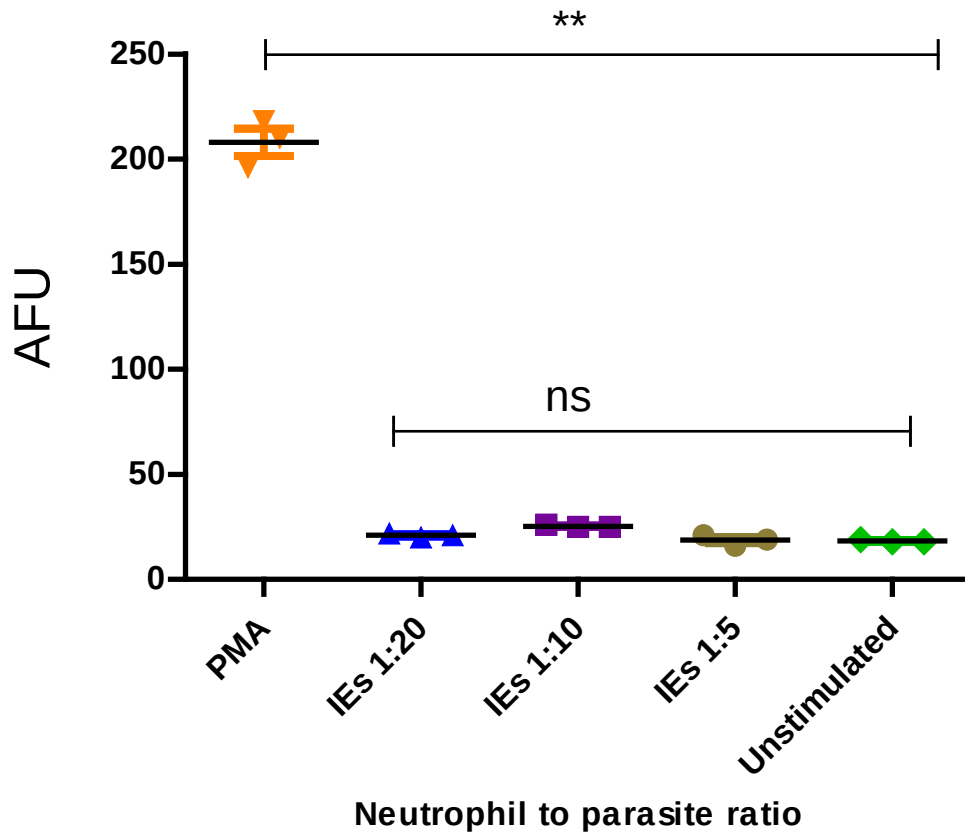


Figure 5 4 NET formation did not increase with parasite density. PMA-stimulated neutrophils and unstimulated neutrophils were used for positive and negative controls respectively. Neutrophils were incubated with varying ratios of IE for 5 h. The signal of SYTOX green-stained extracellular DNA in the solution was measured. The amount of fluorescence corresponds to the amount of extracellular DNA present as a result of NET formation. Three separate experiments were performed using neutrophils from 3 different donors, with triplicate assays performed within each experiment. Data presented as fluorescence relative to unstimulated neutrophils; mean $\pm$ SEM; ns = no significant difference ($P \geq 0.05$), paired T-test.

5.3.2 *P. falciparum* inhibits PMA-induced NET formation

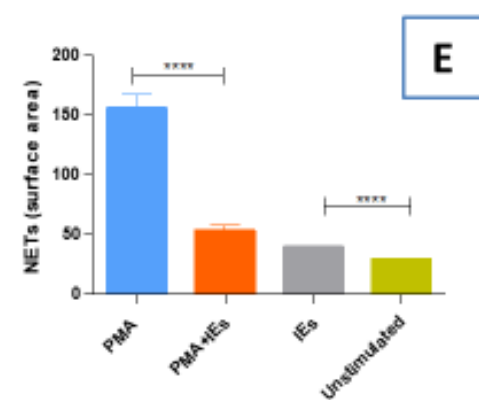
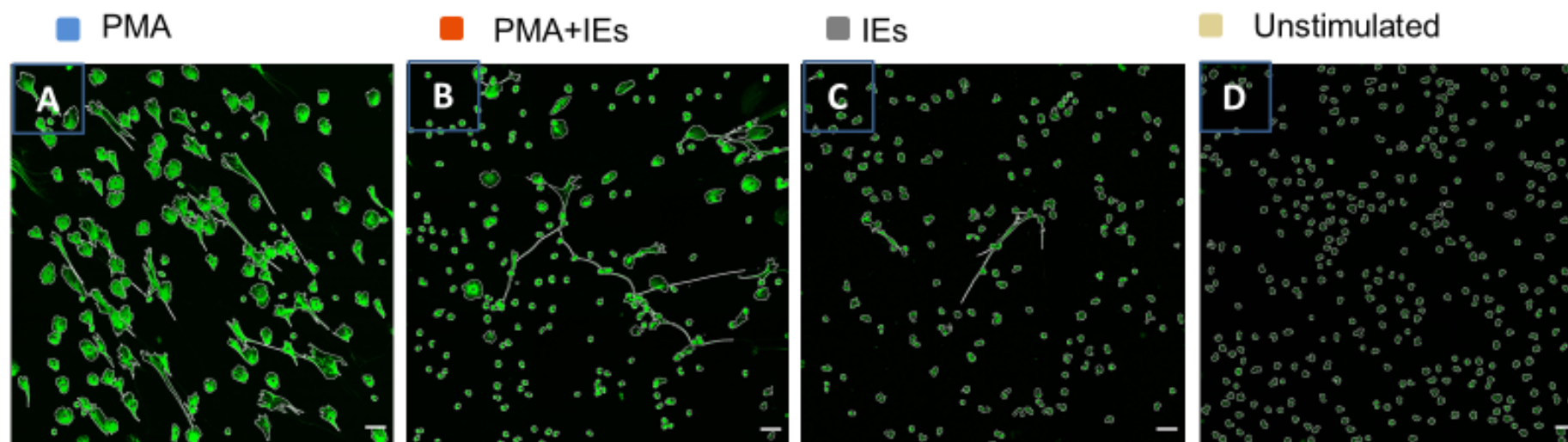
5.3.2.1 *Microscopic Quantification of PMA-induced NET inhibition*

To investigate whether IEs have an inhibitory effect on NET formation, human neutrophils were stimulated with IEs, PMA, both or neither for 3 h on a cover glass slide using 24-well plates. The DNA was stained with SYTOX green (cell membrane-impermeable dye) and DAPI (which stains both intracellular and extracellular DNA), and NETs formation was visualized with confocal microscopy (magnification of 63x). Images were captured (tile scan 3x3 and Z-stack with four steps of 1.5 microns) from different areas of each coverslip to accurately represent the effect of the stimulus. Every image had the same X, Y, and Z dimensions (Figure 5.5).

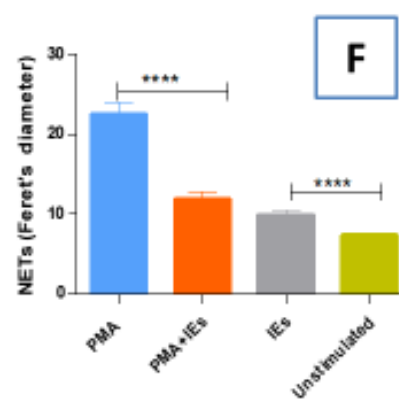
The result of the present study demonstrated that most unstimulated neutrophils stained with DAPI and were SYTOX green negative (figure 5.5D), whereas neutrophils stimulated with IE were mostly positive for SYTOX green, although they displayed normal lobulated nuclei (figure 5.5C). In this study, no evidence of increase in SYTOX green fluorescence was observed when neutrophils coculture IEs suggesting no NET formation. The formation of NETs increased substantially upon stimulation with PMA (figure 5.5A) but NETs formation by IEs was comparable to unstimulated control (figure 5. 5C).

Inhibition of NET formation was observed when neutrophils cocultured with IEs in the presence of PMA (figure 5.5B), compared with neutrophils stimulated with PMA alone (figure 5.5A). The percentage of PMA-induced NETs formation inhibition (based on cell total area, Feret diameter, and perimeter) by IE was quantified, as described in detail in the methods **section 3.3.2.1**. When the total surface area of the cell was measured, neutrophils treated with IEs in the presence of PMA have significantly reduced cell surface area compared to PMA alone (E). PMA-induced NET inhibition by IE was also analyzed based on cell elongations (F) and shape differences (G) (also called Feret's diameter and perimeter respectively) and a significant reduction of NET formation was observed.

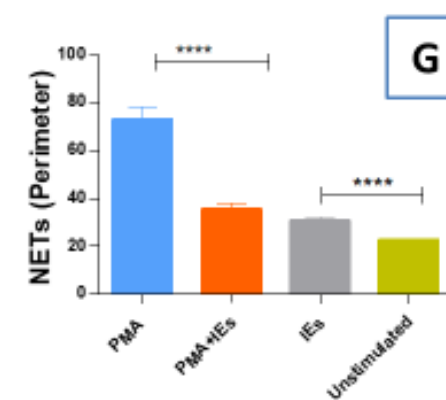
In a microscopic analysis, significantly higher NET formation (larger cell surface area, Feret's diameter and perimeter) was detected on IE stimulated neutrophils than unstimulated neutrophils without affecting nuclear morphology (Figure 5.5E-G).



Surface area



Feret's diameter



Perimeter

Figure 5 5 *P. falciparum*-infected erythrocytes (IEs) inhibit PMA-induced NETs formation. Representative images of NETs visualized by co-staining with SYTOX green and DAPI following treatment with PMA alone (A), PMA + IEs (B), IEs (C) and unstimulated neutrophils (D) are shown. Human neutrophils were treated with IE in the presence of PMA for 3 hours on a cover glass slide and NETs formation was visualized with confocal microscopy (magnification of 63x). Inhibition of PMA-induced NET formation by IEs was quantified using SYTOX green and DAPI (B). NETs formation was quantified by quantifying stained extracellular DNA over 9 Z-stacks. The percentage of NETs formation based on cell total area (E), Feret diameter (F) and perimeter (G) were quantified by Fiji software are shown. Assays were performed in duplicate and are representative of 3 separate experiments using neutrophils from 3 different donors. Results are expressed as mean $\pm$ SEM. * P <0.05, ** P <0.01, *** P <0.001 **** P <0.0001, paired T-test. Scale bars 50 μ m

5.3.2.2 Fluorometric quantification of IE NET inhibition

Next, cell-free supernatant was tested for nucleic acid contents using SYTOX™ Green dye. In this study, no increase of extracellular DNA levels was observed in neutrophils incubated with IE compared with unstimulated neutrophils ($P > 0.05$) (figure 5.6). In agreement with microscopic results, incubation of neutrophils and IEs in the presence of PMA significantly inhibited the release of NETs compared to stimulation of neutrophils with PMA alone, $p = 0.045$, *paired t-test* (figure 5.6A). Similarly, neutrophils were incubated with IEs in the presence of LPS and a similar inhibitory effect on LPS-induced NET formation was observed, $p = 0.0013$, *paired t-test* (figure 5.6B). To investigate whether NET inhibition is parasite-specific, neutrophils were incubated with IEs or uRBC in the presence of PMA. We observed no effect on PMA induced NET formation when neutrophils were cocultured with uRBC in the presence of PMA (figure 5.6A).

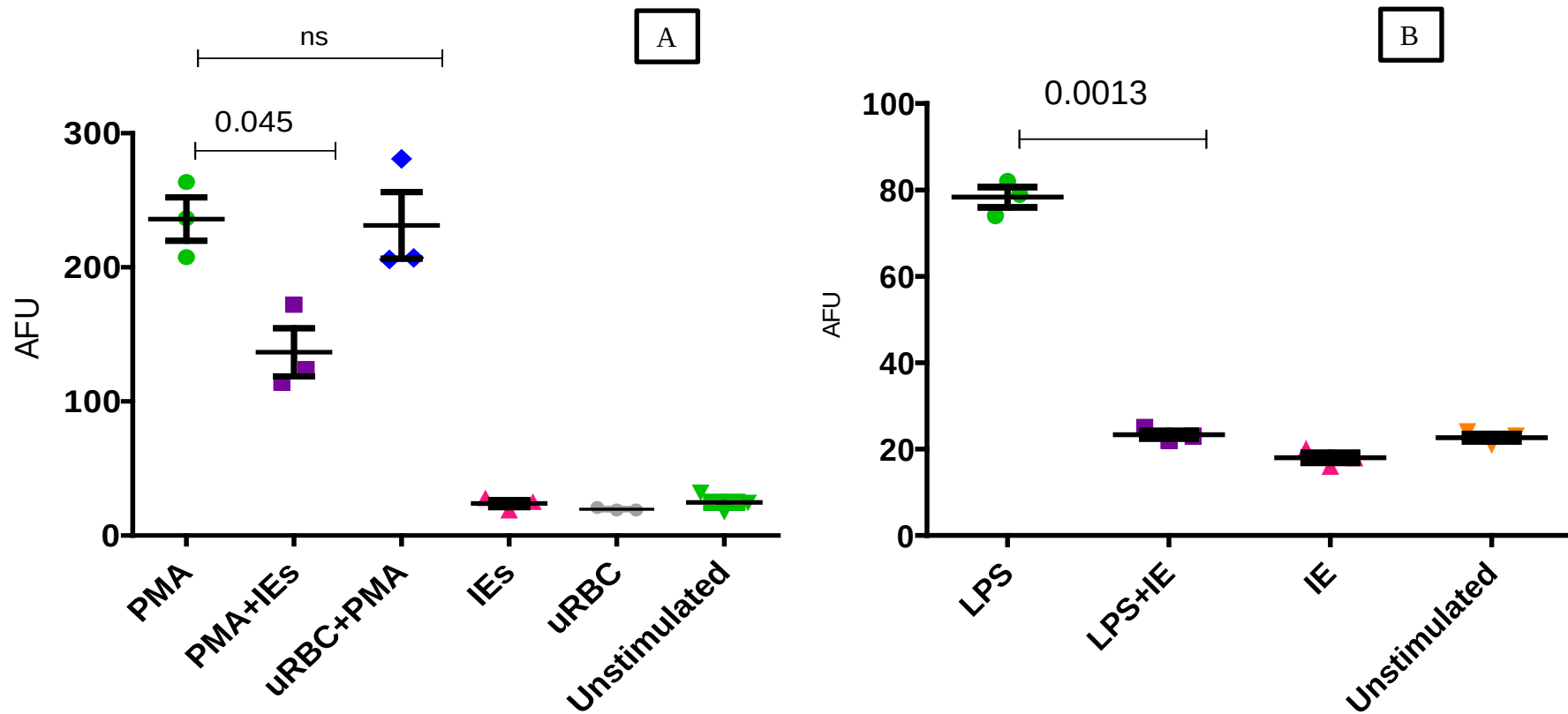


Figure 5 6 Effect of IE on PMA-and LPS-induced NET formation. Neutrophils were stimulated with PMA or LPS for 4 h with or without IEs (10:1 ratio). Unstimulated neutrophils were used as negative controls and PMA and LPS alone stimulated neutrophils were used as a positive control. Three separate experiments were performed using neutrophils from 3 different donors, with triplicate assays performed within each experiment. Data presented as mean $\pm$ SEM; $P < 0.05$ (A) and $P < 0.0013$ (B), paired T-test.

We next aimed to investigate whether the parasite disintegrates the already formed NET. To do this, neutrophils were stimulated with PMA for 4 h, and then IEs were added to PMA stimulated neutrophils for 2 hours and the signal of SYTOX green-stained extracellular DNA was measured. As observed, PMA-induced NET was not degraded by IEs after 2 h of exposure to IE (figure5.7).

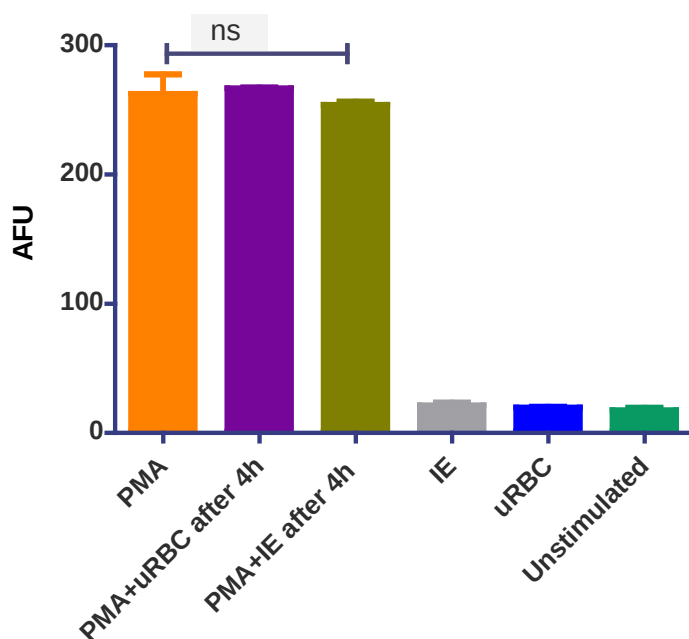


Figure 5 7 IE did not degrade PMA- induced NETs. The bar graph shows one triplicate assay result performed using one neutrophil donor. Neutrophils were stimulated with PMA, and IEs or uRBCs were added after 4 h of incubation. Neutrophils stimulated with PMA alone were used as positive control and unstimulated neutrophils or uRBCs as a negative control. The signal of SYTOX green-stained extracellular DNA in the supernatant was measured after 2 h of incubation with IEs. The amount of fluorescence corresponds to the amount of extracellular DNA present as a result of NET formation and data was presented as mean $\pm$ SEM; ns=no significant difference ($P > 0.05$), paired T-test.

5.3.2.3 *P. falciparum* IEs inhibit PMA-induced NET formation in a time and dose-dependent manner.

To investigate whether IE could inhibit NETs formation in a time and dose-dependent manner, neutrophils stimulated with PMA were co-cultured with varying ratios of IEs (figure 5.8). To quantify the amount of the released extracellular DNA, the signal of SYTOX green-stained DNA in the medium was measured at different time points for 5 h. The amount of extracellular DNA increased after PMA stimulation, however, the degree of NETs inhibition increases with increasing parasite density and the time of incubation (figure 5.8).

PMA is a potent NET inducer frequently used to induce NETosis by activating protein kinase C (PKC) and NADPH oxidase, which generates ROS (116), whereas some other inducers induce NETosis in a receptor-mediated fashion (307) and NADPH oxidase independent manner (181). To evaluate whether IEs inhibit the other pathways, neutrophils were incubated with IEs in the presence of PMA or LPS. Our finding showed that IE inhibited PMA-induced, but not LPS-induced, NET formation, figure 5. 9.

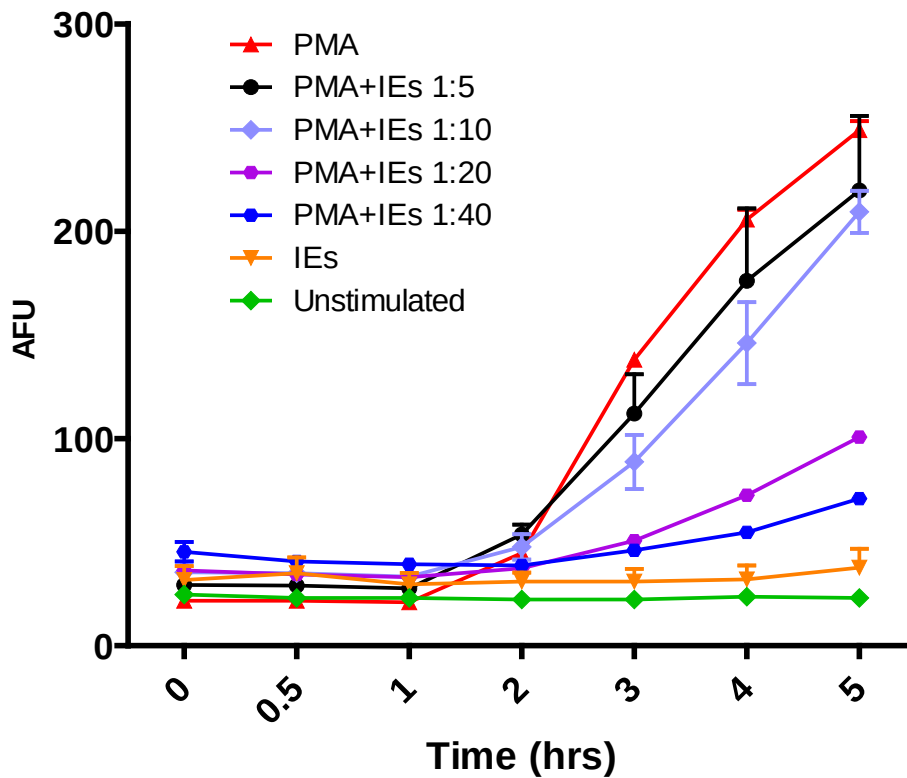


Figure 5 8 Parasite density-dependent inhibitory effect of IE on PMA induced NET formation over time. Neutrophils were cocultured with increasing ratios of IEs (MOI of 5, 10, 20 and 40) in the presence of PMA for 5 h. Neutrophils stimulated with PMA alone were used as positive control and unstimulated neutrophils as a negative control. The signal of SYTOX green-stained extracellular DNA in the supernatant solution was measured every 1 h for 5 h. The line graph shows the inhibitory effects of IE on PMA-induced NETs formation in a dose and time-dependent manner. Three separate experiments were performed using neutrophils from 3 different donors, with triplicate assays performed within each experiment. Comparative analysis was done by Two-way analysis of variance (ANOVA). Data was presented as mean $\pm$ SEM and statistical significance determined as p value < 0.05 . Asterisks indicate significance: ***P < 0.001 , ****P < 0.0001 .

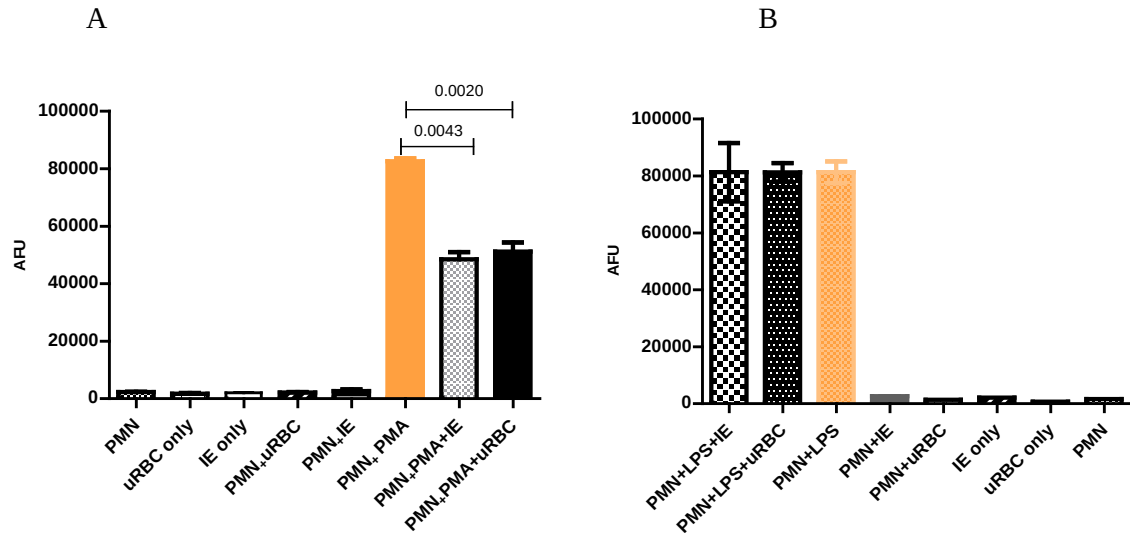


Figure 5 9 Effect of IE on PMA- and LPS-induced NET formation. Neutrophils were stimulated with PMA (A) or LPS (B) for 5 h with or without IEs (10:1 ratio). Unstimulated neutrophils were used as negative controls and neutrophils stimulated with PMA or LPS alone were used as a positive control. One experiment was performed in triplicate using neutrophils isolated by negative selection. Mann-Whitney test was used to compare two independent groups. Data was presented as mean $\pm$ SEM and statistical significance determined as p value < 0.05 . Asterisks indicate significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

5.3.3 Merozoites have no effect on NET formation and do not inhibit PMA induced NET formation

We next evaluated the effect of purified merozoites on NET formation or PMA-induced NET inhibition. Human neutrophils were stimulated with purified merozoites with or without PMA. To quantify the amount of the released extracellular DNA, the signal of SYTOX green-stained DNA in the solution was measured at different time points for 5 h. PMA was used as a positive control and unstimulated neutrophils and merozoite alone were used as a negative control. When assessing the effect of merozoites on NET formation neutrophils were exposed to positive controls, IEs and merozoites parallelly. The results of the present study showed that merozoites have no effect on NET formation, and merozoites did not inhibit PMA-induced NET release (figure 5.).

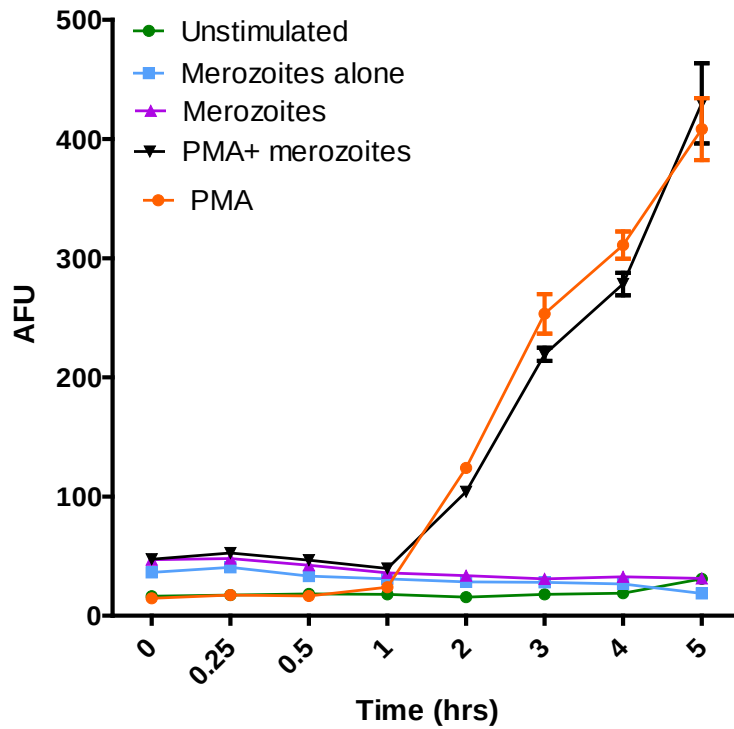


Figure 5 10 Time course of NET formation and the effect of merozoites. Neutrophils were cocultured with purified merozoites and/or in the presence of PMA for 5 h. The line graphs show the merozoite time-dependent effects on extracellular DNA signal, after staining with SYTOX green. Neutrophils stimulated with PMA alone were used as positive control and unstimulated neutrophils as a negative control. Neutrophils were exposed to positive controls, IEs and merozoites parallelly. The signal of SYTOX green-stained extracellular DNA in the supernatant was measured every 1 h for 5 h. Three separate experiments were performed using neutrophils from 3 different donors, with triplicate assays performed within each experiment. Data was presented as mean $\pm$ SEM and statistical significance determined as p value < 0.05 . Asterisks indicate significance: ***P < 0.001 , ****P < 0.0001 .

5.3.4 Effects of skeleton binding protein 1 knock out (SBP1KO) *P. falciparum* IEs on NET formation

Inhibition of NET formation was evaluated using skeleton-binding protein 1 knock out (SBP1KO) parasites IEs. The skeleton binding protein 1 (SBP1) has been shown to be required for transport of PfEMP1 to the red cell membrane. We hypothesized that the PfEMP1 on the surface of IE may influence IE-induced NET formation. To evaluate the hypothesis, neutrophils and *P. falciparum* IEs (SBP1KO) with varying ratios of IEs were cocultured with or without PMA at 37 °C, 5% CO₂. Neutrophils stimulated with PMA alone was used as positive control and unstimulated cells and IE alone as a negative control. To quantify the amount of extracellular DNA release as components of NETs, the signal of SYTOX green-stained DNA in the supernatant solution was measured for up to 5 h. The result of this study showed that the coculturing of SBP1KO IEs with human neutrophils was unable to induced NET formation, but IE (SBP1KO) was effective in inhibiting PMA-induced NETs formation. The amount of extracellular DNA increased after PMA stimulation, however, the degree of PMA induced NETs inhibition increases with increasing parasite density (figure 5.11).

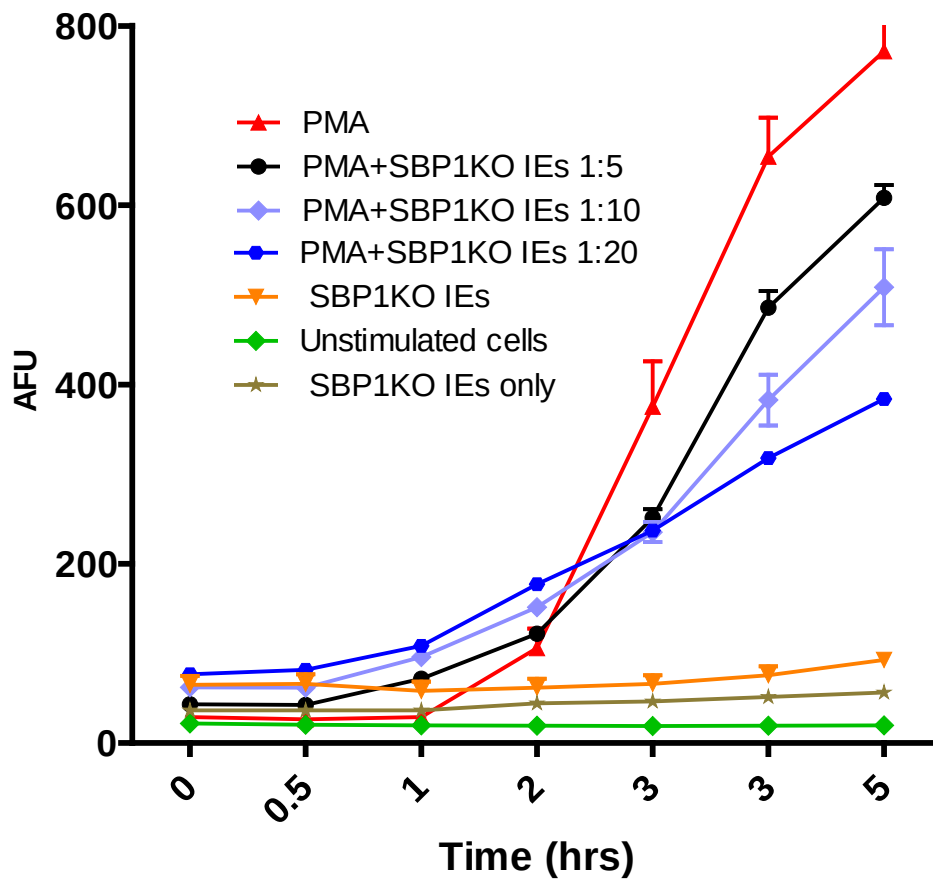


Figure 5 11 Skeleton binding protein 1 knockout (SBP1KO) IE inhibit PMA-induced NETs formation. Neutrophils and increasing ratios of SBP1 KO (MOI of 5, 10, 20 and 40) were cocultured with or without PMA. The signal of SYTOX green-stained extracellular DNA in the supernatant solution was measured every 1 h for up to 5 h. The line graph shows the inhibitory effects of IE on PMA-induced NETs formation in a dose and time-dependent manner. Three separate experiments were performed using neutrophils from 3 different donors, with triplicate assays performed within each experiment. Data was presented as mean $\pm$ SEM and statistical significance determined as p value < 0.05 . Asterisks indicate significance: ***P < 0.001 , ****P < 0.0001 .

5.3.5 In HL-60 cells, *P. falciparum* IE induce NET formation and do not inhibit PMA-induced NET formation

Next, the NET inhibitory effect of IE was evaluated using the human leukemia cell line HL-60 cells, which can be induced to differentiate into mature, neutrophil-like myeloid cells when treated with dimethyl sulfoxide (DMSO) (308). The HL-60 cell line is considered an alternative cell culture model to study neutrophil functions. HL-60 cells and different ratios of IEs were cocultured with or without PMA. The signal of SYTOX green-stained extracellular DNA in the supernatant was measured, and the amount of extracellular DNA release was quantified up to 6 h and after overnight incubation.

IE stimulated HL-60 cells formed NETs to a similar extent to PMA after 5 h of incubation. After overnight incubation, IE induces significantly higher NET formation compared to PMA. NETs formation by HL-60 cells in response to IE correlates with an increase in incubation time but was not dependent on parasite density. Interestingly, we observed no inhibitory effect of IEs on PMA-induced NETs formation by HL-60 cells (figure 5.12 A and B). Since the timeline limited us to repeat this assay experiments could be designed to check the repeatability of the results.

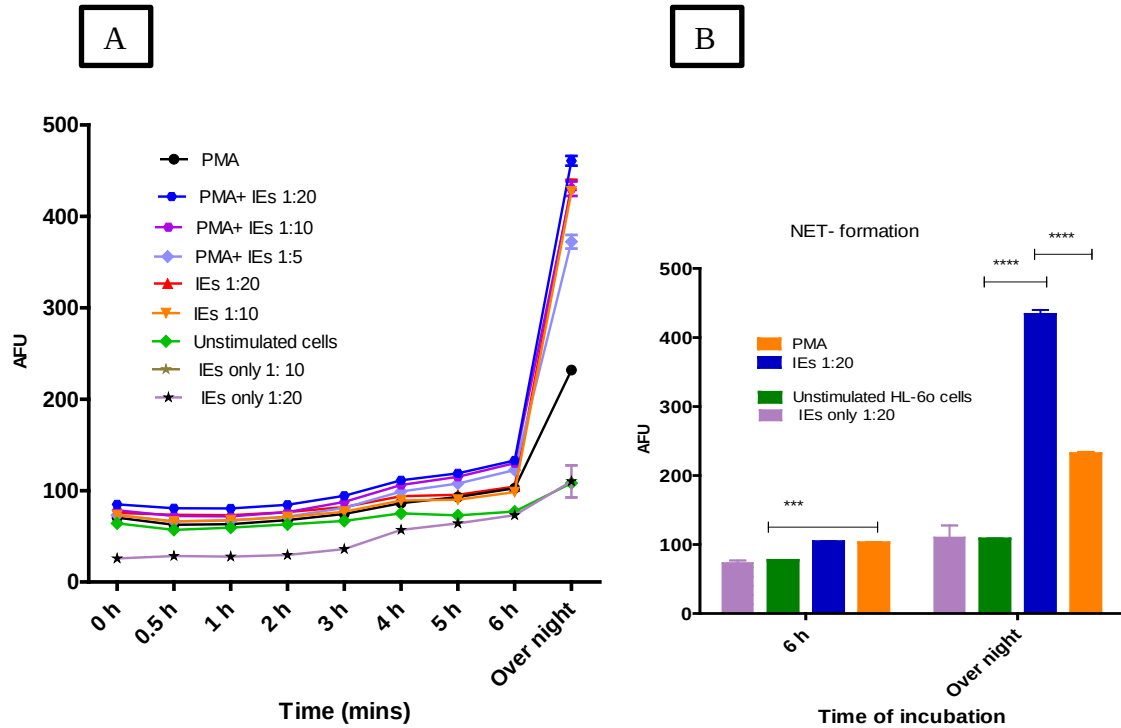
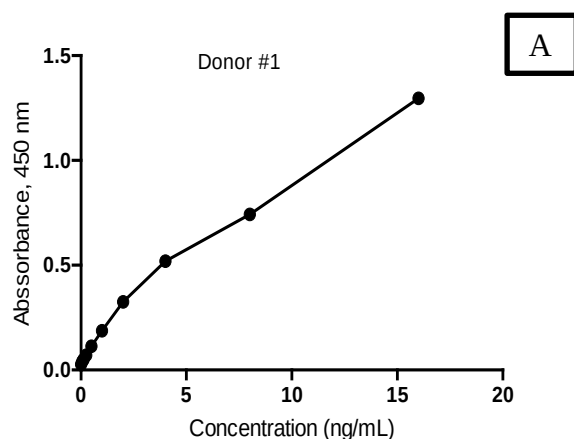


Figure 5 12 Differentiated HL-60 cells form extracellular traps in response to *P. falciparum* IE. HL-60 cells were differentiated into neutrophil-like cells with DMSO and then cocultured with IEs. The signal of SYTOX green-stained extracellular DNA in the solution was measured. (A) Time-course analysis of NET formation. (B) Results from one experiment are shown as mean and SEM. Comparisons between unstimulated and stimulated cells were performed by Two-way analysis of variance (ANOVA). Asterisks indicate significance: *** $P < 0.001$, **** $P < 0.0001$.

5.3.6 IEs, but not merozoites, inhibit myeloperoxidase (MPO) release by human neutrophils

We next investigated the effect of *P. falciparum* IEs and purified merozoites on activation of neutrophils by PMA, by using MPO as a marker of neutrophil activation. Extracellular MPO release was measured by ELISA in supernatants of IE and merozoite stimulated human neutrophils. To do this, neutrophils were stimulated with IEs or merozoites in the presence of PMA for 4 h and supernatants were collected. Neutrophils stimulated with PMA were used as positive controls and unstimulated neutrophils as negative controls. IE inhibited the extracellular release of MPO by PMA-stimulated neutrophils, as compared to neutrophils stimulated with PMA alone ($P < 0.05$) (figure 5.13B). By contrast, merozoites did not inhibit the release of MPO from PMA-stimulated neutrophils incubated for 4h ($P > 0.05$). The concentration of MPO in samples was calculated by extrapolation from a standard curve (figure 5.13A).



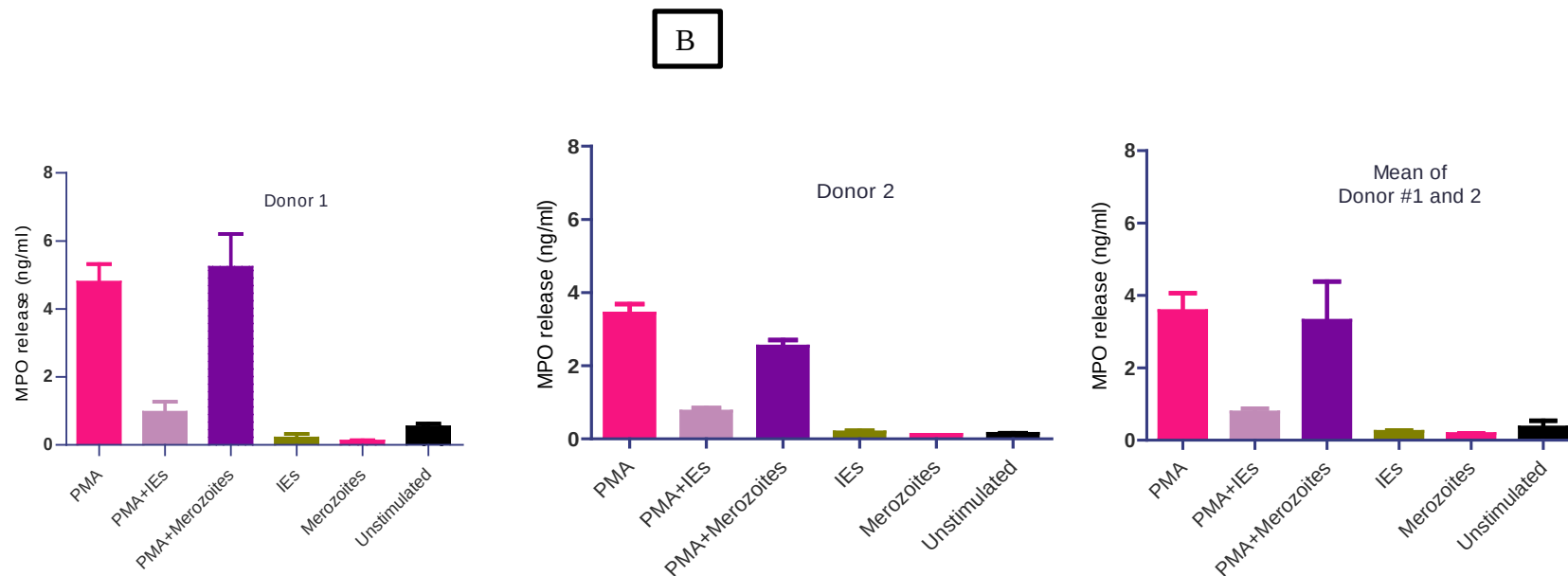


Figure 5 13 *P. falciparum*-infected erythrocytes, but not merozoites, inhibit neutrophil degranulation. Neutrophils were stimulated with mature IEs or merozoites in the presence of PMA for 4 h. Neutrophils stimulated with PMA alone was used as positive controls and unstimulated neutrophils as a negative control. The supernatant was collected and the level of MPO was measured by ELISA. Two separate experiments were performed using neutrophils from 2 different donors, with duplicate assays performed within each experiment. The concentration of MPO in samples was calculated by extrapolation from a standard curve

5.4 Discussion

In the present study, we investigated *in vitro* human neutrophil responsiveness to *P. falciparum* parasites (both IEs and merozoites), focusing on NET formation. The ability of IE to inhibit NET formation was also investigated. The NET release was monitored through a combination of microscopy (to assess changes in neutrophil morphology), ELISA (to quantify MPO release as a measure of neutrophil degranulation) and fluorometric-based assays (to assess extracellular DNA released from neutrophils). We identified that (i), incubation of *P. falciparum* IEs with neutrophils did not cause significant DNA release. By confocal microscopy, neutrophils stimulated with IEs displayed normal lobulated nuclei and showed only very limited NET formation; (ii), IEs were effective in inhibiting PMA-, CaI- and LPS-induced NET formation in a dose and time-dependent manner; (iii), IEs also inhibited neutrophil degranulation, decreasing PMA-induced MPO release; (iv), by contrast, *P. falciparum* merozoites did not inhibit NET formation or neutrophil degranulation, and (v), *P. falciparum* induced NET formation by HL-60 cell lines.

5.4.1 *P. falciparum* IE induce very limited NETs formation *in vitro*

Neutrophils, the most abundant white blood cells and the first phagocytic cells to be recruited to the site of infection, play a critical innate defense mechanism against malaria caused by *P. falciparum*. However, the specific mechanism by which neutrophils could be eliminating *P. falciparum* is far from clear. The *in vitro* study of neutrophil-*P. falciparum* interaction could lead to a better understanding of early aspects of innate immunity against *Plasmodium* parasites. Following the discovery of NETs as a neutrophil antimicrobial mechanism, which is an important component of extracellular killing, the list of microorganisms able to trigger NETs formation has increased (116), but very little is known regarding *P. falciparum* parasites to induce NETs.

In the current study, the microscopic evaluation demonstrated that neutrophils stimulated with IE displayed normal lobulated nuclei. Most unstimulated neutrophils stained with DAPI and were SYTOX green negative (Figure 5.5), whereas neutrophils stimulated with IE were mostly positive for SYTOX green. However, incubation of human neutrophils with PMA induced dramatic changes in morphology, resulting in NETs that stained positive with DAPI and SYTOX green, and demonstrated typical morphology of diffuse, spreading NETs. The possible reason why neutrophils stimulated with IE displayed normal nuclear

morphology but were positive for the cell membrane-impermeable dye, SYTOX green (309), could be that IE may impair the cell membrane of neutrophils without affecting the nuclear membrane, suggesting that cell membrane disintegration alone may not lead to NET release without nuclear decondensation and degranulation. During NET formation, nuclear morphology changed, and the nuclear membrane and the granule membranes disintegrate leading to the mixing of the NET components (148, 310), and release into the extracellular environment.

The result of our study showed that IEs induces only very limited NET formation by microscopy, and on fluorometric measurements, incubation of IEs and neutrophils did not cause significant extracellular DNA release suggesting no NET formation. Although our study revealed very limited NET induction by IEs alone, previous studies have reported apparent IE induced NET formation by human neutrophils *in vivo* (120, 176). For example, the presence of NETs was reported on Giemsa stained blood smears of children with uncomplicated *P. falciparum* malaria (120), adults with symptomatic malaria infection with *P. falciparum*, *P. vivax* or *P. malariae* (176), and in the mouse malaria species *P. berghei* (174) and *P. yoelii* NL (303). The possible reason for the very limited NET formation *in vitro* compared to the presence of NETs *in vivo* could be related with neutrophil priming with cytokines or some other factors in the circulation. The *in vivo* formation of NETs by human neutrophils could be triggered by either the production of cytokines or heme or a combination of some other factors. During malaria infection, free heme is released into circulation because of haemoglobin degradation and may induced NET formation. A high concentration of tumor necrosis factor (TNF)- α is present in the circulation of malaria patients and it might also have a stimulatory effect on NETs formation (304). According to Knackstedt *et al.*, when neutrophils primed with TNF- α were incubated with free merozoites, parasite digestive vacuoles or heme, only heme robustly induced NETs (304).

The other reason for very limited NET formation might be due to some parasite factors that might inhibit or degrade NET formation to escape the immune system. Previously, DNase 1 has been shown to degrade extracellular DNA released from neutrophils to prevent the accumulation of NET aggregates (209), and it has been suggested that TatD-like DNase is released by *P. falciparum* (PfTatD) (183) and *P. knowlesi* (PkTatD) (311), which degrades NETs. Thus, to confirm whether the TatD-like DNase is related with NET formation

inhibition or degradation, future research needs to include PfTatD knock out parasites to stimulate neutrophils and compare them with the non-knock out parasite for ability to affect NET formation.

5.4.2 Parasite density and opsonization of IE didn't change neutrophil responses towards NET liberation

The result of our study demonstrated that neither opsonization of IE nor increasing ratios of IEs to neutrophils enhanced NET formation and release following neutrophil-IE coinubation. Studies reported that both opsonized and unopsonized microbial pathogens induced NETosis. For instance, it was previously reported that opsonized *Nagleria fowleri* trophozoites (253) and *Staphylococcus aureus* (312) induced more pronounced NET release by neutrophils compared to unopsonized controls. Another study showed that various ratios of *Leishmania* promastigotes (*L. donovani* LV9, *L. donovani* 1S and *L. major*) induce dose-dependent NETs formation independent of the opsonization status (172), suggesting that unlike *P. falciparum* IE, *Leishmania* parasites on their own can stimulate neutrophils to form NETs. Together, the results of this study indicated that varying ratios of IEs and opsonization of IE with human purified IgG from plasma of malaria infected pregnant women didn't change neutrophil responses towards NET formation.

5.4.3 IE inhibit PMA-induced, but not LPS-induced, NET formation

To date, bacteria, fungi, viruses, and protozoan parasites have been shown to induce NET production in neutrophils (177), while an inhibitory effect on NET formation is rarely reported (178, 179). However, the mechanism of how *P. falciparum* IE inhibits PMA-induced NET formation is unknown. In the current study, to assess the inhibition of NET formation by IEs and merozoites, neutrophils and IEs and merozoites were incubated with or without PMA, and NET formation was evaluated. When neutrophils were co-cultured with IE in the presence of PMA the formation of NETs was inhibited compared to PMA stimulation alone. The degree of NET inhibition increases with increasing parasite density and time of incubation. To our knowledge, we reported for the first time that IEs, but not merozoites, inhibit PMA-induced NET formation. To evaluate the inhibitory effect of IEs on the different pathways of NET formation, neutrophils were exposed to IEs with or without the PMA and LPS. The results of our study showed that IEs inhibit PMA-induced,

but not LPS-induced, NET formation (figure 5.9). It was previously reported that there are multiple pathways to induce NET formation. For example, PMA induced NETs formation requires PKC, NADPH oxidase for producing ROS (181, 313), which disintegrate the granular and nuclear membranes. Whilst typically contained inside azurophilic granules, NE MPO are now free to interact with the nucleus where histones can be cleaved and chromatin decondensation facilitated (181). By contrast, LPS initiates a TLR4-dependent, JNK-mediated sensing mechanism to initiate NADPH oxidase-dependent NET formation (313), while calcium ionophore produces NETs without the need for PKC and NADPH oxidase, with mitochondria also acting as a source of ROS in NET formation in response to this stimulus (181). Huge quantities of PAD4 are already present in the cytosol and translocate into the neutrophil nucleus until bound with calcium from the influx induced by the calcium ionophore. The PAD4 mediates histone citrullination and leads to chromatin decondensation (314). Components of the response to *Plasmodium spp.* infection including inflammatory TNF and IL-8 cytokines produced by leukocytes and crystal uric acid released on IE rupture can induce neutrophils to produce NETs (121, 315), which depends on PAD4.

Although both PMA and LPS mediate NADPH oxidase-dependent NET formation, IE have no effect on LPS-induced NET formation but inhibit PMA-induced NET formation. One explanation could be that IE may have an effect on the activation of the PKC or Raf/ERK/MEK pathway without affecting JNK. The future study should compare IE-related NET inhibition with a range of known inhibitors against stimuli involved in NETosis. Future studies should systematically measure the level of ROS, MPO and NE from neutrophil supernatants stimulated with IEs in the presence of PMA/LPS and compare with neutrophils incubated with PMA/LPS alone. Furthermore, it is also important to compare the levels of inhibition with known ROS, MPO and NE inhibitors.

Previously, pharmacological and microbial inhibition of NET formation was reported elsewhere (181, 187). For example, microorganisms such as HIV-1 (169), *Bordetella pertussis* (188), *Lactobacillus rhamnosus* strain GG (178), *dengue virus* serotype-2 (189), and *Cryptococcus neoformans* and the capsular component GXM (190) have been shown to inhibit NET formation. Another study has also shown that agaphelin, a mosquito salivary protein, secreted upon infection with *P. falciparum* inhibits PMA-induced NET formation (182). Many neutrophil surface molecules could be changed upon stimulation and the

mechanism of inhibition may be dependent on the engagement of cell membrane receptors. Different microorganisms appear to use different mechanisms to inhibit or induce NET formation. For instance, opportunistic fungus *Cryptococcus neoformans* and the capsular component GXM (glucuronoxylomannan) inhibit NETs liberation in human neutrophils (190). *Bordetella pertussis* adenylate cyclase toxin suppresses NET formation by inhibiting the oxidative burst (188), and HIV-1 induces the production of IL-10, which may suppress ROS production, thus inhibiting the ROS-dependent NET formation (169), *dengue virus* serotype-2 inhibits glucose uptake as a metabolism-based way to suppress the formation of NETs (189) and *Acinetobacter baumannii* inhibits NET formation by suppressing neutrophil cell adhesion (185).

Glucose, ROS, the Raf-Mek-Erk signaling pathway, and histone citrullination are required for the NETs formation (316). The effect of IE on ROS production has been controversial as different studies have presented conflicting results. For example, it has been reported that *P. falciparum* and *P. vivax* malaria (317) and malaria-induced immunomodulatory haem oxygenase 1 (HO-1) (221) impaired neutrophil function, by reduced oxidative burst activity (221, 317), suggesting that HO-1 might inhibit NET formation by impairing ROS production. Another study has also indicated that phagocytosis of parasite digestive vacuoles leads to impairment of neutrophil functions such as inhibition of ROS production (133) suggesting DVs inhibit NET formation.

Previous studies demonstrated that levels of the anti-inflammatory cytokine IL-10 were significantly higher in *P. vivax* (318) and *P. falciparum* (49) infected individuals as compared to healthy controls, and IL-10 inhibits ROS production (169) suggesting that *P. falciparum* might inhibit ROS dependent NET formation. Chang *et al.*, reported that IL-10 suppresses the immune response by blocking the activation of the Toll-like receptor (TLR) signaling pathway, specifically MyD88-dependent signaling (319). Saitoh *et al.*, showed that neutrophils detect HIV-1 by TLRs 7 and 8, which recognize viral nucleic acids and engagement of TLRs 7 and 8 induces NET formation, leading to NET-dependent HIV-1 elimination (169). In this same study, authors reported that IL-10 inhibits TLR 7- and TLR8-signaling pathways, resulting in the suppression of NADPH oxidase-dependent NET formation (169). A recent study demonstrated that *P. falciparum* RNA (PfRNA) and IEs specifically activate TLR8 (320). Thus, the mechanism that IE inhibits NET formation might be by inhibiting ROS production or producing IL-10 which results in inhibition of

TLR signaling and suppression of NADPH oxidase-dependent NET formation. Altogether, HO-1, DVs and IL-10 inhibit ROS production, which in turn decreases the release of MPO and neutrophil elastase from primary granules. Both ROS and primary granules are vital for nuclear membrane disintegration and chromatin decondensation, which are crucial for NET formation (148, 310). The NET inhibition effect of IEs, but not merozoites, in this study could be related to HO-1 and DVs.

Remijnsen and colleagues demonstrated that both autophagy and ROS are required for NET formation, and inhibition of autophagy by wortmannin prevented chromatin decondensation without affecting ROS production (321). They showed that inhibition of either autophagy or NADPH oxidase stops chromatin decondensation and defects in either pathway result in apoptotic cell death instead of NETosis (321), suggesting that IE might inhibit NET formation by affecting the autophagy pathway, although further study is needed to confirm this phenomenon. Autophagy is an evolutionarily conserved intracellular degradation system aiming to maintain cell homeostasis in response to cellular stress, which is vital for neutrophil degranulation, ROS production, and NET formation (322). Using myeloid-specific autophagy-deficient mice, it has been shown that autophagy deficiency in neutrophils significantly reduced degranulation *in vitro* and *in vivo* (323). In the same study, the authors demonstrated that ROS generation was also reduced in autophagy-deficient neutrophils (323). Another study reported that chloroquine inhibits NETs formation by inhibiting neutrophil autophagy (324, 325). However, whether neutrophil autophagy occurs during *Plasmodium* parasite infection is unclear.

5.4.4 IE, but not merozoites, inhibit MPO degranulation

In the current study, we evaluated neutrophil degranulation as a measure of NET formation. ELISA was used to quantify MPO release and we found that the level of MPO release in neutrophils stimulated with IE and merozoites was similar to unstimulated neutrophils, but PMA stimulated cells released significantly high levels of MPO. This is, to the best of our knowledge, the first report on IE inhibition of neutrophil degranulation. MPO release in neutrophils stimulated with IE, but not merozoites, in the presence of PMA was significantly lower than for cells stimulated with PMA alone. It was reported previously that *P. falciparum* inhibits ROS production (133, 169, 221, 317). ROS production is responsible for granule lysis (which leads to the release of neutrophil elastase and MPO),

nuclear membrane disintegration and histone citrullination (133). Like ROS, neutrophil elastase and MPO also cause nuclear membrane disintegration and chromatin decondensation that leads to NET formation (148, 207, 326). MPO is abundant in the azurophilic granules of neutrophils and inhibition of neutrophil degranulation by IE may help to maintain neutrophils nuclear morphology and decrease NET formation, which might be an important parasite escape mechanism from the host immune system. Future research needs to evaluate whether IE inhibits the degranulation of other granular proteins such as neutrophil elastase, and measuring specific granular proteins from IE stimulated neutrophils or plasma of malaria infected patients might help to better understand the effect of IE on neutrophil degranulation. In an animal model, it has been shown that autophagy deficiency in neutrophils significantly reduced neutrophil degranulation *in vitro* and *in vivo* (323).

5.4.5 *P. falciparum* IE induce NETs formation by HL-60

The HL-60 cell lines are considered an alternative cell culture model to study neutrophil functions (305). Previous evidence showed that the responses of HL-60 cell lines could be different depending on the stimuli used to their differentiation. For example, DMSO-differentiated HL-60 cells induce NET formation only upon A23187 calcium ionophore stimulation, all-trans retinoic acid-differentiated HL-60 cells only upon PMA stimulation, while dimethylformamide-differentiated HL-60 cells formed NETs in response to either stimulus (327). Another study demonstrated that DMSO-differentiated HL-60 cells induced NET formation upon PMA and *Staphylococcus aureus* stimulation (305).

In this study, we aimed to examine whether DMSO differentiated human leukemia cell lines (HL-60 cells) could produce NETs in response to *P. falciparum* IEs. Furthermore, the inhibitory effect of IEs on PMA-induced NET formation by HL-60-cell lines was also assessed.

The results of the current study showed that stimulation of DMSO-differentiated HL-60 cell lines with IE released NETs in similar amounts to PMA at 5 h, and HL-60 cells induced significantly more NETs upon overnight incubation with IEs than with PMA. Incubation of HL-60 cell lines and IEs in the presence of PMA released significantly higher NETs compared to PMA alone stimulated HL-60 cell lines, suggesting no inhibition of PMA-induced NET formation by PMA (figure 5.12). While PMA-induced NET formation in

human neutrophils was already evident after 4 h of incubation, no HL-60 cells released NETs at similar time points, which is supported by a recent study (305).

It is known that the differentiation of HL-60 cells to neutrophil-like cells is somewhat incomplete as they lack secondary granules and secretory vesicles, which contain high amount of cytochrome *b₅₅₈* (305). Cytochrome *b₅₅₈* is the membrane component of the phagocyte NADPH oxidase enzyme complex located in the wall of secondary granules. On activation, it fuses with phagolysosome or plasma membrane (305), and mediates ROS production. We showed that IE inhibits PMA-induced NET formation by human neutrophils, but it is interesting that IEs are unable to inhibit PMA-induced NET formation by HL-60 cell lines. A possible hypothesis for the formation of NETs in HL-60 cell lines in response to IE, but not in human neutrophils, may be that the secondary granular proteins and secretory vesicles present in human neutrophils could either individually or together mask the action of the primary granules towards NET formation. But further investigation is required to elucidate whether secondary granules and secretory vesicles do influence the role of primary granules in IE induced NET formation or how the *Plasmodium* parasites block the NET formation in human neutrophils.

5.4.6 Strengths and weaknesses of this study

One strength of the current study was the evaluation of the effect of IEs on NET formation or NET formation inhibition *in vitro*. Another strength was the use of a direct neutrophil isolation kit which allowed to get pure and unstimulated neutrophils. Other strengths of this work included the use of different stages of the parasite (trophozoites, merozoites), as well as different methods of measuring NETs. In addition, testing the effect of IE on different potent NET stimulants was also another strength of this study.

One potential limitation of this study was the measurement of neutrophil degranulation, MPO release, at a single time point (4 hours post stimulation). Evaluation of MPO release at multiple time points (e.g. 6 12, 24 and 48 hrs.) could have been more informative in better understanding of neutrophil degranulation. Another limitation of this study was the measurement of only MPO release. Neutrophil elastase release, cytokine release and ROS production were not assessed in the current study. In addition to MPO, analysis of other granular proteins, levels of cytokines and ROS production would have been more important in understanding the effect of IEs on neutrophils function. Another limitation, the parasite

killing ability of neutrophils (by NET release or degranulation) was not investigated. The current study used only placental binding IE to investigate NET formation or inhibition effect of Ies (although similar effects were observed with SBP-1 knockout IE). Testing the other parasite lines (other than CS2) in future studies could be more informative for generalization.

5.5 Future directions

The role of neutrophils in response to *Plasmodium* parasites remains poorly understood, and undertaking the future research priorities will help to clarify this role of neutrophils. It is important to evaluate whether IE influence neutrophil autophagy, which is required for NET formation. Further research needs to investigate and compare whether IE induces NET formation or inhibit autophagy and cause apoptosis or necrosis in neutrophils. Our finding demonstrated that IE inhibit PMA-induced NET formation and release by human neutrophils, but the mechanism of how IE inhibit NET formation is not clear. NET formation occurs through different signaling pathways (181), thus, it might be essential to evaluate the different NET formation pathways using different stimulants to clearly understand whether IE inhibit the PMA pathway or influence other pathways like LPS and CaI induced NET formation. Furthermore, the levels of cytokine secretion, ROS production and specific granules need to be evaluated. To understand the implications of these findings for malaria pathogenesis, the beneficial or the harmful effect of NET formation needs to be investigated further. The results of our study showed that IE inhibited NET formation induced by PMA, but not LPS induced NET production. Therefore, the future study should compare NET inhibition by IE with a range of known inhibitors against stimuli involved in NETosis. Since IEs induce NET formation by HL-60 cell lines and exhibit no inhibition of PMA-induced NET formation on this cell line, the future study should also test the effect of IE on NET formation/inhibition by human monocytes.

5.6 Conclusion

In summary, the inhibition of neutrophil degranulation and NETs formation by IE may be an important mechanism that allows the parasite to escape NETs-mediated killing. Thus, identification of the mechanism by which *P. falciparum* inhibits NET formation could contribute to the development of new treatment strategies for malaria.

6 Chapter 6: Infiltration of neutrophils and neutrophil extracellular traps (NETs) in malaria-infected placentas

6.1 Background

Malaria during pregnancy is caused by the sequestration of *P. falciparum*-infected erythrocytes (IEs) in the growing placenta (263). Histopathology of placental malaria may reveal parasite sequestration (76) and infiltration of leukocytes into the placenta (76, 202, 328).

Neutrophils are the most abundant blood leucocytes of the innate immune system comprising around 50-70% of the white blood cells (122). During infection, neutrophils are the first cell types to be recruited to inflammatory sites, where they have a crucial role in the clearance of microorganisms (119). It was reported previously that in *Plasmodium* infections, the total number of phagocytes is decreased in the circulating blood during pregnancy (200), suggesting the migration of these cells into the placenta to clear the *Plasmodium* parasites. However, the role of neutrophils in malaria is understudied and the involvement of neutrophils and NETs in placental malaria pathogenesis has not been established. In this study, the accumulation of neutrophils and the presence (or absence) of NETs in the intervillous space (IVS) of placentas with and without malaria was assessed.

6.2 Hypothesis and Aims

Hypothesis: Infiltration of neutrophils and formation of NETs in the intervillous space (IVS) of the placenta are features of malaria-infected placentas

Aim: To assess infiltration of neutrophils and the presence of NETs in the IVS of malaria-infected and uninfected placentas

Specific Aims:

- 6.2.1** To evaluate total leukocyte populations in the IVS of malaria-infected and uninfected placentas
- 6.2.2** To evaluate neutrophils infiltrations into the IVS of malaria-infected and uninfected placentas
- 6.2.3** To evaluate the presence (or absence) of NETs in the IVS of malaria-infected and uninfected placentas

6.3 Materials and methods

6.3.1 Ethics, study site, study populations, and sample collection

Placental samples were collected from women who underwent delivery at Queen Elizabeth Central Hospital, Blantyre, Malawi. The calculation was for comparing neutrophils between malaria infected and uninfected placentas and sample size calculation was based on looking for changes in the number of neutrophils in the placenta. We calculated the sample size based on means and SD of neutrophils in uninfected placentas from a cohort in Brazil (74). The percentage of neutrophils was approximately 1% of cells in the IVS and the SD was 0.8% (2 sided, 95% Confidence). For this study, we matched samples according to age and gravidity between malaria-infected and uninfected women, and 10 malaria-infected placentas were compared with placentas from 10 uninfected controls. The Ethics, study site, study populations, and sample collection are described in detail in section 3.4.1 of the general methods.

6.3.2 Placental examination and development of a classification system.

Paraffin-embedded placental tissues were examined following standard histological techniques and the presence of neutrophils and NETs in the IVS were assessed by conventional microscopy, immunohistochemistry, and immunofluorescent staining. The details of placental examination and classification system are described in **section 3.4.2**.

6.3.3 Preparation, staining and examination of paraffin-embedded placental sections

Sections from paraffin-embedded placental tissues were cut at a thickness of 5 microns (μm). Deparaffinisation and rehydration were performed with standard xylene-to-ethanol washes. The staining of placental sections is described in **sections 3.4.3, 3.4.4.2 and 3.4.5**.

On conventional Giemsa microscopy of placental sections, neutrophils and NET-like structures were identified as described in **section 3.4.3**. Two microscopists, who were blinded to the status of infection, individually examined the microscopic slides. Any

discrepancy between the two microscopists was settled by a third senior person in the lab, whose readings were then taken final. Neutrophils, NET-like structures (extracellular DNA stained by Giemsa) and other leucocytes were differentially counted in the IVS of the placenta. Fields of Giemsa stained placental sections were scanned with 40x objective (magnification = 400) to check cell distribution and evaluated with 100x objective (magnification = 1,000) with cell counts obtained in 30 consecutive fields, where the maternal IVS was identified. The criteria used for counting NET-like structures included the following points (i) the NET-like structures should be stained blue with Giemsa, (ii) extracellular DNA should be detected (not only decondensed nuclei inside the cells), (iii) NET-like structure involving multiple cells were counted as a single NET (whatever the source of the NETs), (iv) NET-like structures can be filamentous or fuzzy and (v) NET-like structures should be in the IVS of the placenta.

In immunohistochemistry staining, neutrophils and NETs were identified as described in **section 3.4.5**. For this study, a neutrophil was defined as ‘intact’ if elastase staining was confined within the cytoplasm of the cell and if an intact, multilobulated nucleus was present based on the morphology of stained cells. When extracellular elastase was detected in the presence of a disrupted, decondensed nucleus these structures were counted as ‘NETs’ and if there is unclear nuclear-cytoplasmic integrity, neutrophil was defined as ‘indeterminate’. For NET quantification, intact and indeterminate neutrophils were considered as non-netting neutrophils (198).

In immunofluorescence staining (MOP-NE-DAPI overlap), NETs were classified as **cell-free NETs** (NETs that are released away from neutrophils), and **anchored NETS** (NETs that appeared attached to neutrophils). For this purpose, anchored NETs were characterized as reticular structures anchored to neutrophils, spot-like structures anchored to neutrophils,

membranous structures on the surface of neutrophils and linear structures anchored to neutrophils, whereas cell-free NETs were characterized as fragmented or cotton-like structures. This classification is based on a previous study done in various organs of a murine sepsis model (329).

6.3.4 Data analysis

Statistical analyses were performed using GraphPad Prism software (Version 5). Comparisons between 2 groups were performed using the non-parametric Mann Whitney test and a p-value of < 0.05 was regarded as significant. In figures, p values are indicated as follows: (* < 0.05 , ** < 0.01 , *** < 0.001 , **** < 0.0001).

6.4 RESULT

Infiltration of leukocytes into the placenta and the presence of NETs in the IVS were assessed by light microscopy of Giemsa stained placental sections (section 6.4.1). Next, placental sections were stained by a neutrophil specific marker and neutrophils and NETs in the IVS were assessed by immunohistochemistry staining, section 6.4.2. Then, the presence of NETs was confirmed by immunofluorescence staining which is important to observe the colocalization of NET markers (confocal microscopy) (section 6.4.3).

6.4.1 Total leucocyte, neutrophils and NET-like structures in the IVS

In conventional Giemsa microscopy (magnification = 1,000; high- power field), the types of leukocytes were identified by their size, shape, and nuclear morphology. Total leukocytes, neutrophils, and NET-like structures were counted separately (in the same microscopic field) in 30 consecutive fields where the maternal IVS was identified and were presented as number per field.

A representative Giemsa -stained placental section is shown in figure 6.1. In this study, densely populated infected erythrocytes, massive infiltration of leukocytes and dilated IVS were observed in malaria-infected placentas (figure 6.1 B) compared to healthy placentas (figure 6.1 A). Another interesting finding in the current study was that in healthy placentas (malaria uninfected, figure 6.1 A, back arrow) the white cell morphology was normal, while in malaria-infected placentas the morphology of leukocytes was severely affected (figure 6.1 B, green arrow), making it hard to differentiate neutrophils from other cells.

The results of our study showed that the number of total leukocytes ($p = 0.037$) (figure 6.2A) and NET-like structures ($p = 0.037$) (figure 6.2C) were significantly higher in the IVS of malaria-infected placentas as compared to controls. However, the numbers of neutrophils did not differ significantly between malaria-infected and malaria uninfected groups ($p = 0.47$) (figure 6.2 B).

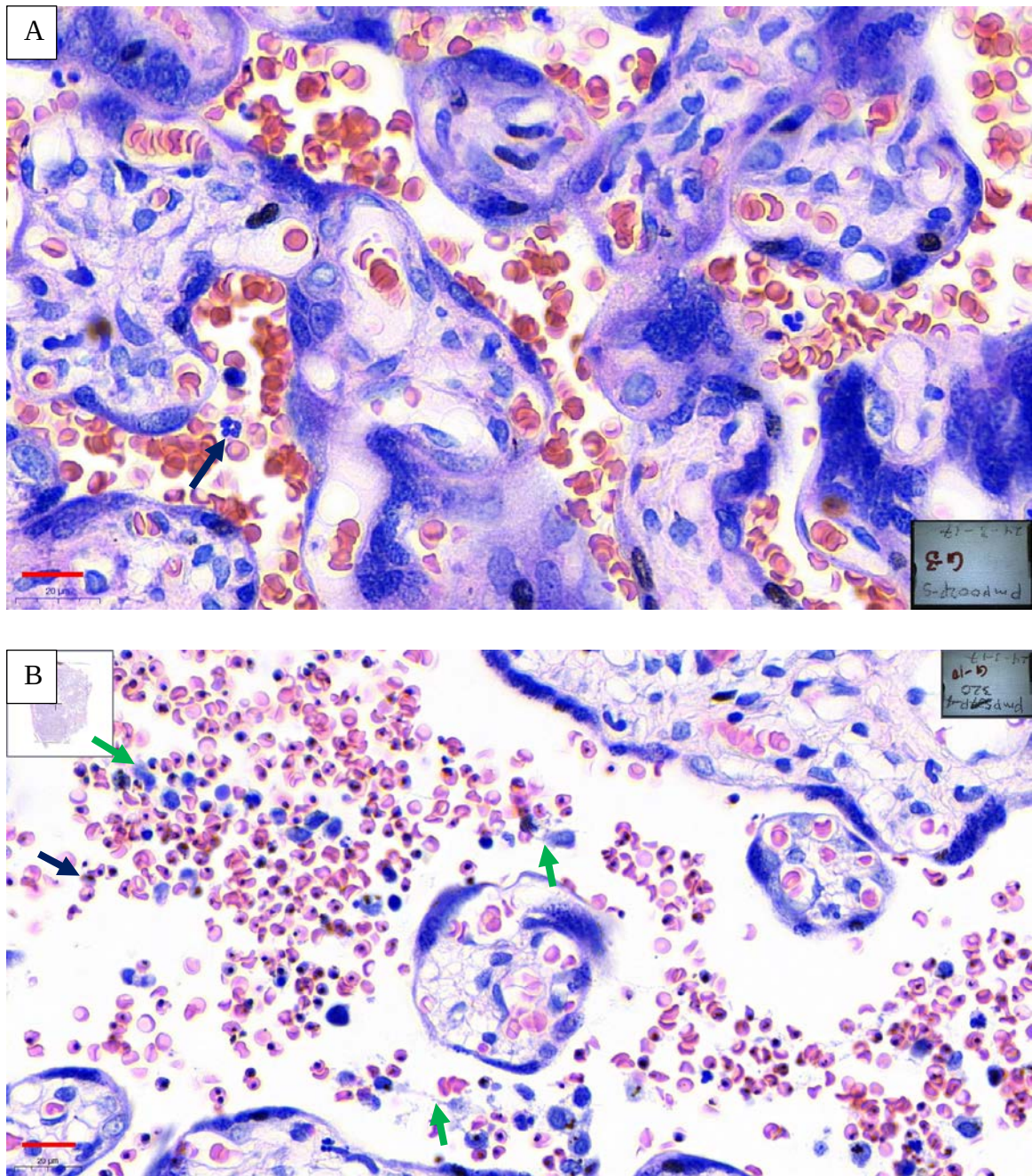


Figure 6 1 Representative Giemsa-stained images from *Plasmodium falciparum* uninfected (A) and infected placentas (B). Placental sections were stained with 10% Giemsa for 2 hours at 37°C. Images were captured by Metasystem V-Slide scanner microscopy (Australian Phenomics Network, APN), generating digitized high-resolution whole slide images. (A) unstimulated leukocytes, black arrow. (B) malaria-infected erythrocytes, black arrow and stimulated leukocytes, NETs (green arrow). Giemsa stains DNA blue. Scale bar = 20 µm

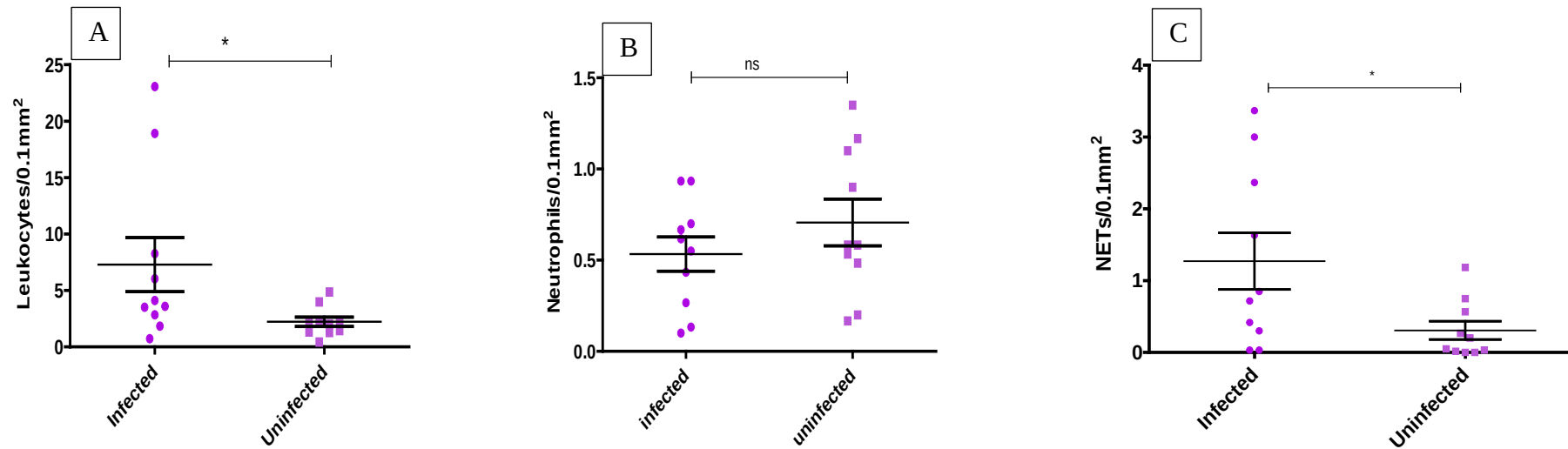


Figure 6 2 Total leukocytes, neutrophils, and NET-like structures in the intervillous space of the placenta. Paraffin-embedded placental tissues were cut at a thickness of 5 microns, stained with Giemsa, and examined by light microscopy under 100x objective. Total leukocytes (A), neutrophils (B) and NET-like structures (C) were counted in 30 consecutive fields where the maternal intervillous space was identified, and are presented as average number per field (0.1mm² = 1 field). The y-axis is the counts and the x-axis is the study groups. Comparisons between 2 groups were performed using the non-parametric Mann Whitney test and a p-value of < 0.05 depicted significance, ns= no significance. p-value < 0.05: (*< 0.05, **< 0.01, *** < 0.001, **** < 0.0001).

6.4.2 Neutrophils infiltration into the placenta and the presence of NETs in the IVS were associated with malaria infection

To evaluate neutrophil infiltration into the maternal blood space during malaria in pregnancy, immunohistochemical staining of placental sections was performed using neutrophil elastase as a neutrophil-specific marker, and all cells positive for neutrophil elastase were considered as neutrophils. A representative neutrophil elastase-stained neutrophil in placental IVS is shown in figure 7.3.

Using neutrophil elastase to detect neutrophils, the number of neutrophils in placental IVS was significantly higher in malaria-infected placentas as compared to uninfected controls ($p = 0.023$) (figure 7.4A). Similarly, a significantly higher number of NETs in placental IVS were detected as, anchored NETs cell-free NETs, in the infected placentas compared to uninfected placentas ($p = 0.0005$) (figure 7.4B).

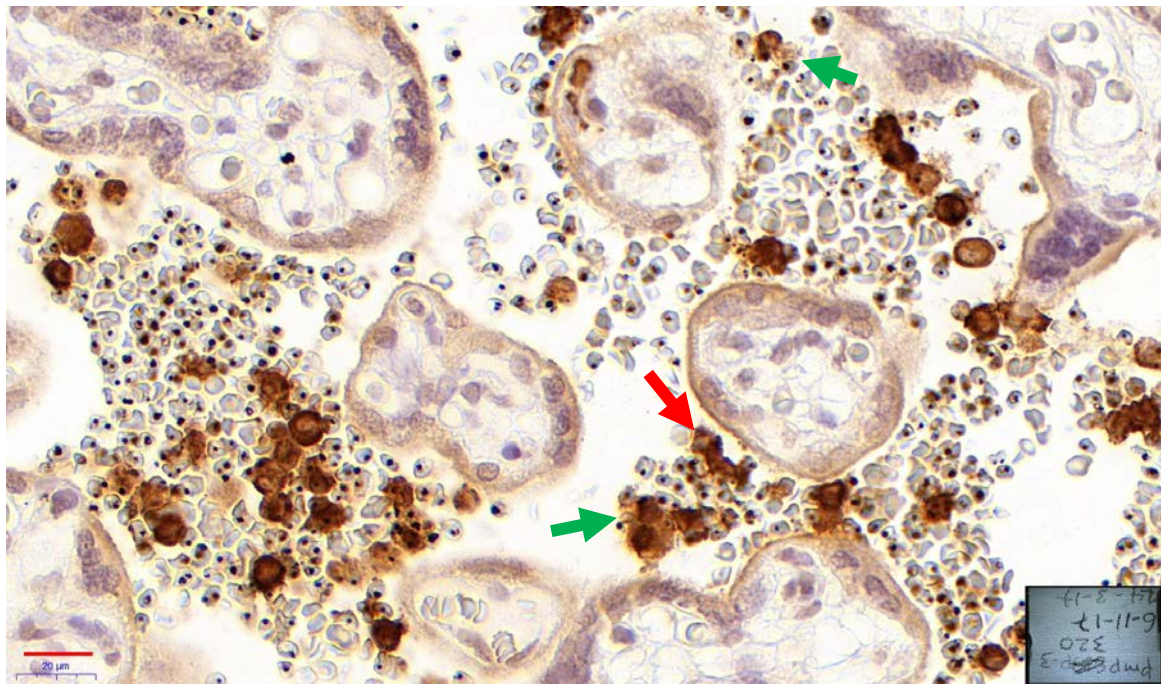


Figure 6 3 Neutrophils and neutrophil extracellular traps (NETs) in the intervillous space of the malaria-infected placenta. Representative histological examples of extracellular NETs (green arrows) and cells with decondensed nuclei (red arrows) in placental intervillous space. Placental sections were stained with the neutrophil marker elastase (elastase Immunohistochemistry staining) and images were captured by Metasystem V-Slide scanner microscopy (Australian Phonemics Network, APN), generating digitized high-resolution whole slide images. Scale bar = 20 μ m.

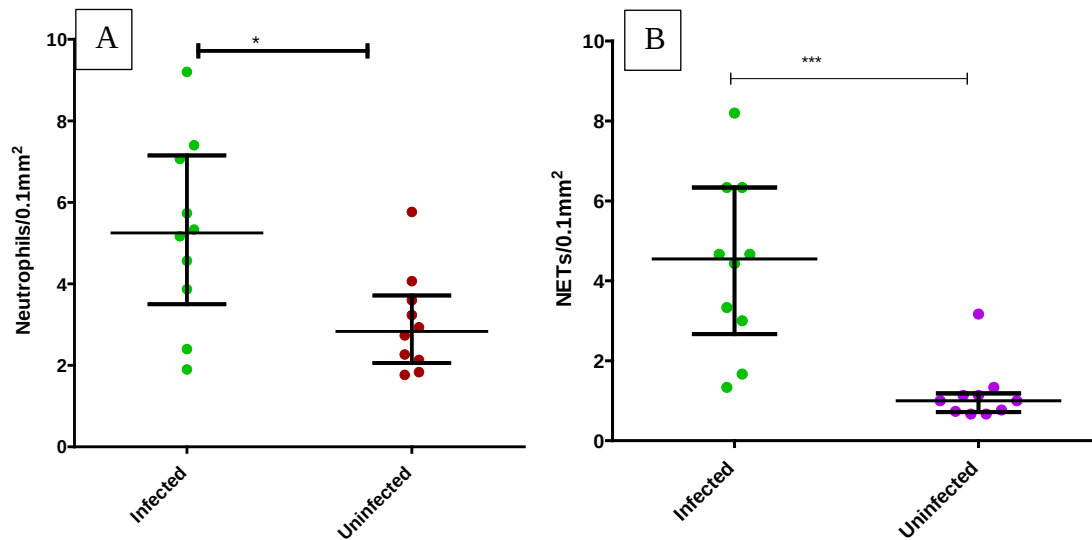


Figure 6 4 Neutrophils infiltration and placental NETs are higher in malaria-infected placentas. Paraffin-embedded placental tissues were cut at a thickness of 5 microns, stained with anti-human neutrophil elastase by immunohistochemistry with 3,3'-Diaminobenzidine (DAB) to detect elastase, and examined by microscopy under 100x objective (magnification = 1,000; high- power field). (A) neutrophils and (B) NETs were counted in 30 consecutive fields, where the maternal intervillous space was identified and was presented as number per field ($0.1\text{mm}^2 = 1$ field). The y-axis is looking at the counts and the x-axis is the study groups. The non-parametric Mann Whitney test was done and statistical significance determined as p-value < 0.05. p-value < 0.05: (*< 0.05, **< 0.01, *** < 0.001, **** < 0.0001).

6.4.3 Immunofluorescence staining of placental NETs

The formation of NETs in the placental IVS was confirmed by immunofluorescence staining with anti-human neutrophil elastase, anti-human MPO and DAPI, demonstrating the colocalization of NET markers (NE-MPO-DAPI overlaps) (figure 7.5).

On immunofluorescence staining, two different forms of NETs were identified: anchored NETs that are anchored to neutrophils, while the second is cell-free NETs that are released away from neutrophils and circulating in the IVS of the placenta (figure 6.5). Morphologically they have been characterized as described **above** in **section 6.3.4**.

Using immunofluorescence staining, a higher number of neutrophils and NETs were reported in *P. falciparum*-infected placentas as compared to controls. Although NETs were observed in *P. falciparum* uninfected placentas, the number of both total neutrophils and neutrophil NETs in IVS were significantly higher in *P. falciparum*-infected groups compared with controls, figure 6.6.

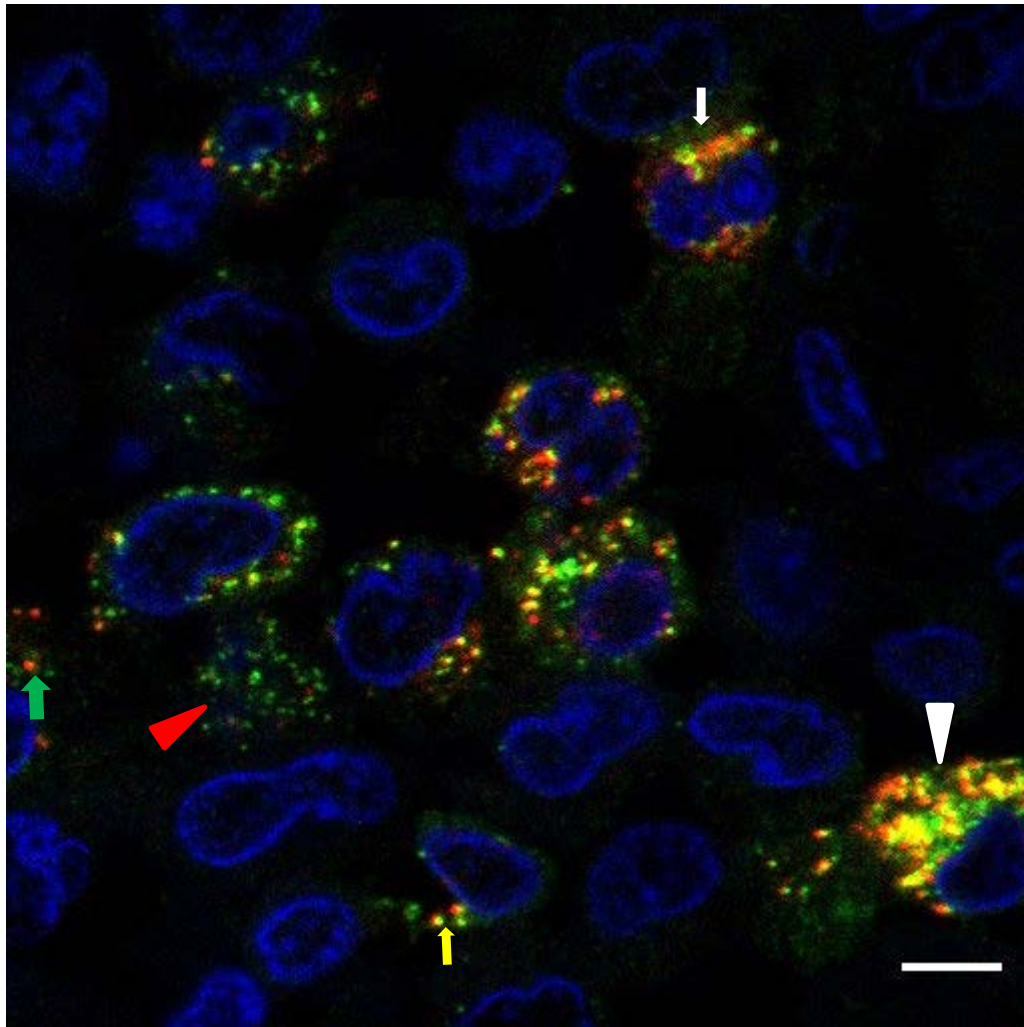


Figure 6 5 Representative image of NETs in intervillous spaces of malaria-infected placentas. Immunofluorescence staining for nuclei (blue, DAPI) and granular components (red, neutrophil elastase and green, myeloperoxidase). Cells lose their distinct granule pattern and show colocalization (pink) of neutrophil elastase (red), myeloperoxidase (green) and DAPI (blue). Nuclei and granules are individually visible because the cell has flattened out and some cells already released cell-free NETs. NETs were classified as cell-free NETs and anchored NETS. Anchored NETs were characterized as spot-like structures anchored to neutrophils (yellow arrow), membranous structures on the surface of neutrophils (white arrow) and linear structures anchored to neutrophils (white arrowhead). Cell-free NETs were characterized as fragmented (green arrow) or cotton-like structures (red arrowhead). Scale bar = 50 μ m.

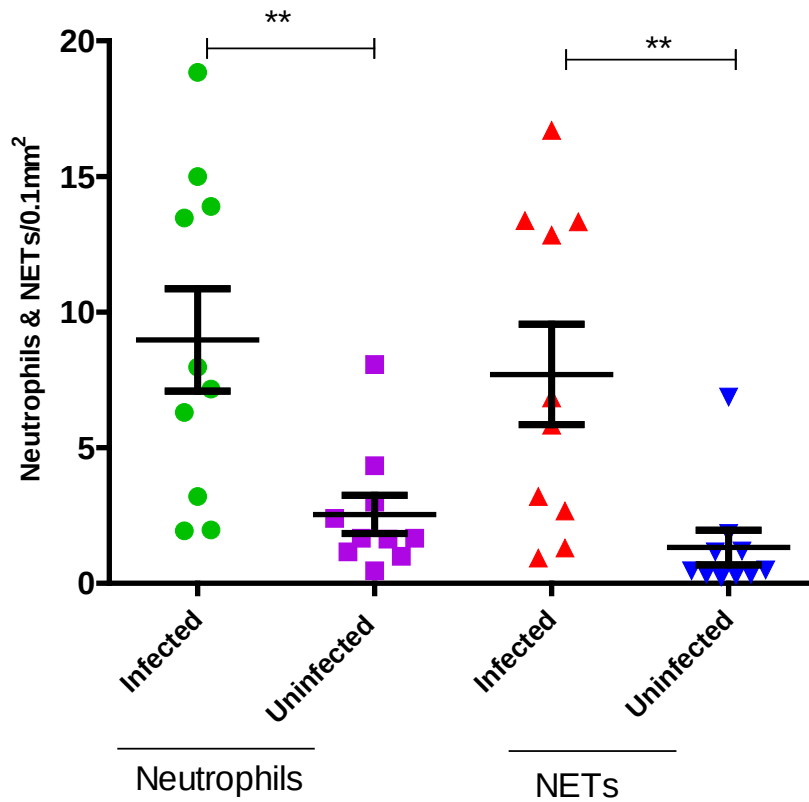


Figure 6 6 Neutrophils and placental NETs were higher in malaria-infected placentas. Paraffin-embedded placental tissues were cut at a thickness of 5 microns, stained with anti-human neutrophil elastase, anti-human myeloperoxidase, and DAPI and examined by confocal microscopy under 60x objective and/ or scanned by Metasystem V-Slide scanner microscopy. Neutrophils and NETs were counted in 30 consecutive fields, where the maternal intervillous space was identified, and are presented as number per field ($0.1\text{mm}^2 = 1 \text{ field}$). The y-axis is the counts and the x-axis is the study groups. Comparisons between 2 groups were performed using the non-parametric Mann Whitney test and a p-value of < 0.05 depicted significance ($* < 0.05$, $** < 0.01$, $*** < 0.001$, $**** < 0.0001$).

6.5 Discussion

Placental malaria is characterized by the sequestration of IEs and the infiltration of immune cells within the IVS in the placenta (191). Massive infiltration of leukocytes (monocytes and macrophages) into the infected placenta during malaria in pregnancy was reported (258) and active infection can be accompanied by leucocytes infiltrates, particularly monocytes (330). Before this project, the knowledge of the infiltration of neutrophils to the placenta during malaria in pregnancy was fragmented. Neutrophils are the predominant innate immune cells that are vital in immune defence against invading pathogens and have been regarded as the first line of defense in the innate arm of the immune system, but their migration to the placental IVS may also cause inflammatory pathology (191, 202), which might be caused by neutrophil degranulation and NET formation. The role of neutrophils and NETs in placental malaria pathogenesis is largely unexplored. Thus, in this study, we aimed to evaluate the infiltration of neutrophils and the presence of NETs in the IVS of placentas infected with malaria.

In this chapter, for the first time, we reported NETS in malaria infected placentas. The number of neutrophils and placental NETs are significantly higher in malaria-infected placentas compared to controls.

6.5.1 Infiltration of leukocytes to the placental blood space

On Giemsa microscopy, a significantly higher number of total leukocytes and NET-like structures were observed among malaria-infected placentas as compared to the controls. However, the numbers of neutrophils appeared similar between malaria-infected and uninfected placentas. Our findings support previous studies demonstrating frequent infiltration of leukocytes into the infected placenta during pregnancy-associated malaria (202, 258). It was reported that in *Plasmodium* infections during pregnancy, the total number of granulocytes, monocytes, and lymphocytes are decreased in the circulating blood, which includes a significant reduction in circulating neutrophil count (200, 202). This could be driven by migration and accumulation of neutrophils in the IVS of the placenta, potentially because during placental infection syncytiotrophoblasts produce high amounts of neutrophil chemoattractants like IL-8 (202), which could be associated with neutrophil migration into the placenta. The maternal blood volume of the placenta is relatively small- maybe 200 mls at most- compared to 5 L total circulating blood volume-

so you would need a very high concentration of leukocytes in the IVS to explain such a difference.

The possible reason that neutrophil numbers did not differ between infected and uninfected groups in the current study could be that when *P. falciparum* activates neutrophils in the IVS of the placenta, this results in nuclear round-up and chromatin decondensation that leads to morphological changes, as a result, the neutrophils may be counted as other types of leukocytes such as monocytes when using Giemsa stained sections. Although there is no study assessed neutrophil morphology in malaria in pregnancy, it has been reported that changes in leukocyte number and morphology in the peripheral blood of malaria-infected patients are common (331), and band cells and toxic granulation of the neutrophils were more frequently noted in malaria-infected patients (332). Because of this uncertainty, we used neutrophil specific markers to evaluate neutrophil infiltration to the placenta.

6.5.2 Neutrophil infiltration into the IVS of malaria placentas.

Neutrophil elastase IHC staining was used to assess neutrophil numbers and neutrophil NETs in the placenta. Our results demonstrated a significantly higher number of neutrophils and NETs in the placental IVS of malaria-infected placentas than the uninfected controls. The high number of neutrophils infiltrating into the placenta in this study suggests they could be involved in the clearance of *Plasmodium* parasites either by phagocytosis and/ or formation and release of NETs, but their effect on placental tissue or pregnancy outcomes requires further investigation. Although a higher number of neutrophils is reported in malaria infected placentas in the current study, frequent migration of monocytes into the placenta have been documented during malaria in pregnancy (330). Some work has used the monocyte/macrophage specific marker CD68 and inflammatory infiltrate were positive for CD68, suggesting a monocyte-macrophage infiltration (333).

The first-ever published data on *P. falciparum* parasite-induced NETs was reported by Baker VS *et al* in 2008, and they suggested that NETs are an important structure in entrapping *P. falciparum* IEs within blood vessels (120). The sequestration of *P. falciparum* in the placenta triggers neutrophil migration into the placenta in order to phagocytose the parasites there (201), suggesting that placental formation of NETs may occur in the course of the disease.

6.5.3 NETs in the IVS of malaria infected placenta.

For the first time, we reported significantly more NETs in *P. falciparum*-infected placentas compared to controls. The presence of placental NETs was confirmed by immunofluorescence staining of NET contents (colocalization of MPO, NE, and DAPI). Although there are no published data on NETs in placental malaria, placental NETs were reported previously, for instance, Zhong XY *et al* confirmed that circulating NETs were significantly elevated in pregnancies complicated by preeclampsia (334). NETs in the placental IVS were also detected from frozen placental tissues collected from pregnancies affected by pre-eclampsia (180, 196, 197) and from pre-eclampsia and pre-eclampsia plus lupus pregnancies (198). Although they are not placental NETs, *in vivo* NET formations were also reported from American tegumentary leishmaniasis (149), sections of *E. bovis*- and *E. arloingi*-infected intestinal tissue samples (199), a mouse intranasal infection model infected with *T. gondii* (170), suggesting migration of phagocytic cells to the site of infection and NETosis plays a role against protozoan parasites.

In the current study, although NETs were significantly more common in malaria-infected placentas, NETs were also observed in uninfected placentas. The explanation for the presence of NETs in malaria uninfected placentas could be due to other confounding factors, as we did not control for other infections. In agreement with our findings, NETs were reported in normal placenta located near the syncytiotrophoblast layer (196, 197), which could be associated with the deportation of syncytiotrophoblast microparticles during the third trimester of normal pregnancy (180). Marder *et al* reported NETs in the controls but the numbers of NETs in placental IVS was significantly higher in the preeclampsia and SLE groups compared with controls. Syncytiotrophoblast microparticles from the placenta will be shed into the maternal circulation and could interact with maternal leukocytes and induce mononuclear cell production of the proinflammatory cytokines like TNF- α (335). Moormann AM *et al* reported an increase in expression of TNF- α and IL-8 in malaria infected compared to uninfected placentas (203). Placentally-derived IL-8 and syncytiotrophoblast microparticles triggered NETs formation by stimulating neutrophils (180), with similar kinetics to what had been previously observed using bacterial agents (116). This suggests that malaria infection, by inducing the production of these cytokines, leads to more pronounced placental NETs compared to uninfected placentas.

Although the role of NETs in the pathogenesis of placental malaria is unknown, the association between *in vitro* NET formation and contribution to pathogenesis in falciparum malaria was reported (176). The number of NETs has increased significantly in proportion to severity of malaria disease (176, 304), which suggests that NETs formulation can contribute to malaria pathogenesis (176, 201). Circulating NETs and antinuclear IgG antibodies to double-stranded DNA were reported from malaria infected children, suggesting NETs and antinuclear IgG antibodies contribute to malaria pathogenesis in children (120). Knackstedt *et al* recently reported a high concentration of neutrophils and NETs in the blood of malaria infected mice and found that the presence of NETs causes liver damage (304). The authors of this study also measured circulating NET components (NE/DNA, MPO/DNA complexes) by ELISA in plasma samples from Gabonese malaria patients and found that the number of NETs was significantly higher in severe malaria compared to uncomplicated malaria. They indicated that soluble NET components facilitate parasite sequestration in the liver and lung and mediate tissue destruction (304). Boeltz S *et al*, reviewed the association between NET formation and severity of clinical malaria and their contribution to malaria pathogenesis, suggesting that an increase in NETs may exacerbate intravascular thrombosis and promote ischemia and reduced tissue perfusion (201). NETs associated damage to the maternal endothelium was observed in preeclampsia since endothelial cells are susceptible to cell death induced by NET releasing neutrophils (336).

Malaria infection increases NETs in the placenta, but parasites inhibit PMA related NET formation *in vitro*. Those two observations seem in conflict, but our findings demonstrated that IE inhibit PMA-induced NET formation, rather than degrading NETs formed by PMA stimulation. During malaria infection, the parasite or the components of *Plasmodium spp.* infection including inflammatory (TNF and IL-8) cytokines produced by white blood cells and crystal uric acid released on IE rupture can induce neutrophils to produce NETs (121). The pathway of NET formation in response to TNF and IL-8 and crystal uric acid is NADPH oxidase-independent, but PMA-induced NET formation is NADPH oxidase-dependent (181), suggesting that IE have effects on the activation of PKC or Raf/MEK/ERK, which further activate NADPH oxidase and generate ROS. Thus, since IEs have no NET degradative effect it could be possible to detect NETs in the placenta.

Further studies of whether parasites are likely inhibiting or promote NETs by assessing whether spatially NETs are positively or negatively associated with parasite sequestration are warranted. Furthermore, *in vitro* NETs might not be related to the parasite itself but rather it could be associated with cytokines or parasite byproducts, which we recommended future researchers to look at.

6.5.4 Strength and limitation of this study

The strength of the current study was the evaluation of neutrophils infiltration and NET formation in the IVS for the first time using different techniques such as Giemsa, immunohistochemistry and immunofluorescence staining. The other strength of this chapter was the use of specific markers to stain neutrophils and NET contents. The use of age and gravidity matched malaria infected and uninfected placentas was another advantage of this chapter. One limitation could be this study only looked at the placental NETs but didn't measure circulating NET components by ELISA from the plasma of malaria-infected pregnant women. The presence of NETs in the placenta does not necessarily mean that these NETs are associated with *Plasmodium* infection only because we did not control for other infections, this could be a further limitation of this type of study.

6.5.5 Future directions

Although important findings are reported in this chapter, some experimental areas need further investigation. As discussed before, only the presence or absence of neutrophils and NETs in the placenta were assessed. For future researches, the circulating NET components such as NE/DNA or MPO/DNA complexes should be assayed by ELISA using the plasma of malaria-infected pregnant women for a complete picture. Furthermore, the levels of cytokines released in response to malaria infection should also be tested to understand their priming effect and NET formation. Diverse stimuli, including inflammatory cytokines, induce NET release (180). Priming of neutrophils from healthy subjects with cytokines such as TNF α , IL-8 provides synergistic signals for NET formation (304). Although neutrophils are abundant immune cells that are crucial for immune defence (123, 137), their migration and the formation of placental NETs might be pathological, causing endothelial and placental injury (191, 198, 202, 336, 337). Thus, the presence of neutrophils and NETs

in the IVS of the placenta and pregnancy outcomes should be assessed. Since there are no published works that assess placental NETs in the context of malaria infection, the outcomes from this chapter can be used as the basis for further studies to explore the role of neutrophils and NETs in pregnancy and to relate these observations to birth outcomes during malaria in pregnancy. Furthermore, exploring the spatial association between NETs and pathological processes in the placenta like fibrin deposition or endothelial damage would be important in future studies.

6.6 Conclusions

In this chapter, we show, for the first time, that neutrophils infiltrate into the IVS of the placenta, where they release NETs. Overall, a higher number of neutrophils and placental NETs were observed in *P. falciparum*-infected placentas as compared to control.

7 Chapter 7: Overall Discussion, Future Directions and Conclusion

7.1 Discussion

Malaria remains a major health challenge worldwide (3), and pregnant women are at high risk of developing the disease, especially the first-time mothers due to the lack of protective antibody against placental binding parasites, which express PfEMP1 VAR2CSA protein on the surface of IEs (3, 338). Naturally acquired antibodies against VAR2CSA are produced during successive pregnancies, and VAR2CSA is also an important vaccine candidate. Two clinical studies, PRIMALVAC (NCT02658253) and PlacMalVac (NCT02647489) are assessing the potential of VAR2CSA as a vaccine candidate in humans, aiming to improve pregnancy outcomes by inducing antibodies that block the adhesion of IEs to the placenta (80) and/or trigger removal by phagocytic cells, particularly monocytes (339). A better understanding of innate immunity to malaria may help identify alternative strategies to reduce morbidity and mortality linked to malaria in the absence of an effective vaccine. Furthermore, understanding the collaboration of antibody and phagocytic cells for the phagocytic clearance of *Plasmodium* parasites is also important for malaria control strategies. Before this project, knowledge of the role of neutrophils in malaria infection was fragmented. This thesis has investigated the role of neutrophils in the immune response in malaria, mainly focussed on antibody-dependent neutrophil phagocytosis (ADNP); NETs formation *in vivo*; and how IE modify NETS formation *in vitro*.

7.1.1 Principal findings

The main findings of this Ph.D. thesis are (i) functional antibodies induced neutrophil phagocytosis of placental binding IEs, (ii) opsonizing antibodies to IEs and DBL2 coated beads, but not DBL3 and DBL5 coated beads, were associated with protection from placental malaria, ADNP was higher in NPM when comparing the two infected groups which highlights the importance of controlling for exposure when looking at antibody responses and protection from malaria, (iii) in placental tissue sections, numbers of NETs were higher in malaria-infected placentas compared to uninfected placentas, (iv) incubation of neutrophils with IEs induced only very limited NET formation *in vitro*, and (v) IEs inhibit NET formation, neutrophil degranulation, and MPO release.

7.1.1.1 Antibody-dependent neutrophil phagocytosis of IE and beads coated with DBL antigens

Antibodies play significant roles in facilitating opsonic phagocytosis (98, 292, 340), however, neutrophil mediated phagocytosis of CSA binding IEs or VAR2CSA DBL antigens is not investigated in detail. We hypothesized that antibodies could opsonize CSA binding IEs or VAR2CSA DBL antigen-coated beads and promote antibody-dependent neutrophil phagocytosis (ADNP). We also hypothesized that these antibodies are associated with protection against placental infection (chapter 4). To evaluate whether functional antibody responses to pregnancy-specific IE and VAR2CSA DBL antigens were associated with protection against placental malaria (which is still not well established (262)), we used plasma samples from PNG pregnant women collected upon enrollment into a randomized controlled trial intermittent preventive treatment in pregnancy. Women were followed up until delivery and were grouped into placental malaria (PM) and infected but no placental malaria (no PM) and noninfected at delivery, based on light microscopy of Giemsa stained placental sections and peripheral blood smear together with PCR of peripheral blood. Samples were selected for a case control study based on the presence of parasites in the periphery and placenta at delivery. For the purpose of this study, antibody responses in women with PM and no PM were compared to investigate the possible protective and functional roles of antibodies. To measure ADNP activity in test plasma, neutrophils were isolated from whole blood collected from healthy donors by negative selection.

Several papers have investigated measurement of opsonizing antibodies against IE as a correlate of protection against malaria in pregnancy and in non-pregnant adults, however the interaction of these antibodies with neutrophil mediated clearance of placental binding parasite is not assessed. Although antibody-dependent phagocytosis has been evaluated using full-length recombinant VAR2CSA antigen and IE (71, 97, 99-101, 292) as a target and monocytes and THP-1 cell lines (71, 97, 99-103, 272, 283) as phagocytes, an ADNP assay using IE is still lacking. Hence, for the first time, we developed a high-throughput ADNP assay using IEs or beads coated with VAR2CSA antigens. Because neutrophils are the most abundant blood leukocytes and are rapidly recruited into the site of infection,

assays that can dissect their contribution to antibody-driven immunity provides new insights into vaccine design and evaluation.

Results of our study demonstrated that ADNP for IE and DBL2 coated beads, expressed as the average of ADNP by three neutrophil donors, differed between pregnant women with no PM and women with PM, whereas we observed no evidence of a difference between no PM and PM groups for DBL3 and DBL5 coated beads, suggesting that DBL2 domain of VAR2CSA is an important target of protective immunity. The lack of a protective association between antibodies to DBL3 and DBL5 antigens and placental malaria could indicate that antibody response to DBL3 and DBL5 antigens may be correlated with active and /or past placental infections, rather than associated with protection from PM. A recent systematic review demonstrated that antibody responses to pregnancy-specific IE and VAR2CSA antigens were associated with the presence of placental malaria, rather than being good correlates of protection (262), but this review didn't include neutrophil based assays. The evidence of an association between antibody response to IE and DBL2 antigen and protection from placental malaria suggests that neutrophils may be important in phagocytic clearance of placental binding parasites and protection from PM.

Neutrophils phagocytose IEs (130), and *in vitro* studies have also shown that neutrophil phagocytosis of IEs was increased in the presence of immune sera (124). It was previously reported that antibodies against CSA-binding IEs effectively promoted opsonic phagocytosis of IE by primary human monocytes (292). Another study has shown that antibodies produced against the DBL3 and DBL5 antigens promoted opsonic phagocytosis of IEs by human THP-1 monocyte-like cells (90). Opsonizing antibodies also increased phagocytic clearance of merozoites (130). For example, anti-malarial antibodies promoted phagocytosis of merozoites by human monocytes (340). In the current study, ADNP of IE and DBL2 coated beads was one of features best able to separate our groups (differed between pregnant women with no PM and women with PM). Taking all together, antibodies produced against *P. falciparum* play a crucial protective role in malaria by promoting opsonic phagocytosis of IEs.

The association between neutrophil phagocytosis and protection from placental malaria hasn't been measured. This is the first study examining ADNP in the context of malaria in pregnancy, and our results demonstrated that antibodies to pregnancy-specific IEs and

VAR2CSA antigens (DBL2 coated beads) were associated with protection from placental malaria. This suggests that acquisition of antibody that facilitates ADNP may be an important part of the immune response to malaria in pregnancy. Similar data in other malaria syndromes are almost non-existent, therefore ADNP responses should also be assessed in children with severe or uncomplicated malaria, in parallel with validating the associations with pregnancy malaria reported here in different populations. In other contexts, ADNP in HIV infection was measured using beads coated with gp120 and opsonized with HIV specific antibodies, and the results demonstrated that HIV-specific antibodies with Fc-mediated functions may play an important role in protection from HIV (278, 280, 283), suggesting that antibody-mediated neutrophil-based assays could prove useful in dissecting target-specific immunity (280).

In the present study, nonopsonic neutrophil phagocytosis (phagocytosis of IE in the absence of antibody or antibodies from uninfected controls) was observed in IE-based neutrophil phagocytosis (figure 4.14), but this was not observed in bead-based assays. The possible explanations for this could be due to the different particle characteristics such as size and composition; to the presence of other antigens on IE; or to differences in the density of Ag on beads compared to IE. Using the THP1 cell line, Lloyd YM and colleagues demonstrated higher percent phagocytosis of beads compared to IE, suggesting that the density of VAR2CSA is higher on the surface of recombinant antigen-coated beads compared to IE (97), but they didn't identify nonopsonic phagocytosis in their study. The nonopsonic neutrophil phagocytosis of IE reported in Chapter 4 could be explained by the binding of placental binding IE to CSA expressed by neutrophils (301). Neutrophils express cell surface glycosaminoglycan (GAG) and CSA is the major part of it (302). IEs bind to neutrophils in serum-free assays and the binding was varied with the neutrophil donor (a previous Honors student project in Rogerson's lab), suggesting that future experiments should be designed to evaluate in detail the binding of IEs to CSA expressed by neutrophils. The other explanation could be related with altered red blood cell antigens on IE, such as modified Band 3, that lead to non-opsonic clearance.

7.1.1.2 Effects of IEs on NET formation by human neutrophils

We hypothesized that *P. falciparum* blood stages induce NET formation by human neutrophils. We used isolated neutrophils to evaluate the ability of IEs or purified

merozoites to stimulate neutrophils to form and release NETs *in vitro* (chapter 5). The results of this study showed only very limited IE- or merozoite-induced NET formation (by confocal microscopy; figure 5.1). The microscopic evaluation also demonstrated that neutrophils stimulated with IE displayed normal lobulated nuclei. Most unstimulated neutrophils stained with DAPI and were negative for SYTOX green, a dye impermeable to the cell membrane (309) (Figure 5.8), whereas neutrophils stimulated with IE were mostly positive for DAPI and SYTOX green. The finding of our study suggested that *P. falciparum* IEs affect the cell membrane of neutrophils, which allows the entry of the SYTOX green, without affecting the nuclear envelope as well as nuclear morphology. Nuclear membrane disintegration, chromatin decondensation, and mixing of nuclear and cytoplasmic contents are vital for NET formation and release (116, 148), and nuclear membrane disintegration and chromatin decondensation are mainly dependent on the activity of NE, MPO and ROS (116, 148, 310). NE and MPO are released from granules and enter the nucleus of the cells, where they can promote chromatin decondensation (310). However, the present study (chapter 5) demonstrated that incubation of neutrophil and IE did not cause chromatin decondensation or affect the nuclear morphology of neutrophils. This finding suggests that IE might have some factor that maintains neutrophil nuclear morphology to protect themselves from the antimicrobial effects of histone, by inhibiting either neutrophil degranulation (NE and MPO release) or ROS production.

In the present study, NETs formation in response to IE was not enhanced by the opsonization of IE with antimalarial antibodies. We have shown that antibodies to pregnancy specific IE enhance ADNP of placental binding IE (chapter 4), suggesting that antimalarial antibodies are more important for phagocytic clearance of IE by neutrophil than forming NETs. Next, the effect of parasite densities (IE: neutrophil) on NET formation was also evaluated. Our result demonstrated that increasing ratios of IEs didn't enhance NET formation, suggesting that parasite density have no effect on neutrophils to form NETs. A study showed that various ratios of *L. promastigotes* (*L. donovani* LV9, *L. donovani* 1S and *L. major*) induce a dose-dependent NETs formation independent of the opsonization status (172), suggesting that unlike *P. falciparum* IE, a component of the leishmanial parasite is sufficient to activate neutrophils to form NETs. Taken together, the results of this study indicated that both varying ratios of IEs and opsonization of IE with antimalarial antibodies didn't change neutrophil NET formation. An additional strategy to

investigate could be neutrophil priming with TNF- α and INF- γ which may induce NET formation in response to IE. Neutrophil priming with TNF- α increased NET formation in response to IE (304) by increasing neutrophil ROS production (148).

Studies have shown that *P. falciparum* induces NET formation in the blood of malaria-infected individuals (120, 176, 304), but we observed very limited NET formation *in vitro* in the current study, indicating that IE alone might not be sufficient to stimulate neutrophils towards pronounced NET formation. The *in vivo* NET formation in response to *P. falciparum* could be a process mediated by some factors like cytokines (120), parasite byproducts (heme) or heme in combination with cytokine (TNF- α) priming (304). The levels of cytokines such as IL-6, TNF- α and INF- γ are increased during malaria infection (120). Hence, the secretion of cytokines in response to malaria infection might facilitate NET formation *in vivo*, but our *in vitro* model lacks cytokine priming. Furthermore, concomitant infection or secondary infection with other microbial pathogens might be the cause of *in vivo* NET formation and release in *Plasmodium spp.* infected individuals.

7.1.1.3 Inhibitory effect of IE on NETs formation and neutrophil degranulation

The initial hypothesis of the present study was that human neutrophils form NETs in response to *P. falciparum* *in vitro*. However, we observed only very limited NETs when neutrophils were stimulated with IEs compared to the positive control. Based on this finding, we formulated another hypothesis that the parasites may have some factor(s) that inhibit NET formation by human neutrophils. Many microbial pathogens induce NET formation (116, 162), while an inhibitory effect on NET formation is hardly reported though not unheard of (178, 179). In this study, the NET inhibitory effect of IE was investigated by coculturing neutrophils with IE in the presence of potent NET stimulants (PMA and LPS) (chapter 5). In line with the hypothesis, the findings of this study showed that incubation of neutrophils with IEs in the presence of potent NET inducers (PMA), but not LPS, inhibits NET formation compared with neutrophils stimulated with potent NET inducers alone. When neutrophils were cocultured with uRBC controls in the presence of

PMA we observed inhibition of PMA-induced, but not LPS-induced, NET formation, suggesting that PMA-induced NET inhibition might be associated with IE or parasite byproducts or by haemoglobin released from both infected and uninfected erythrocytes. Both PMA- and LPS- induced NET formation are NADPH oxidase dependent but IE only inhibit PMA-induced NET formation. PMA induced NETs formation requires PKC, Raf/MEK/ERK pathway to activate NADPH oxidase (181, 313), whereas for LPS a TLR4-dependent, JNK-mediated sensing mechanism initiates an NADPH oxidase-dependent NET formation (313), suggesting IE or uRBCs might affect either the activation of PKC and /or the Raf/MEK/ERK with no effect on JNK activation (figure 7.1). LPS produces vital NET (allows NET release with no membrane rupture and and conventional host defense to coexist) within just 30 minutes (159). The inhibition of NET formation by IE is very interesting, this could be a whole PhD in itself, a more detailed analysis of how this occurs should be considered in future studies.

Furthermore, IEs also inhibited neutrophil degranulation, decreasing PMA-induced MPO release. By contrast, *P. falciparum* merozoites did not inhibit NET formation or neutrophil degranulation. MPO is abundant in the azurophilic granules of neutrophils and inhibition of MPO release by IE may help to maintain neutrophils nuclear morphology and decrease NET formation. It is not clear how IE inhibits NET formation and neutrophil degranulation, but it may be an important parasite escape mechanism from the host immune system. The possible reason for the inhibition of NETs formation and neutrophil degranulation by IEs, but not merozoites, could be related to the PfEMP1 protein expressed on the surface of IEs, haemoglobin in IEs, hemozoin or other bioactive molecules. All these possibilities require exploration in future studies.

Neutrophil dysfunction occurs in *P. falciparum* malaria related to hemolysis and heme oxygenase-1 (HO-1) induction, which reduced the production of ROS (317, 341, 342). ROS production and histone citrullination are required for the NETs formation (316). NETs are extracellular web-like structures that consist of decondensed chromatin decorated with

histones and antimicrobial proteins released into the extracellular environment to prevent dissemination and eventually kill bacterial, fungal and viral pathogens (116, 162, 307). However, the inhibition of NET formation by IEs might predispose to invasive bacterial diseases (secondary infection), which are well described in the immunocompromised host following malaria infection (342-344). Induction of HO-1 and IL-10 during malaria infection in humans contributes to the increased susceptibility to bacterial coinfection (343). It has been shown that IL-10 (169) and HO-1 (221, 317) inhibit NET formation by inhibiting ROS production. In addition to inhibition of ROS production, the anti-inflammatory effects of IL-10 reduce neutrophil recruitment and mobilization into infected tissues (343). IL-10 is a potent anti-inflammatory cytokine that is elevated in inflammatory diseases such as malaria. Cytokines that are involved in malaria pathology are inhibited by IL-10 (341, 345). Uncontrolled activation of neutrophils during malaria infection impairs function (133, 221, 317). Leoratti FM *et al* documented that neutrophils from malaria patients display impaired chemotactic activity (50), which increases susceptibility to secondary bacterial infection during malaria (50, 343). Since NETs play an important role in trapping and limiting the dissemination of bacterial, viral and fungal pathogens, IE inhibition of NETs may predispose the susceptible host to secondary infection in endemic areas of malaria.

Overall, inhibition of NET formation might help the malaria parasite escape host immune responses, contributing to parasite survival. However, the mechanism of NET formation inhibition by *P. falciparum* IEs is not known. Thus, further investigation is needed to better understand the NET formation inhibition pathways. Identification of the mechanism by which *P. falciparum* inhibits NET formation could contribute to the development of new treatment strategies for malaria. Therefore, future studies should test the effect of PfEMP1 protein expressed on the surface of IEs, haemoglobin in IEs, hemozoin or other bioactive molecules on NET formation and degranulation inhibition.

The observations that IE inhibit PMA-induced NET formation and the presence NETs in malaria infected placentas seem in conflict, but they are not conflicting each other because our finding demonstrated that IE inhibit, but do not degrade, PMA-induced NET formation. It was reported that the parasites or the components of *Plasmodium spp.* infection including inflammatory (TNF and IL-8) cytokines produced by white blood cells and crystal uric acid released on IE rupture can induce neutrophils to produce NETs (121). Thus, since IEs have

no NET (formed by TNF and IL-8, and uric acid crystals) degradative effect it could be possible to detect NETs in the tissue/placenta. Unlike PMA, the pathway of NET formation in response to TNF, IL-8 and crystal uric acid is NADPH oxidase-independent, operating instead by activating the PAD4 (181), suggesting that IE have an effect on the activation of PKC or Raf/MEK/ERK pathway but not on PAD4 activation (figure 7.1). NET formation is increased in illnesses associated with infection and inflammation, which may be beneficial or detrimental. The primary role of NETs is to contain pathogens and to prevent infection spread, being protective (346). However, NETs were subsequently found to be harmful in several diseases, including lung disease, thrombosis, cancer and autoimmune disease (346). The presence of neutrophils and NETs in the placenta may trap, immobilize and kill the parasite, but the released NETs contain elastase, which might cause placental damage. Further studies assessing NETs and NET components, both *in vitro* and *in vivo*, are warranted to systematically investigate in detail this paradox.

7.1.1.4 Placental malaria and NETs

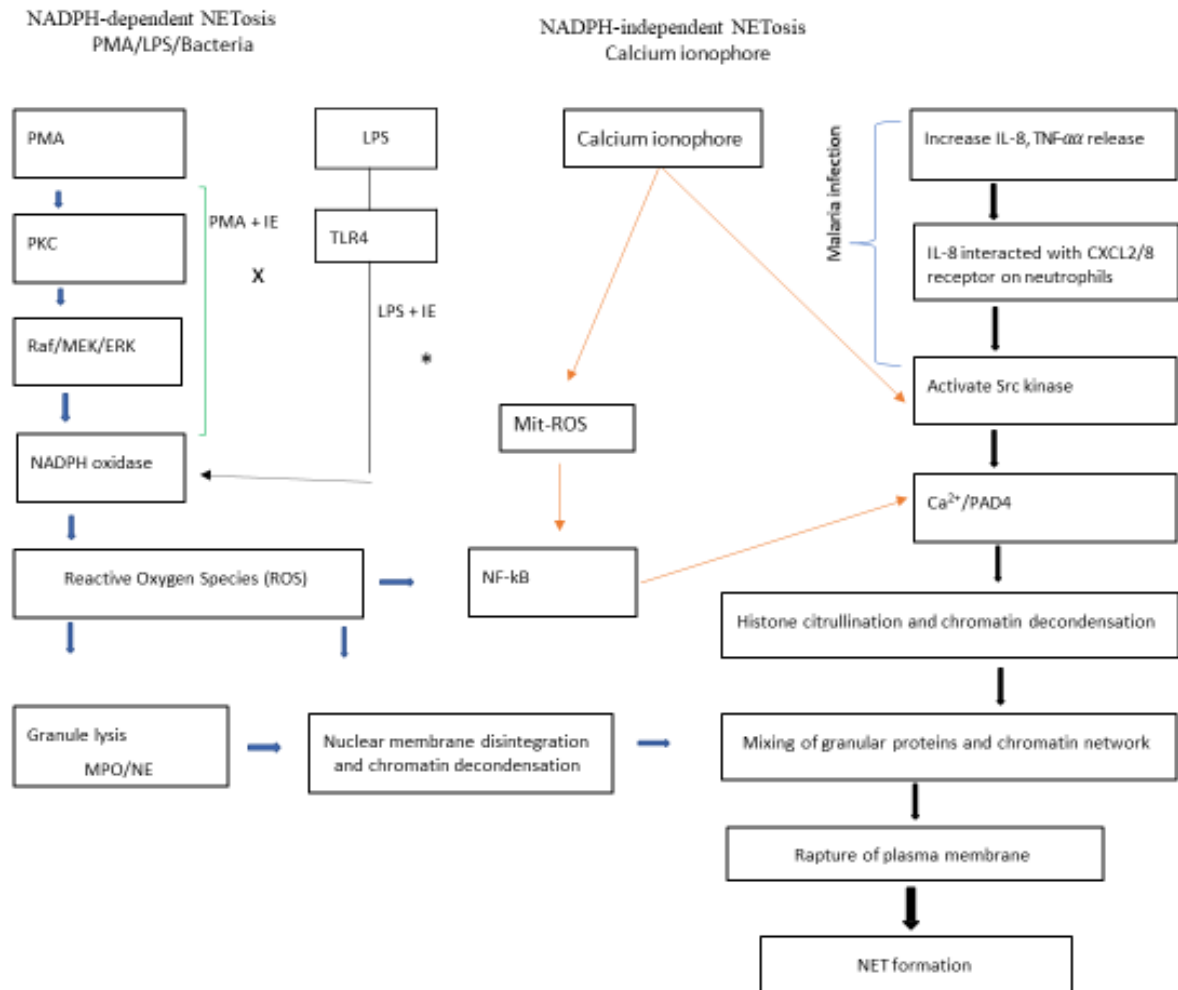
Researchers have shown that *P. falciparum* induces NET formation in the blood of malaria infected children (120, 176, 304), however, migration of neutrophils to the placenta and the presence (or absence) of NETs in the IVS of the placenta was not reported in malaria infection during pregnancy. We hypothesized that malaria during pregnancy increases neutrophils migration to the placenta, where they form NETs to trap and kill the parasite in the IVS of the placenta. To assess neutrophils migration and the presence of NETs, paraffin-embedded placental tissues were evaluated (chapter 6). Migration of leukocytes into the placenta and the presence of NETs in the IVS were assessed by light microscopy of Giemsa stained placental sections. The result indicates a significantly higher number of total leukocytes and NET-like structures were identified in the IVS of malaria-infected placentas as compared to controls. However, the numbers of neutrophils did not differ significantly between malaria-infected and malaria uninfected groups. The possible reason that neutrophil numbers did not differ between infected and uninfected groups when using Giemsa stained sections could be that when *P. falciparum* activates neutrophils in the IVS of the placenta, this results in nuclear round-up and chromatin decondensation leading to morphological changes, as a result of which the neutrophils can be mistaken for other forms

of leukocytes such as monocytes, which prompted us to use neutrophil specific markers to evaluate neutrophil migration to the placenta.

Placental sections were stained by a neutrophil specific marker and neutrophils and NETs in the IVS were assessed. Our findings showed that more neutrophils migrate into the IVS of the malaria-infected placentas compared to uninfected controls, and form NETs. Although there are no related malaria studies to compare our findings, placental NETs were reported from placentas with pre-eclampsia and lupus pregnancies (180, 198, 202, 336). Gupta *et al* reported that placentally-derived IL-8 and placental micro-debris triggered NETs formation by stimulating neutrophils (180). In the current study, the presence of NETs in the placenta suggests that NET formation could be mediated by priming of neutrophils with cytokines released in response to the parasites (120, 176, 304), or it could be like pre-eclampsia and lupus, IEs could stimulate placenta to release bioactive molecules that then induce neutrophils to form NETs.

Previously, NETs were reported on Giemsa stained blood smears of children with uncomplicated *P. falciparum* malaria (120) and adults with symptomatic malaria infection with *P. falciparum*, *P. vivax* and *P. malariae* (176). The triggers of *in vivo* NET will require further investigation. One possibility for the formation of *in vitro* NETs could be related to secretion of cytokines in response to the parasites. The other possible mechanism underlying the formation of NETs in the blood could be related to formation of heme because of haemoglobin degradation by the parasites. Coinfection of malaria and other microbial pathogens could also be the trigger for *in vivo* NET formation (341). Of relevance to this hypothesis, a recent study showed that NETs in malaria infection are triggered by a combination of extracellular heme with TNF- α priming (304), but not by the parasite itself or the heme alone. In the present study, a significantly higher number of NETs were identified in placentas infected with malaria compared to uninfected placentas (chapter 6), whereas incubation of neutrophils and IEs alone *in vitro* induces very limited NETs or inhibit NET formation induced by potent stimulants which could be cytokines, heme, histones or other parasite factors that weren't encountered under the *in vitro* conditions in our experiments (chapter 5). We recommend these factors need to be considered and investigated in *in vitro* conditions in future studies

The association between NET formation in the blood and contribution to pathogenesis in severe falciparum malaria has been reported (176), but there is no data on placental malaria. Our results showed that more NETs in the IVS of the malaria-infected placentas compared to uninfected controls. In published studies, the number of NETs in the circulation was increased significantly in proportion to the severity of malaria disease (176, 304), which suggests that NET formation can contribute to malaria pathogenesis (176, 201). Circulating NETs and antinuclear IgG antibodies (ANA) to double-stranded DNA were reported from malaria infected children, suggesting NETs and ANA contribute to malaria pathogenesis in children (120). Boeltz S *et al* reviewed the association between intracellular NET formation and severity of clinical malaria and the contribution of NETS to malaria pathogenesis, observing that the increase in NETs may exacerbate intravascular thrombosis and promote ischemia and reduce tissue perfusion (201). Knackstedt *et al* recently reported a high concentration of neutrophils and NETs in the blood of malaria-infected mice and found that the presence of NETs causes liver damage (304). The authors also measured circulating NET components (NE/DNA, MPO/DNA complexes) by ELISA in plasma from malaria patients in Gabon and Mozambique and the number of NETs in the plasma was significantly higher in severe malaria compared with healthy controls from the same region. The authors of this same study reported that soluble NET components facilitate parasite sequestration in the liver and lung and mediate tissue destruction (304). NETs associated damage to the maternal endothelium was also observed in preeclampsia since endothelial cells are susceptible to cell death induced by NET releasing neutrophils (336). The result of the current study suggests that the presence of NETs in malaria infected placentas might cause maternal endothelium and placental damage by NET releasing neutrophils, which might be associated with poor pregnancy outcomes. Neutrophil degranulation produces myeloperoxidase, elastase, and metalloproteases; they are all enriched in NETs, can degrade extracellular matrix proteins, and can destroy the integrity of the endothelial vascular cells (346). MMPs further activate barrier disrupting cytokines and chemokines, such as IL-1 β , TNF- α , and CXCL8 (346), which can amplify the signalling of hyperpermeability. Activation of neutrophils leading to production of toxic ROS molecules and elastase can cause placental damage and eventual demise of the foetus (347). Hence, future studies should examine the relationship between the presence of placental NETs and NET components with pregnancy outcomes.



NB: * = IE inhibit LPS-induced NET formation. X = IE inhibit PMA-induced NET formation by affecting either PKC and/ or Raf/MEK/ERK pathway. PMA +IE = incubation of neutrophils with IEs in the presence of PMA. LPS + IE= incubation of neutrophils with IEs in the presence of LPS.

Figure 7.1 Schematic representation of NET formation pathways. Different agonists induce either NADPH oxidase-dependent (agonists: PMA, LPS, bacteria etc.) or NADPH oxidase-independent NET formation (agonists: Calcium ionophore, uric acid crystals etc). These agonists induce different forms of reactive oxygen species (ROS) (NADPH-ROS and Mitochondrial-ROS). Induction of PMA receptor leads to activation of protein kinase C (PKC) which activates the Raf/MEK/ERK pathway. PMA and LPS induce NET formation via the activation of NADPH oxidase. Calcium ionophore mediates NADPH oxidase-independent NET formation. ROS result in nuclear membrane disintegrations and granule lysis (MPO/NE release), as well as NF-κB activation. The PAD4 mediates histone citrullination and leads to chromatin decondensation. IL-8 and TNF-α released during malaria infection interact with CXCL2/8 receptor on neutrophils and trigger NET formation *in vivo*. Coincubation of neutrophils with IE in the presence of LPS has no effect on LPS-induced NET formation (*), however, when neutrophils are stimulated with PMA in the presence of IE, IE inhibited PMA-induced NET formation and MPO release, possibly by affecting the PKC or Raf/MEK/ERK pathway (x).

7.2 Study limitations and future directions

Since most of the assays performed in this Ph.D. thesis (ADNP, NET inhibition, and placental NET evaluation), in the context of neutrophils and malaria, there are several gaps, and the limitations of this study should be addressed in future works.

These novel studies in malaria pathogenesis have revealed that neutrophils play an important role in the immune response in malaria. ADNP for DBL2 coated beads and IE differed between pregnant women with no PM and PM (**In chapter 4**). ADNP was measured only using placental binding IEs and some selected recombinant DBL antigens (DBL2, DBL3 and DBL5 antigens) in this study. For further experiments, all the 6 recombinant DBL domains, as well as the full length VAR2CSA, should be incorporated. In addition, future research should also evaluate ADNP with other types of parasite lines, and other antigens. Furthermore, future studies should test ADNP in children with severe or uncomplicated malaria as well as validating associations with pregnancy malaria in different populations.

For the first time, we reported that IEs, but not merozoites, inhibit NET formation as well as neutrophil degranulation (**chapter 5**). The possible reason for the inhibition of NETs formation and neutrophil degranulation by IEs but not merozoites could be related to IE surface antigens, haemoglobins in IEs, hemozoin or other bioactive molecules. The other possible reason could be that the inhibition of PMA-induced NET formation may be associated with erythrocytes or the haemoglobin they contain which may have an effect on the activation of PKC, however, free merozoites may lack this action. Therefore, future experiments should be designed to test these, as well as identifying the mechanism. In addition, it could be important to compare IE NET inhibition with known inhibitors of the different pathways of NET formation. IEs, inhibition of MPO release, given that the supernatants were collected at 4 h post stimulation, stimulation time may not be enough to release MPO (348). Future studies should test neutrophil degranulation at multiple time points (e.g. 6, 12, 24 and 48 hrs). As a measure of degranulation, the release of NE, cytokine release and ROS production were not assessed in the current study. Analysis of other granular proteins, levels of cytokines and ROS production from IE stimulated neutrophil

supernatants would have been of value in understanding the effect of IEs on neutrophil function. Since we reported the inhibitory effect of IE on NET formation for the first time, the results from this Ph.D. thesis can be used to further investigate all the NET formation pathways using different stimulants.

In chapter 6 of this Ph.D. thesis, we only looked at the placental NETs but didn't measure circulating NETs. Given that NETs are composed of nuclear DNA and granular proteins, future studies should consider measuring the levels of NET components by ELISA from the plasma of malaria-infected pregnant women. It was previously reported that NETs appear to be involved in various disorders of the reproductive cycle including infertility, excessive placental damage and fetal loss (347, 349). In an animal model, it has been documented that NETs capture sperm in the female reproductive tract during their transfer to the oocyte resulting in infertility. The presence NETs in placental blood may lead to thrombotic events resulting in severe placental damage and consequent loss of the fetus (347, 349). Previous studies have revealed the association between NETs and severity of malaria disease, and the contribution of NETs to tissue damage (120, 176, 201, 304). The result of the current study demonstrated the presence of NETs in IVS of malaria infected placentas, suggesting that NETs in IVS might cause maternal endothelial and placental damage and might be associated with poor pregnancy outcomes. However, this study didn't assess the effect of placental NETs on placental injury and pregnancy outcomes. Hence, future studies should compare the presence of placental NETs with pregnancy outcomes. In the present study, we didn't control other infections. The presence of NET in the placenta does not necessarily mean that these NETs are associated with *Plasmodium* infection alone, suggesting future studies could control for other infections or inflammatory diseases which are known to induce NET formation.

7.3 Conclusion

Parasite sequestration and inflammation are the two main mechanisms of malaria pathogenesis and complications. Our work added to our current understanding of malaria immunity by identifying the role of neutrophils in immune response in malaria. The results from this Ph.D. thesis provided several pieces of evidence, and have demonstrated that

functional antibodies induce neutrophil phagocytosis of placental binding IEs. Antibodies to pregnancy-specific IEs and VAR2CSA antigens (DBL2 coated beads) were associated with protection from placental malaria. This suggests that acquisition of antibody that facilitates ADNP may be an important part of the immune response to malaria in pregnancy. Similar data in other malaria syndromes are almost non-existent, therefore ADNP responses should also be assessed in children with severe or uncomplicated malaria, in parallel with validating the associations with pregnancy malaria reported here in different populations.

Neutrophils migrated into the placenta during malaria in pregnancy, where they form NETs in IVS of malaria infected placenta, suggesting neutrophils play a protective role against placental malaria via opsonic phagocytosis and *in vivo* NETs formation.

We report, for the first time, that IEs inhibit NET formation and neutrophil degranulation *in vitro*. Inhibition of NET formation might help the malaria parasite escape host immune responses, contributing to parasite survival. Identification of the mechanism by which *P. falciparum* inhibits NET formation could contribute to the development of new treatment strategies for malaria.

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9 Appendices