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Biological properties of the digestive vacuole of *Plasmodium falciparum*: Activation of complement and coagulation

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LIST OF ABBREVIATIONS

DV : Digestive vacuoles

CD55 : Decay accelerating factor

DBP : Duffy binding-like protein

DBL-EBP : Duffy binding-like erythrocytic binding protein

ER : Endoplasmic reticulum

GPI : Glycosylphosphatidylinositol

IHC : Immunohistochemistry

IgG : Immunoglobulin G

LMW-DXS : Low molecular weight dextron sulfate

NTS : Non-typhoidal *Salmonellae*

MAC : Membrane attack complex

MSP 1 : Merozoite surface protein 1

PfHRP : *Plasmodium falciparum* histidine-rich protein

PCR : Polymerase chain reaction

PMN : Polymorphonuclear granulocytes

PNH : Paroxysmal nocturnal hemoglobinuria

PPM : Parasite plasma membrane

pRBC : Parasitized red blood cells

PVM : Parasitophorous vacuole membrane

QBC : Quantitative buffy coat

RDTs : Rapid Diagnostic Tests

ROS : Reactive oxygen species

TF : Tissue factor

TVN : Tubo-Vesicular Network

ABSTRACT

Plasmodium falciparum is an intracellular protozoan parasite that has been associated with humans since the dawn of time and causes severe forms of malaria. It is a major health problem around the globe and causes highest toll of death among children less than five years of age in developing countries. An infected female *Anopheles* mosquito injects malaria sporozoites into the skin while taking a blood meal. The sporozoites, which are released into the blood stream, reaches the liver where they undergo exoerythrocytic schizogony. After exoerythrocytic schizogony, millions of merozoites are released into the blood stream and infect new red blood cells, where they undergo erythrocytic schizogony in a cyclic manner. The erythrocytic schizogony stage of *Plasmodium* life cycle is where all clinical manifestations of malaria as a disease become apparent. The clinical symptoms like fever, headache, jaundice, vomiting have been associated with hyperparasitemia and these clinical symptoms coincide with the cyclical release of malaria parasites during schizonts rupture. A severe form of malaria develops as a consequence of capillaric sequestration of parasitized red blood cells (pRBC) and rosetting of pRBC with uninfected red blood cells which obstruct the blood flow to the brain. Activation of complement and coagulation, and increase in vascular permeability further aggravates severity of the disease which can lead to microcirculatory disturbances with comatous death as the ultimate outcome.

Rupture of each *Plasmodium falciparum* infected red blood cell releases 8-32 infective merozoites along with a single digestive vacuole into the blood stream. The released digestive vacuole is an organelle in which hemozoin is surrounded by an intact membrane. We have discovered that the digestive vacuoles have the capacity to dually activate the complement and coagulation systems. Activation of complement and coagulation requires an intact DV membrane. The complement and coagulation activating properties of the DV are inhibited by low molecular weight dextran sulfate. In non-immune serum, DVs are opsonised with complement C3b and rapidly phagocytosed by polymorphonuclear granulocytes (PMN). Upon rupture, DVs lost its functional activities and the extracted malaria pigment from the DV organelle is not engulfed by the PMN. Liberated merozoites are not opsonized in non-immune serum and escape phagocytosis. High titered anti-malarial antibodies from immune patients mediate some uptake of the merozoites, but to an extent that is not sufficient to markedly reduce re-invasion rates. Engulfment of DVs by PMN induces a respiratory burst, but the generated reactive oxygen species (ROS) are unable to suppress the infective capacity of invading merozoites. Finally, ingested DVs drive the PMN into a state of functional exhaustion. Upon challenging of PMN with bacteria after DV ingestion, the ability to phagocytose bacteria prevails, but their capacity to mount a respiratory burst is reduced

and microbicidal activity is compromised. We propose that these events might be linked to the development of septicemic episodes in patients with severe malaria in sub-Saharan African countries.

ZUSAMMENFASSUNG

Die lebensbedrohliche Form der Malaria wird durch Plasmodium falciparum verursacht und geht einher mit schwerwiegenden Störungen des Gerinnungs- und Komplementsystems- Bei der Ruptur eines infizierten Erythrozyten werden 8 bis 32 Merozoiten und eine Nahrungsvakuole in die Blutbahn freigesetzt. Die Nahrungsvakuole (digestive vacuole = DV) ist ein membranumhülltes Organell, in dem das Abbauprodukt des Hämoglobins, das sog. Hämozoin, verpackt ist. Die Dissertationsarbeit befasst sich mit bislang unbekannten biologischen Eigenschaften der DV. Wir haben entdeckt, dass das freigesetzte Organell die bemerkenswerte Fähigkeit besitzt, den intrinsischen Gerinnungsweg und das Komplementsystem simultan zu aktivieren. Beide Vorgänge lassen sich mit niedermolekularem Dextransulfat inhibieren. Bindung von C3b bewirkt, dass DVs von neutrophilen Granulozyten selektiv phagozytiert werden. Die Phagozyten werden dabei aktiviert und es kommt zur Bildung von reaktiven Sauerstoffradikalen. Dieses treibt die Zellen in einen hypofunktionellen Zustand: während ihre Phagozytosefunktion erhalten bleibt, können aufgenommene Bakterien nicht mehr effizient abgetötet werden. Die DV stellt möglicherweise einen neuen Pathogenitätsfaktor des Malariaparasiten dar und könnte unmittelbar für tiefgreifende Störungen des Gerinnungssystems und des natürlichen Immunsystems verantwortlich sein.

1. INTRODUCTION

1.1 Historical outline

Malaria is an ancient disease that has been associated with humans since the dawn of time. For thousands of years malaria was attributed to the inhalation of noxious vapours or miasmas from wetlands, and hence, the word malaria has been derived from Italian “Mal Air" meaning bad air. Malaria antigens were detected in the skin and lungs of Egyptian mummies from 3200 and 1304 B.C. (Miller et al. 1994). Ancient accounts of malaria date back to Vedic writings of 1600 B.C. and autumnal fevers were referred to as “king of diseases” described from Vedic period in India. Tertian and quaternary fevers along with other malaria symptoms were also described in Chinese medical classic Nei-ching (Bruce-Chwatt et al. 1988). The great Greek physician Hippocrates (400 BC) characterized malaria as a disease with intermittent and periodic fevers, and he considered it was connected to living in proximity to marshes. The descriptions in Roman medical literature also describes about malaria that it was associated with stagnant water and led the Romans to develop drainage programmes which was the first documented preventive measure against malaria. In the book *Little House on the Prairie,* Laural Ingalls Wilder clearly described the impact of malaria on the history of United States and it is commonly known as “fever and ague”. By the early eighteenth century, malaria was distributed worldwide due to growing population and increased movement of people across the globe.

By seventeenth century, the research on malaria was further extended. Thomas Sydenham (1624-1689) studied the differences in the periodicity of malaria fever and described clinical manifestation of malaria in detail. In the middle of 17th century, the Peruvian bark known by the natives as “Quina –quina” was introduced to Europe. For the first time in England around 1660, Thomas Sydenham and Richard Morton (1637-1698) confirmed its efficacy against malaria fever. This Peruvian bark was later called Cinchona bark and contained the active anti-malarial agent quinine.

1.2 Global Distribution

Malaria is one of the world’s most widespread disease. According to the World Health Organisation (WHO) report on malaria in 2011, malaria is prevalent in 106 countries in the tropical and subtropical regions of world where *Anopheles* mosquitoes can thrive and multiply. Malaria is caused by five *Plasmodium* species that affect humans i.e. *P. falciparum, P. vivax, P. Malariae, P. ovale,* and *P. knowlesi.* Malaria due to *P. falciparum* is the most common and deadly in African countries. As temperature plays a vital role in *Plasmodium falciparum* below 20°C (68°F) it cannot complete life cycle in the *Anopheles* mosquito, and thus cannot be transmitted. *Plasmodium vivax*

is less dangerous but more prevalent because it can tolerate low ambient temperature (world malaria reports 2011). According to the world malaria report 2011, an estimated 3.3 billion people were at risk of malaria in 2010. Eighty one percent of malaria cases and 91% of deaths over the world were estimated to occur in African countries, children less than five years of age and pregnant women are being most severely affected.

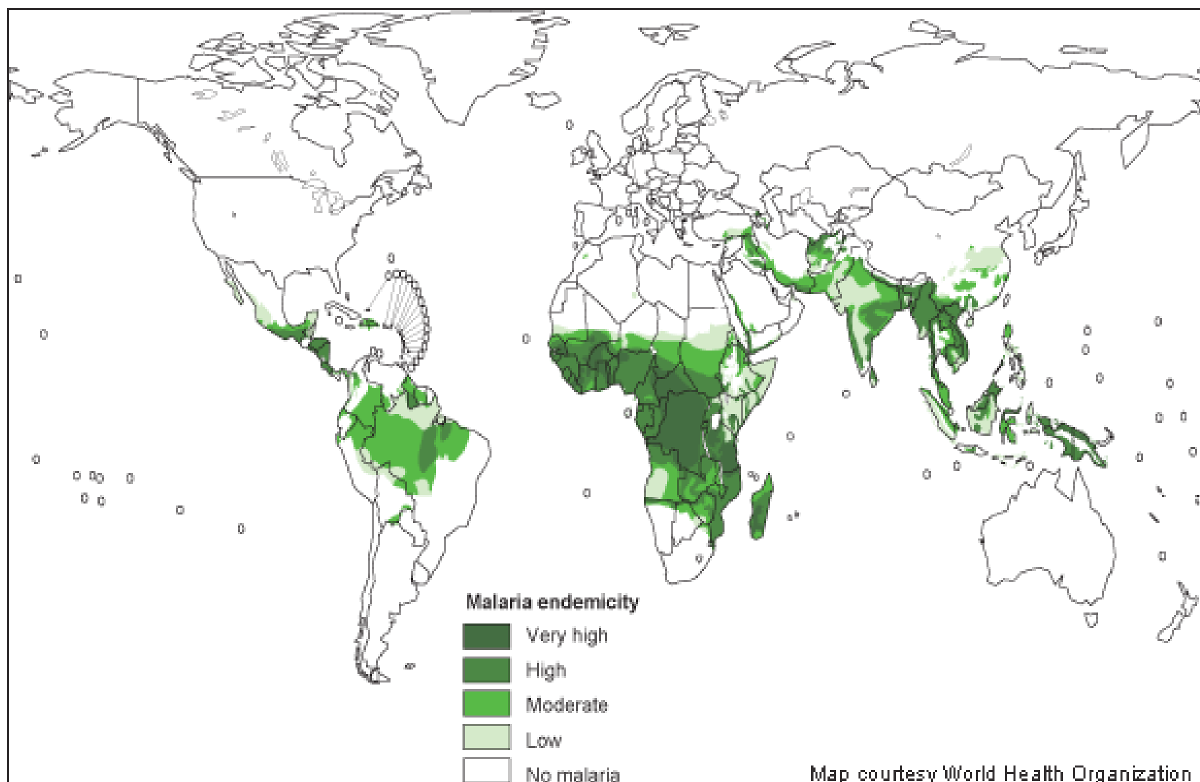

Figure 1. Global distribution of malaria: The intensity of malaria transmission and endemicity in different countries around the globe. Source: - World Malaria Report 2011.

1.3 Taxonomy

The Phylum Apicomplexan consists of a wide spectrum of eukaryotic organisms. It includes protozoan parasites that infect both humans and veterinary animals such as *Plasmodium, Toxoplasma, Theileria, Eimeria, Cryptosporidium,* and *Babesia* species. Laveran coined the name "Oscillaria malariae" without knowing the biological function of the parasite, and he believed that flagellate bodies are more important stage (Laveran et al. 1881). Since the spores develop at the end of the life cycle, they have been classified in the Class Telosporea. Within the Class Telosporea, the Order Eucoccidiorida comprises parasite of red blood corpuscles of vertebrate

animals. Danilewsky observed similar parasite in the birds and he placed the parasites into new group Haemosporidia. The parasite is unable to pass any part of their life cycle outside the body of the host, so it belongs to the order Haemosporina in which they constitute the family Plasmodiidae. The family Plasmodiidae contains a single Genus *Plasmodium* that undergoes schizogony in the vertebrate host and sporogony in mosquito.

There are about 200 species of *Plasmodium*, 22 of them infect primates. Five species infect humans*: P. falciparum, P. Ovale, P. Vivax and P. malariae*, *P. knowlsi*. Of these, *P. falciparum* is the deadliest and important in public health as it is the parasite responsible for the high mortality observed in malaria endemic regions.

1.4 Life cycle

The malaria parasite has a complex, multistage life cycle, which completes in two living organism, the invertebrate vector mosquito and the vertebrate host. Infection of the host is initiated by the bite of infected female *Anopheles* (=Greek; hurtful, harmful*)* mosquito. Invasive sporozoites (Greek; Sporos =seeds) contained in the mosquito's saliva are inoculated into host skin. Following intradermal deposition of sporozoites, some are destroyed by local macrophages and other reaches the blood vessels. The sporozoites that reach a blood vessel infect the liver within few hours after infection. A mean of 15 sporozoites are inoculated (Rosenberg et al. 1990) and they remain in the circulation for 15-60 minutes (Sihden and Smith 1982) then sporozoites migrate into hepatocytes, where they undergo exo-erythrocytic schizogony within the parasitophorous vacuole. The entire pre-erythrocytic stage of the parasite lasts about 5-10 days depending on the parasite species, and thought to occur only once in the case of mammalian malaria parasite: on an average 5-6 days for *P. falciparum*, 9 days for *P. ovale,* 8 days for *P. vivax,* 13 days for *P. malariae,* and 8-9 days for *P. knowlesi.* In *P.vivax* malaria, some of the sporozoites develop dormant forms known as hypnozoites in the liver for months and infection can recur later causing relapses of clinical infection after few weeks to months. (Krotoski et al. 1982).

The merozoites released from the liver into the circulation rapidly invade red blood cells, where they begin part of the life cycle called erythrocytic schizogony. Merozoites recognize, attach, and invade the red blood cell through the presence of species-specific receptors in/on erythrocyte membrane in as little as 60 seconds. *P. vivax* depends on two different ligands for its invasion into erythrocyte i.e. Duffy binding-like protein (DBP) that binds to Duffy blood group antigen (Miller et al. 1976) and reticulocyte homology protein that binds to unknown receptors on reticulocytes (Galinski et al. 1992). *Plasmodium falciparum* can use highly redundant and alternate invasive pathways that utilize several different receptors to enter the red blood cell. For example, *P. vivax*

has only one Duffy binding-like erythrocytic binding protein (DBL-EBP) family of gene, whereas *P. falciparum* has four DBL-EBP genes (Mayer et al. 2009). It has been known that some species of malaria parasite preferentially infects young erythrocytes. An in vitro study shows that *P. berghei* preferentially infects reticulocytes (McNally et al. 1992). Similarly, *P. vivax* has preference for infection of reticulocytes (Garnhamet et al. 1966) and in *P.falciparum* reticulocytes and young RBC are more susceptible than old RBC (Pasvol et al. 1980).

Within the RBC, the merozoite develops into ring form, trophozoite stages, and the nucleus divides asexually to form a schizont containing between 8-32 merozoites. The erythrocytic schizogony occurs every 48 hours in case of *P. falciparum, P. ovale, P. vivax,* and 72 h in case of *P. malariae*, while in *P.knowlesi* it lasts 24 hours. Finally, the rupture of erythrocytic schizont releases new merozoites which invade new RBC. The parasite density can rise rapidly to the level as high as 10^{13} per host (Greenwood et al. 2008).

A small fraction of merozoites differentiate into male or female gametocytes. Gametocytes may be rounded or banana/crescent in shape depending on the species. Gametocytes complete their sexual life cycle in the female mosquito. Most female mosquitoes have to feed on animals and get sufficient blood meal as a protein source to nourish their eggs and lay 30-150 eggs every 2-3 days. Mature female and male gametocytes ingested by the mosquito reach the mid gut of the insect, where the male gametocyte undergoes a rapid process of DNA replication and nuclear segmentation. The progression from haploid genome to octaploid genome with the formation and migration of eight nuclei into highly motile, flagellated microgametes is called exflagellation (Janse et al. 1986a; Janse et al. 1986b). This process is completed within 10 to 20 minutes after ingestion of blood meal. Fertilization of female gametes by free swimming male gametes leads to formation of the zygote. Gametogenesis, formation of male and female gametocytes and exflagellation of male gametes is triggered mainly by drop in temperature (Ogwan'g et al. 1993; Billker et al. 1997) accompanying the transition from warm-blood host to the mosquito gut. Change in pH, carbon-dioxide tension (Carter and Nijhout 1977), and mosquito mid gut factors (Billker et al. 1997; Garcia et al. 1997) also significantly contribute to the induction of gametogenesis.

The zygote develops into elongated slowly motile ookinete which actively penetrates the gut epithelial cells and develops into an oocyst. The oocyst undergoes multiple rounds of asexual replication leading to formation of as many as 10,000 new individual nuclei within a mature oocyst of 40 to 60 µm in diameter. The time required for the establishment of mature oocyst from initial zygote depends on the parasite species, the *Anopheline* species, and on the ambient temperature: it varies from 7 to 30 days. The products of the mature oocyst are the **sporozoites**, which are narrow, curved in shape, actively motile and 10-15 µm in length. Rupture of the mature

oocyst releases the sporozoites into the haemocelomic space of the mosquito. The sporozoite migrates and reaches the salivary glands, where it penetrates the basal membrane and settles in the salivary duct, thus completing the life cycle (Lobo and Kumar 1998).

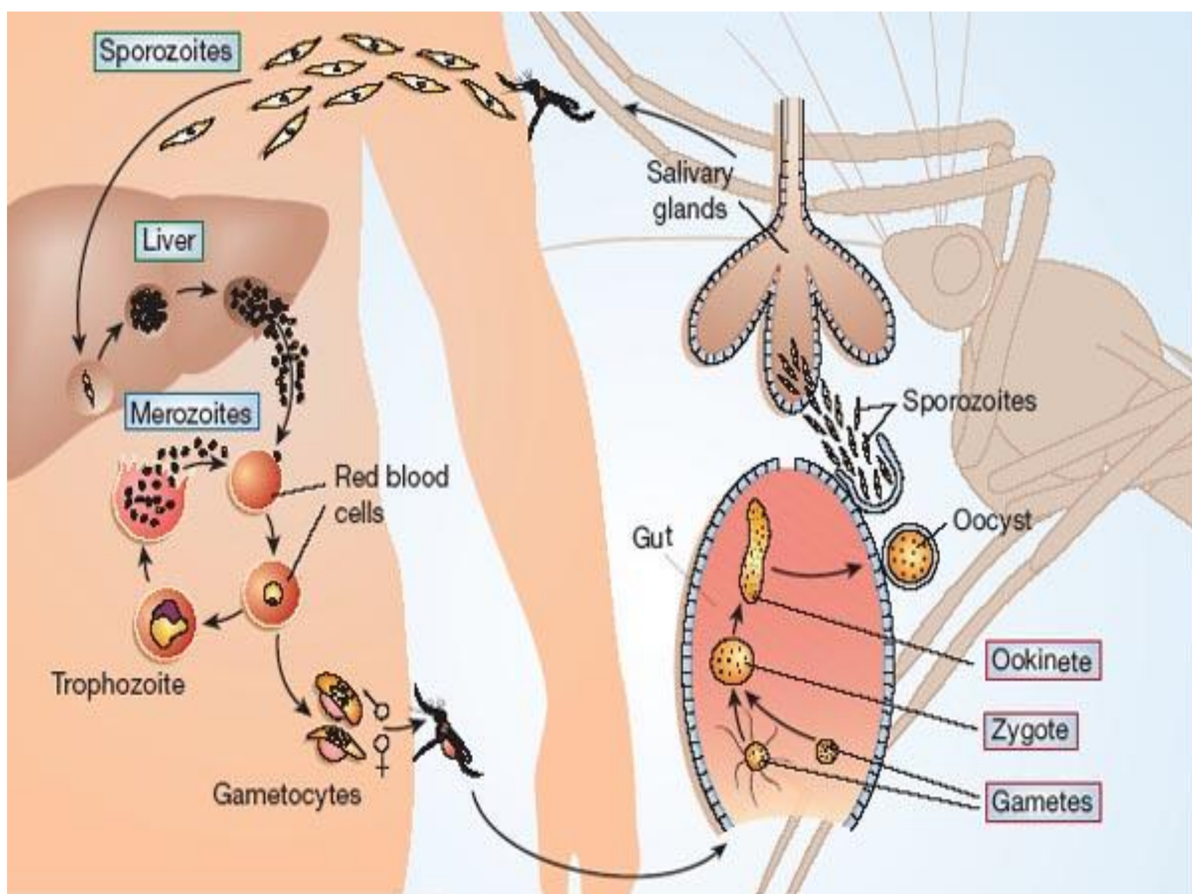

Figure 2. Life cycle of the human malaria parasite: Both the sexual and asexual phase of the life cycle, alternating between the mosquito and human hosts are shown. First, the sporozoites are injected by the infected mosquito into the skin, and reach the liver, where they differentiate into merozoites. Second, after exoerythrocytic schizogony, merozoites enter the blood stream and infect new red blood cells and continuous erythrocytic schizogony. Source: - **Ménard, R** (2005). Medicine: Knockout malaria vaccine? *Nature* **433**, 113-114.

1.5 Ultrastructures of different stages of the erythrocytic cycle

The merozoite stage

The free invasive merozoite is very small, <1.6 mm long and 1.0 mm wide, ovoid in shape with a low, flat ended projections at one end (the apical prominence) (Langreth et al. 1978). The apex contains three sets of secretory vesicles used in invasion and these are; a pair of rhoptries,

numerous small micronemes and small rounded dense granules. The nucleus is placed basally and a plastid and a mitochondrion lie along one side of the merozoite (Bannister et al. 2000a). The merozoite coat is made up of merozoite surface protein (MSP) 1 which comprises most abundant protein of the merozoite coat and it is known to be cleaved during invasion (Holder 1994). After invasion, the dense granule releases their contents in to the parasitophorous vacuole and increases the area of its membrane (Culvenor et al. 1991), and probably facilitates the change in shape of the parasite to the ring stage (Bannister et al. 2000a).

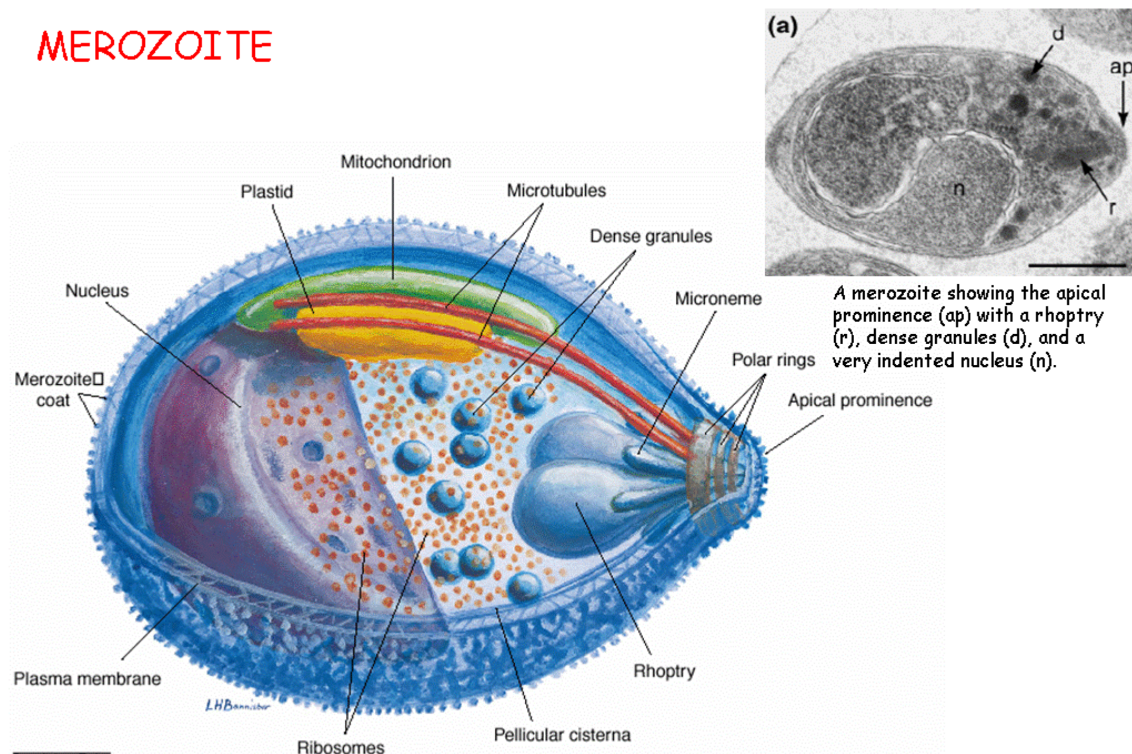

Figure 3. Three-dimensional organization of a *Plasmodium falciparum* merozoite, with the pellicle partly cut away to show the internal structure. Source: - **Bannister et al. (2000).** A brief illustrated guide to the ultrastructure of Plasmodium falciparum asexual blood stages. *Parasitol. Today,* **16:** 427–433.

The ring stage

After invasion the parasite morphs itself into a thin discoid, flat or cup-shaped ring form (Langreth et al. 1978). At the early ring stage the parasite starts importing haemoglobin through a small dense ring on the surface of the parasite called “cytostome” (Slomianny 1990) and feeds on

haemoglobin. Through the cytostome, a small digestive vacuole with RBC cytosol forms by pinching off the parasitophorous vacuole membrane (PVM) and the plasma membrane of the parasite (Slomianny 1990). The free haem resulting from haemoglobin digestion is converted into inert black-brown haemazoin pigment crystals that accumulate within the digestive vacuole throughout erythrocytic phage of the cycle. Initially these small vacuoles derived from endocytosis act as an individual digestive vacuole, but in later stages these vesicles fuse to from a single large digestive vacuole (Bannister et al. 2004). As the parasite matures the area of the PVM also increases and the parasite extends membranous structures into the surrounding medium, and finally changes its shape to round or irregular trophozoite (Elford et al. 1995).

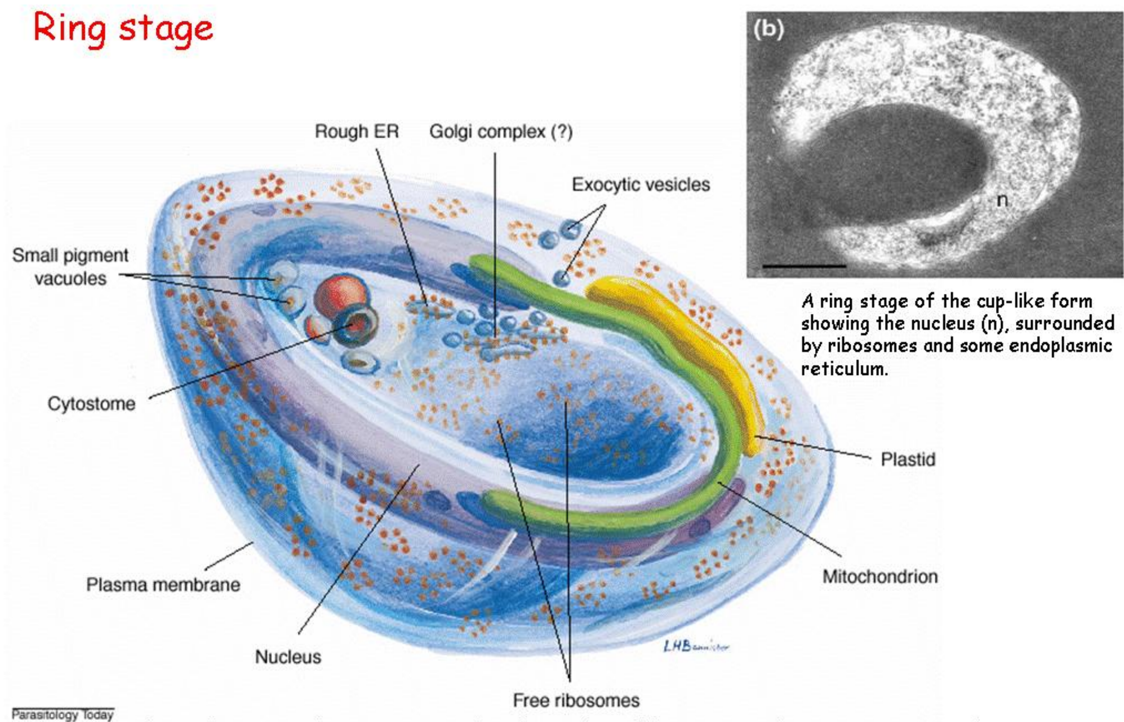

Figure 4. Three-dimensional structure of a *Plasmodium falciparum* early ring stage. Source: - **Bannister et al. (2000).** A brief illustrated guide to the ultrastructure of Plasmodium falciparum asexual blood stages. *Parasitol. Today,* **16:** 427–433.

Trophozoite stage

The parasite feeds more actively on haemoglobin than the ring form and forms a large digestive vacuole in which the degradation products of haemoglobin (hemozoin crystals) accumulates. The parasite surface forms irregular bulges and deep tubular invaginations [Tubo-Vesicular Network (TVN)] within the host cell and some of these invaginations may extend out to the RBC surface (Trelka et al. 2000). These extended membranes structures called as Maurer's clefts. There is

some evidence to suggest that these structures are involved to access large extracellular molecules from the parasite surface into the erythrocyte cytosol and perhaps vice versa, because some fluorescent dyes can readily penetrate to the parasite through the RBC (Pouvelle et al. 1991), (Lauer et al. 1997). By the mid-trophozoite stage, the rough ER and putative Golgi complex have increased in size and complexity (Bannister et al. 2004). Parasite export proteins to the erythrocyte surface to form "knob" like structures in *P. falciparum.* The Knobs help *P. falciparum* trophozoite to strongly adhere to the endothelial cells of various tissues in the body. Sequestration of parasitized erythrocytes in the brain microvasculature blocks the blood supply to the brain, and drives the patient to comate state and leading to cerebral malaria, finally death (Ponsford et al. 2012).

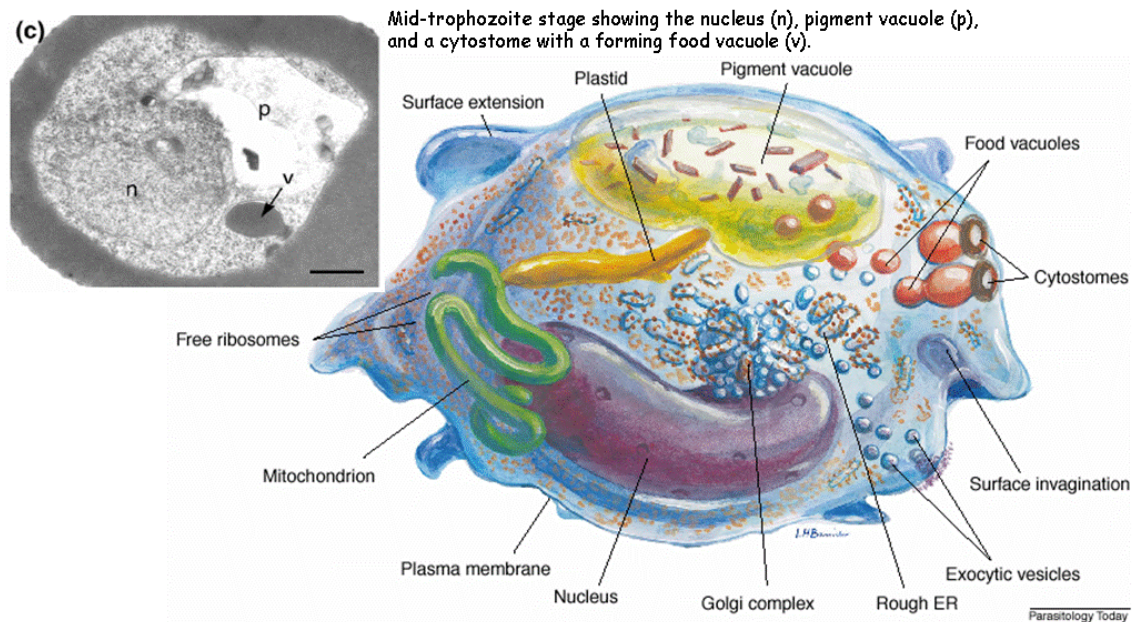

Figure 5. *Plasmodium falciparum*, trophozoite stage characterized by its irregular shape, the increased protein synthesis machinery, increased uptake of hemoblobin through multiple cytostomes, growth of the digestive vacuole, and structures associated with export of parasite proteins (Golgi body, exocytic vesicles). Source: - **Bannister et al. (2000).** A brief illustrated guide to the ultrastructure of Plasmodium falciparum asexual blood stages. *Parasitol. Today,* **16:** 427–433.

The schizont stage

The trophozoite undergoes repetitive nuclear division to become a schizont. Ingestion of hemoglobin lasts until late in the schizont stage and almost complete consumption of haemoglobin while adding further haemozoin crystals to the digestive vacuole. At the same time, the parasite exports proteins to the RBC continuously until the late schizont stage (Bannister et al.

2004). The exported proteins distort the RBC membrane to increase the number of knobs on the cell (Nagao et al. 2000). In *P. falciparum* the nucleus divides and produces 16- 32 merozoites. Nuclear division is endomitotic, a common feature in unicellular eukaryotes (Bannister et al. 2000a). To form the merozoite, the cytoskeletal components including polar rings and microtubles are assembled beneath the merozoite surface and a cleavage furrow now forms around each nascent merozoite for defining its shape (Bannister et al. 2000b). Before complete separation, each merozoite gets a copy of mitochondrion and the plastid. After complete segregation from each other, the merozoites cluster within the PVM and they are released when the erythrocyte membrane and the PVM are ruptured. These last steps are triggered by secretions from the apical complex (Bannister 2001). Merozoite egression from the host erythrocyte has recently been shown to occur in a two step process (Salmon et al. 2001). Using GFP-tagged proteins it was shown that PVM ruptures first followed by a secondary rupture of the erythrocyte membrane. Selective inhibition of the two step escape process was achieved by using specific protease inhibitors (Wickham et al. 2003).

SCHIZONT

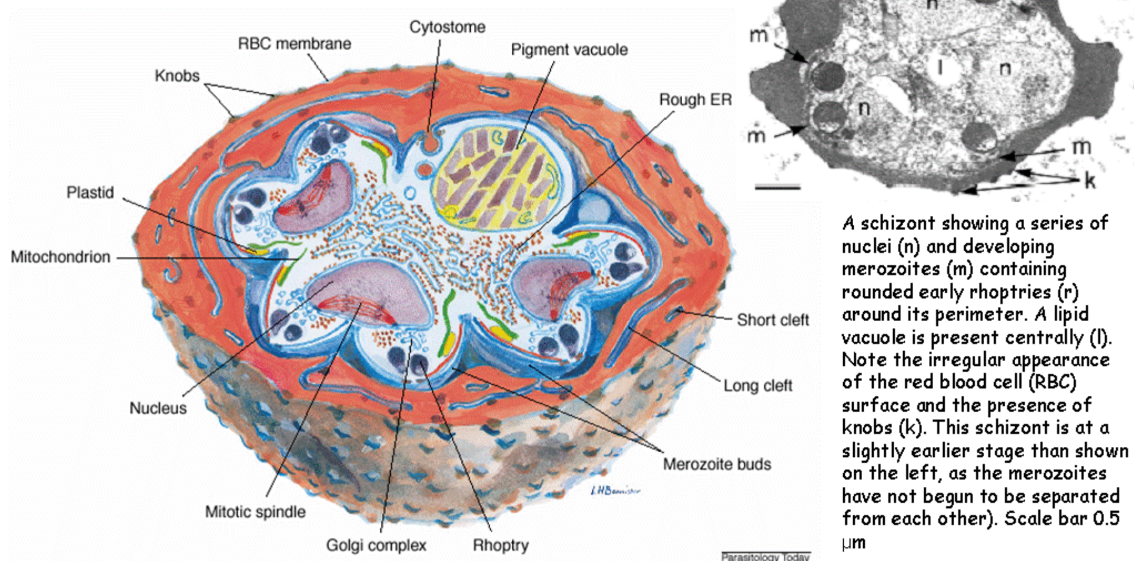

Figure 6. *Plasmodium falciparum* infected RBC at the end of schizont stage, depicted in a schizont-infected red blood cell, to show parasite and RBC structure, Maurer's clefts, and surface knobs. Merozoites are maturing from the surface of the schizont and the apex of each merozoite contains apical organelles, and mitochondria (green) and plastids (yellow) are migrating into the buds. Source: - **Bannister et al. (2000).** A brief illustrated guide to the ultrastructure of Plasmodium falciparum asexual blood stages. *Parasitol. Today,* **16:** 427–433.

1.6 Transmission

The most important milestone in the malaria history was the discovery of malaria transmission. Laveran discovered the malaria parasite in 1880 and these findings were confirmed by Camillo Galgi, Marchiafav, Celli and many other researchers worldwide. Next, the researchers mainly focused on unravelling the mystery of malaria transmission. Laveran and Patrick Manson have demonstrated the role of mosquitoes in the transmission of filariasis while working in South China in 1878. Based his experience on filariasis, Manson speculated that mosquitoes could play a vital role in malaria transmission. Manson passed his experience and finding to his colleague Ronald Ross. Taking this cue, Ross initially used Culex and Aedes mosquitoes to study malaria transmission and infection but he was in vain. Ross was not an entomologist, so he classified the mosquitoes he was studying as gray or barred-back (*Culex* sp), brindled (*Stegomyia* sp) and dappled-winged (*Anopheles* sp) (Ross et al. 1923). He used avian malaria parasite *Proteosoma relictum* (now called *Plasmodium relictum*), commonly infects many species of birds including crows and sparrows and discovered that it was transmitted by 'grey' mosquitoes, probably *Culex fatigans* and published his observations in 1898. When he was working in Secunderabad, India on 20 August 1897, he found pigmented bodies called rods that invade the mosquito's salivary gland in the dapple-winged mosquito after being fed on a malaria patient and he called 20th August as "Mosquito Day" (Ross et al. 1923). Ross discovery was further confirmed by the Italian Scientists and proved that only female *Anopheles* mosquitoes could transmit malaria, and described the whole blood-mosquito life cycles of *P. falciparum* and *P. malariae P. vivax,* (Grassi et al. 1900). Ross was awarded Nobel Prize in 1902 for his discovery of Malaria transmission.

1.7 Clinical symptoms

Malaria infections are asymptomatic during the exoerythrocytic schizogony, and the clinical symptoms do not develop prior to the rupture of infected RBC. In 1886, Camillo Golgi was the first to identify that malaria fever coincides with the cyclical release of malaria parasites during schizonts rupture of red blood cells (Golgi et al. 1886). The mechanisms associated with *P. falciparum* infections are still completely not understood. Malaria infection may take a variety of clinical courses differing in pattern and severity. *P. falciparum* infected erythrocytes have decreased in deformability and sequester to the deep vascular system to escape from splenic clearance. Sequestration and high replication rates are thought to play a key role in falciparum malaria infection. The severity and outcome of the disease depends on age, and immune status of the patients. Several types of malaria fever have been described including intermittent, remittent, and continuous and some are symptomless, others are fatal. Malaria febrile paroxysms last for 5-6

hours and recur every 2- 3 days depending on parasite species and synchrony of the cycle. *Plasmodium falciparum, P. ovale* and *P. vivax* recurs each third day, while P*. malariae* recurs on every fourth day. The severity of the fever is always related to the rupture of mature schizonts and release of antigenic material into the bloodstream. Based on Ronald Ross and David Thomson's observations, a minimum of 200 to 500 parasites per µl of blood in *P. vivax* patients and 600 to 1500 parasites per µl of blood in *P. falciparum* infected patients were necessary to induce fever (Ross et al. 1910). Patients with *P. falciparum* infections can develop organ bleeding, liver or kidney failure and central nervous system problems, which can lead to cerebral malaria and death. WHO recommends criteria to classify malaria infections. These criteria include the presence of the following complications: coma, renal failure, severe anemia, acidosis, respiratory distress syndrome, hypoglycaemia, bleeding, shock, intravascular haemolysis, convulsions, jaundice, and hyperparasitaemia. Severe anemia is very common among children in high malaria transmission regions (Snow et al. 1997). During pregnancy, the clinical symptoms of malaria may vary according to the level of endemicity. In holoendemic areas pregnant women with *P. falciparum* infections have higher parasitemia and more severe clinical symptoms than non-pregnant women. Pregnancy malaria is associated with low birth weight babies and abortions {(Nosten et al. 1994).

1.8 Malaria diagnosis

Malaria is a preventable and treatable disease, must be diagnosed promptly in order to treat the patient within time. Delay in diagnosis and treatment of malaria can lead to death of the patient in non-endemic areas and non-immune patients. Diagnosis of malaria is difficult where malaria is not endemic, because the health-care providers may not be familiar with the disease. Clinical diagnosis gives initial information about the patient, which is based on the clinical symptoms and physical conditions of the patient at the time of examination. The first symptoms of malaria most often includes fever, chills, sweats, headache, muscle pains and vomiting, which are also symptoms of other diseases such as flu and viral fever. These clinical symptoms should be confirmed by one of the below mentioned laboratory test for malaria.

Microscopic diagnosis

Microscopic examination of peripheral blood smear is gold-standard for the confirmation of malaria. Thick and thin smears are prepared from the peripheral blood and stained with Romanovsky stain (most often Giemsa), and examined with a 100X oil immersion objective. These blood smears provide information on the species, the stage, and the density of parasitemia.

Quantitative buffy coat (QBC) technique

The QBC test is a new method for identification of malaria parasite in peripheral blood. The QBC tube is high-precision glass hematocrit tube pre-coated internally with Acridine Orange and Potassium Oxalate. When QBC tube filled with blood from patient and centrifuged at 12,000 rpm for 5 min, blood cells separate according to their densities and form discrete bands. RBCs containing malaria parasite are less dense than normal blood cells and concentrate below the white blood cells at the top of the erythrocyte column. The fluorescing malaria parasites are observed from the red blood cell/white blood cell interface using a standard white light microscope equipped with the UV microscope adapter, an epi-illuminated microscope objective. The QBC method is easy and faster (20-30 minutes), but identification of the parasite species, and quantification of the parasitemia is difficult (Adeoye and Nga 2007).

Antigen diagnosis

A dipstick antigen detection test provides an attractive alternative method for diagnosis of malaria in the field studies, where staining and microscopy equipment are not easily available. These "Rapid Diagnostic Tests" (RDTs) offer a useful alternative to microscopy and are currently used in some clinical settings. Sensitivity and specificity of this method for the diagnosis of malaria infections are between 80% and 100% when compared with blood films (Heutmekers et al. 2012).

Molecular diagnosis

Polymerase chain reaction (PCR) is useful for the detection of species-specific *Plasmodium* genome and it is more sensitive than other tests, and can able to detect as few as 10 parasites/µl of blood sample. PCR is very expensive technique and difficult to take to the rural places, where malaria is more prevalent. Although quantitative PCR method has been described, the nested PCR is most widely used method for epidemiological and clinical research purpose (Singh et al. 1999). Clinical workers prepare the blood sample on a glass fiber membrane and dry the membranes, the dried blood spots are sent to the lab for PCR diagnosis (Curtis et al. 1998). The use of glass fiber membrane is very simple and the sensitivity is excellent, even though the use of filter paper reduces the sensitivity (Färnert et al. 1999). PCR has been regularly used successfully as a gold standard method for epidemiological purpose (Hänscheid and Grobusch 2002).

1.9 Chemotherapy

For nearly two centuries following its introduction, cinchona was largely dispensed as a powder prepared from the bark of *Cinchona.* In 1820, the French chemists Pelletier and Caventou isolated two alkaloids, which were named as quinine and cinchonine. Quinine was soon found to be a more reliable therapeutic agent for malaria treatment. In the middle of 18th century, Ledger found

some species of cinchona, which was found to have high quinine content and it was subsequently named *Cinchona ledgeriana.* It is less effective and potentially more toxic than chloroquine*.* Chloroquine is a prototype of 4-aminoquinolines anti-malaria drug, which also includes amodiaquine, a most widely used drug for all types of malaria fevers (McChesney 1983). The mechanism of action of chloroquine is not yet clear; at high concentration the drug reaches the digestive vacuole and inhibits the haemozoin formation by raising its pH. Primaquine, 8-aminoquinoline inhibits the parasite at schizont stage and is useful to control schizonts in the tissues. In addition, these compounds inhibit parasite growth and effectively act against sexual forms of *P. falciparum*. The metabolic products of primaquine block electron transport disrupting the parasite's energy metabolism. Antifolate drugs, such as pyrimethamine, sulphadoxine block tetrahydrofolate biosynthesis, an important cofactor in the parasite's metabolism. Pyrimethamine is a competitive inhibitor of dihydrofolate reductase, while sulphadoxine blocks dihydropteroate synthetase. These anti malarial drugs also inhibit the parasite at schizont stage and are used in combination as a therapy in the case of chloroquine resistant *P. falciparum* infections. Mefloquine and halofantrine compounds have potent action against blood schizonts. Artemisin is an effective anti-malarial drug isolated from *Artemisia annua*. Artemether, artesunate and dihydroartemisin are the derivatives of artemisin and have more potent anti malarial activity than the parent compound. These compounds act most rapidly than all other antimalarials developed so far. They mainly act against schizonts but the mechanism of action is not completely understood. Atovaquone is a hydroxynaphthoquinone which has anti-protozoan activity. It has a novel mode of action by inhibiting electron transport in *P.falciparum*.

Drug resistant parasites have been reported from most parts of the world as a result of mutations which are selected after frequent use of anti-malarial drugs. Now, new anti-malarial remedies are therefore urgently required. Anti-malarial combinations may increase efficacy of the treatment, decrease the duration of treatment and also decreases the development of drug resistant parasites. Combination therapy is the simultaneous use of two or more anti-malaria drugs with independent mode of anti-malarial activity. The concept of combination therapy is based on synergistic effect of two or more drugs and improves the therapeutic efficiency. Artemisinin based combinations are in clinical trials and are known to improve survival rates, reduce the development of resistance and they might decrease transmission of drug-resistant parasites (Nosten and White 2007), (Bosman and Mendis 2007), (Kamya et al. 2007).

1.10 The discovery of hemozoin

The presence of black-brown pigment was first observed by German physician, Johann Heinrich Meckel in 1847, in the autopsy studies on the body of patient who had died from malaria. However, he did not make connection between the pigment and malaria. Rudolf Virchow, German pathologist in 1848 pictured and described the pigment in the blood of a patient infected with malaria and he is the one who first linked pigment to malaria. Hischel, in 1850, noticed and confirmed the connection between the presence of pigment and intermittent fevers. Planer, in 1854, observed the pigment in the circulating blood. He suggested that the pigment in the circulating blood emanates during the fever and it causes many symptoms. Finally Laveran, a French military surgeon in 1880, discovered the fact that the pigment was mainly contained in the body of a living parasite. Councilman and Abbot, in 1885 performed autopsy studies on two cases of comatose pernicious malaria and described that pigment containing bodies in and outside of the red blood cell. They have mainly observed these pigmented bodies in the capillaries of the brain and also in the liver and spleen, as well as pigmented leukocytes. Golgi is the one who first photographed the pigmented malaria parasite (Sullivan 2002).

Hemozoin structure

In the year 1847, a German Physician Johann Heinrich Meckel, discovered a black-brown pigment in an insane person and subsequently this was linked to malaria by the pathologist Rudolf Virchow in 1849 (Virchow et al. 1849). Initially this pigment was believed to be melanin and in 1911 WH Brown distinguished the malaria pigment from melanin using potassium permanganate bleaching technique (Brown et al. 1911). Later Gosh and Nath in 1934, Gosh and Sinton in 1934 have demonstrated that malaria pigment hemozoin and the alkaline preparations of hemozoin were identical to heme in the spectra (Gosh and Nath et al. 1934, Gosh and Sinton et al. 1934). Other groups (Ashong et al. 1989) speculated the necessity of proteins in the hemozoin formation and identified proteins that co purified with hemozoin. Two individual groups Fitch and Kanjananggulpan et al. (1987), Slater et al. (1991) confirmed that hemozoin contains only haem and not the proteins (Fitch and Kanjananggulpan 1987; Slater et al. 1991). By using infrared and X-ray spectroscopy Slater et al. (1991) postulated that hemozoin contains iron-corboxylate bond between two haem monomers. Iron in the hemozoin crystals contains in the form of 3+ ferric states (Brémard et al. 1993) and it has paramagnetic properties (Nalbandian et al. 1995). By using X- ray powder diffraction method, Pagola et al. (Pagola et al. 2000) concluded that haemozoin contains Fe1-O41 head to tail heme dimers in the crystals rather than the step ladder polymers

(Slater et al. 1991) and the dimers form chains linked by hydrogen bonds in the crystal (Pagola et al. 2000).

Slater and Cerami showed the conversion of iron(III) protoporphyrin IX (Fe(III)PPIX) to hemozoin under acidic (pH 5.5) conditions in the presence of parasite extract and postulated that heme polymerase is responsible for hemozoin formation (Slater and Cerami 1992). Formation of hemozoin under these conditions was inhibited by widely used anti-malarial quninolines. Afterwards, Egan et al., 1994; Dorn et al., 1995 have demonstrated the β-hematin formation in the test tube at 60-70° C in an excess molar concentration of acetate and millimolar concentrations of monomeric heme(Egan et al. 1994; Dorn et al. 1995). β-hematin has identical Fourier Transform Infrared absorbance peaks at 1,207 cm21 and 1,667 cm21, to hemozoin and both have identical properties like, insolubility in water, basic solutions at pH 9 and also in dimethyl sulphoxide (DMSO) which normally solubilises protoporphyrins (Slater et al. 1991). Debate continues over a spontaneous chemical reactions or a parasite guided process in the digestive vacuole. Fitch et al., in 1999 have shown that lipid micelle can also form heme crystals *in-vitro* (Fitch et al. 1999) and in the parasite extracts from *Plasmodium berghei* (Chou and Fitch 1992). In Goldbergs laboratory purified digestive vacuoles immunised to rabbits and the immune serum was used to screen cDNA library (Sullivan et al. 1996). Most of the clones were matched to previously characterised gene Pf histidine-rich protein (PfHRP) II. PfHRP II was shown to localize in the digestive vacuole and demonstrated that PfHRP II binds to heme at an acidic pH and initiates the hemozoin formation (Sullivan et al. 1996). Two other groups, Lynn et al., 1999; Papalexis et al., 2001 also independently showed that PfHRP II initiates crystals formation (Lynn et al. 1999), (Papalexis et al. 2001). Hemoglobin constitutes 95% of total cytosolic proteins of the red blood cell. During intraerythrocytic cycle of malaria parasite, the host hemoglobin is consumed and an estimated 70-80% of it is degraded (MORRISON and JESKEY 1948), (Orjih and Fitch 1993). Degradation of hemoglobin occurs via semi-ordered process by parasite proteases within the lysosome-like organelle, called digestive vacuole and releases heme and amino acids (Goldberg et al. 1990). The heme moiety is stored in the form of an inert polymer as malaria pigment hemozoin in the digestive vacuole (Sherman 1977).

Digestive vacuole (DV) is a highly specialized organelle and is the primary site for hemoglobin degradation (Olliaro and Goldberg 1995). DVs in *Plasmodium falciparum* are acidic organelles with an estimated pH around 5.0-5.4 (Krogstad et al. 1985). Digestive vacuoles are formed by endocytosis from the parasite surface via cytostome. Cytostome is large double membrane invaginations of the parasitophorous vacuole membrane (PVM) and parasite plasma membrane (PPM) and contains electron dense material flanking around the cytostome (Langreth et al. 1978). Vesicles containing hemoglobin buds from the parasitophorous vacuole membrane (PVM) are

ingested through cytostome to from one or more digestive vacuoles and the PVM is subsequently degraded. (Aikawa et al. 1966; Slomianny et al. 1985; Slomianny 1990; Slomianny and Prensier 1990; Lazarus et al. 2008; Dluzewski et al. 2008). Parasite starts importing hemoglobin in the early and mid ring stages through the double membrane vesicles. Each individual endocytic vesicle can import degradative enzymes from the parasite secretory pathways (Klemba et al. 2004) and can break down hemoglobin to forms hemozoin crystals (Klemba et al. 2004). Later these small DVs fuse to form a single large digestive vacuole, which eventually fills with hemozoin as the parasite grows (Bannister et al. 2004; Elliott et al. 2008). In the completely developed blood schizont, the DV remains a separate, distinct organelle, which is separated from the newly formed merozoites. Upon rupture each schizont releases one DV along with 16-32 infective merozoites into the blood stream.

1.11 Immunity to malaria

Natural malaria infections lead to only a partial and short lived immunity that is unable to completely protect the individual from new malaria infection. Children below five years of age are at highest risk of severe malaria and clinical symptoms generally become less severe are rare in adults. In low or unstable transmissions areas, immunity against malaria is not acquired and therefore all age groups are at high risk.

Innate Immunity

Natural immunity against malaria is an obstinate inherent of the host that prevents establishment of infection or an immediate inhibitory responses against the malaria at the beginning of infection. In high malaria transmission regions, individuals can be partially protected against malaria due to alterations in the structure of hemoglobin. For example, sickle cell anemia and beta-thalassaemia make more difficult for *P. falciparum* or *P. vivax* to invade red blood cells (Williams 2006). Glucose 6 phosphate dehydrogenase deficiency confers 50% protection against severe malaria infections (Carter and Mendis 2002; Doolan et al. 2009). Some people lack Duffy antigen in the red blood cells on their surface, which act as a receptor for *Plasmodium vivax* and so individuals without Duffy protein are resistant to *P. vivax* infection (Barnwell et al. 1989). Individual with acute malaria infections induces non-specific early immune responses, which limits the progression of disease. Tissue macrophages and dendritic cells (DCs) expressing pattern recognition receptors are thought to be play very important role in early immune response to malaria. It has recently been described that *P. falciparun* derived hemozoin (Coban et al. 2005) and parasite derived DNA (Gowda et al. 2011) induce proinflammatory cytokines though toll like receptor 9 (TLR9). *P. falciparum* infected erythrocytes expresses erythrocyte membrane protein 1

(PfEMP1), which is recognised via CD36 receptor on phagocytes resulting in the clearance of infected erythrocytes by tissue macrophages (Ayi et al. 2005). Severe malaria infection induces strongly elevated blood concentrations of non-specific malaria immunoglobulin, but the importance of the underlying polyclonal B-cell activation for innate immunity is not understood.

Acquired Immunity

People living in holoendemic regions under intense malaria transmission acquire immunity that protects against severe clinical manifestations. Anti-malarial immunity, which protect against high parasite burden is acquired slowly and not very efficiently and remains species and strain specific. Asymptomatic infections are frequent in children and adults in holoendemic regions. The equilibrium between host immune response and constant low-parasite burden in patients has been termed premunition and it indicates that immunity against malaria is mediated by the presence of parasite (COLBOURNE 1955). Repeated malaria infections induces specific and polyclonal antibody production, predominately IgG and IgM antibodies. During the first months, infants are protected from severe malaria clinical manifestations (Franks et al. 2001) and this protection has been associated with passive transfer of maternal antibody IgG (Riley et al. 2001) Passive transfer of antibodies from immune patients protects by reducing parasitemia and clinical disease. The infants after 5 to 6 months of age become more susceptible to severe malaria and severe malarial anaemia is the common clinical symptom. However, during pregnancy, women are more susceptible to malaria infections. It is thought that this derives from immune suppression during gestation period and sequestration of infected erythrocytes in the placenta especially during first and second pregnancy (Menendez 2006). Antibodies against malaria may protect by inhibiting merozoite invasion of red blood cell and intra-erythrocytic development of the parasite. Opsinization of infected erythrocytes by specific antibodies enhances the clearances form the circulation. Malaria parasite escapes from the host's immune response, due to their antigenic diversity and clonal antigenic variation, and also changes the immune response and causes considerable amount of immune suppression. The adaptive anti malaria immunity does not persist for long time. In the absence of repeated malaria infections, the acquired immunity become fruitless and the individual become vulnerable to the malarial infection once again.

1.12 The complement system

Complement system is one of the most important and evolutionary highly conserved ancient components of the innate immune system. The human complement system consist more than 30 soluble plasma proteins and membrane associated regulator proteins (Sarma and Ward 2011). Plasma contains more than 3 grams of complement proteins per liter and constitutes 15% of globulin fraction (Walport 2001). Many complement proteins circulate in the serum as

proenzymes and are activated by proteolytic cleavage. Complement components are designated by the historical order of the discovery of the proteins: C1-C9, and by letters (e.g. factor D, factor B). Complement activation leads to a sequential cascade of enzymatic reactions resulting in opsonization of pathogens with C3b and their removal by phagocytes, and by lysis of susceptible cells. There are three pathways for activation of the complement cascade, the classical, alternative and lectin pathway. All pathways converge at C3 and result in the formation of membrane attack complex (C5b-9).

Classical complement pathway

The first discovered complement pathway was the classical pathway whose activation occurs only after adaptive immune responses have been recruited. Activation of classical pathway begins with the formation of antigen-antibody complex (immune complex) or with the binding of antibody to a cell surface and end with the lysis of the cell. Binding of specific IgM or IgG (subclasses 1, 2 or 3) to antigen induces conformational changes in the Fc region of the antibody molecule and that exposes a binding site for C1of the complement component. Antigen-antibody complexes containing IgM are more efficient in activating complement than immune complexes containing IgG, because IgM is a pentamer. C1 cleaves the C4 into C4a and C4b and the C4b fragment attaches to the activator surface in the vicinity of C1 and rapidly followed by C2 binding to adjacent C4b, where the C2 is also proleolytically cleaved by C1. The resulting C4bC2a is the classical complement pathway C3 convertase.

Alternative complement pathway

Alternative pathway is evolutionarily more ancient complement pathway than the two other complement activation pathways. Alternative pathway was named because it was identified secondary to the classical pathway. Activation of alternative complement pathway does not require antibody, so the alternative pathway is a component of the innate immune system. Under normal circumstances, the circulating C3 has an unstable thioester bond which is spontaneously become activated by reacting with water, and constantly generates C3b at low level in a process which can be called as complement pathway “tickover”. This spontaneously generated C3b is rapidly inactivated either by interacting factor I using factor H as a cofactor. The spontaneously generated C3b can bind to microbial cell surfaces and then initiates the complement activation. Factor B is then recruited to the bound C3b on microbial surface, followed by Factor D that cleaves C3b-bound factor B. The resulting C3bBb complex on microbial surface is known as alternative complement C3 convertase and this complex is stabilized by plasma properdin (Kemper et al. 2010) that extends its half-life 6-10 folds.

Lectin complement pathway

Mannose binding lectins (MBL) are proteins that bind to specific carbohydrates on the surface of pathogens including yeast, bacteria, parasites and viruses. MBL is an acute phase protein produced in inflammatory response. After binding of MBL to the surface of pathogen, MBL associated proteins MASP-1 and MASP-2 bind to MBL, and induces cleavage of C4 to C4a and C4b and C2 to C2a and C2b. Pathogen surface bound C4bC2a has C3 convertase enzyme activity (Turner 1996).

Three pathways converge at membrane attack complex

Activation of all three complement pathways leads the formation of terminal C5b-9 or membrane attack complex (MAC) on the target cell. Formation of MAC on the target cell membrane enables ions and small molecules diffuse freely across the membrane and leads to the cells lysis. Assembly of C5b-9 complex in the membrane lipid bilayer of complement activating target cell starts with cleave of C5 α to generate C5a and C5b (Tegla et al. 2011). The C5b component is extremely unstable and binding of C6 to it stabilizes its activity. Binding of C5b6 to C7 undergoes a structural change that exposes a hydrophobic region which enables the C5b67 complex to insert in to the phospholipid bilayer. The subsequent interaction of C8 and C9 with C5b67 results in the formation of C5b-9 complex and this complex referred to terminal complement complex (Bhakdi and Tranum-Jensen 1987).

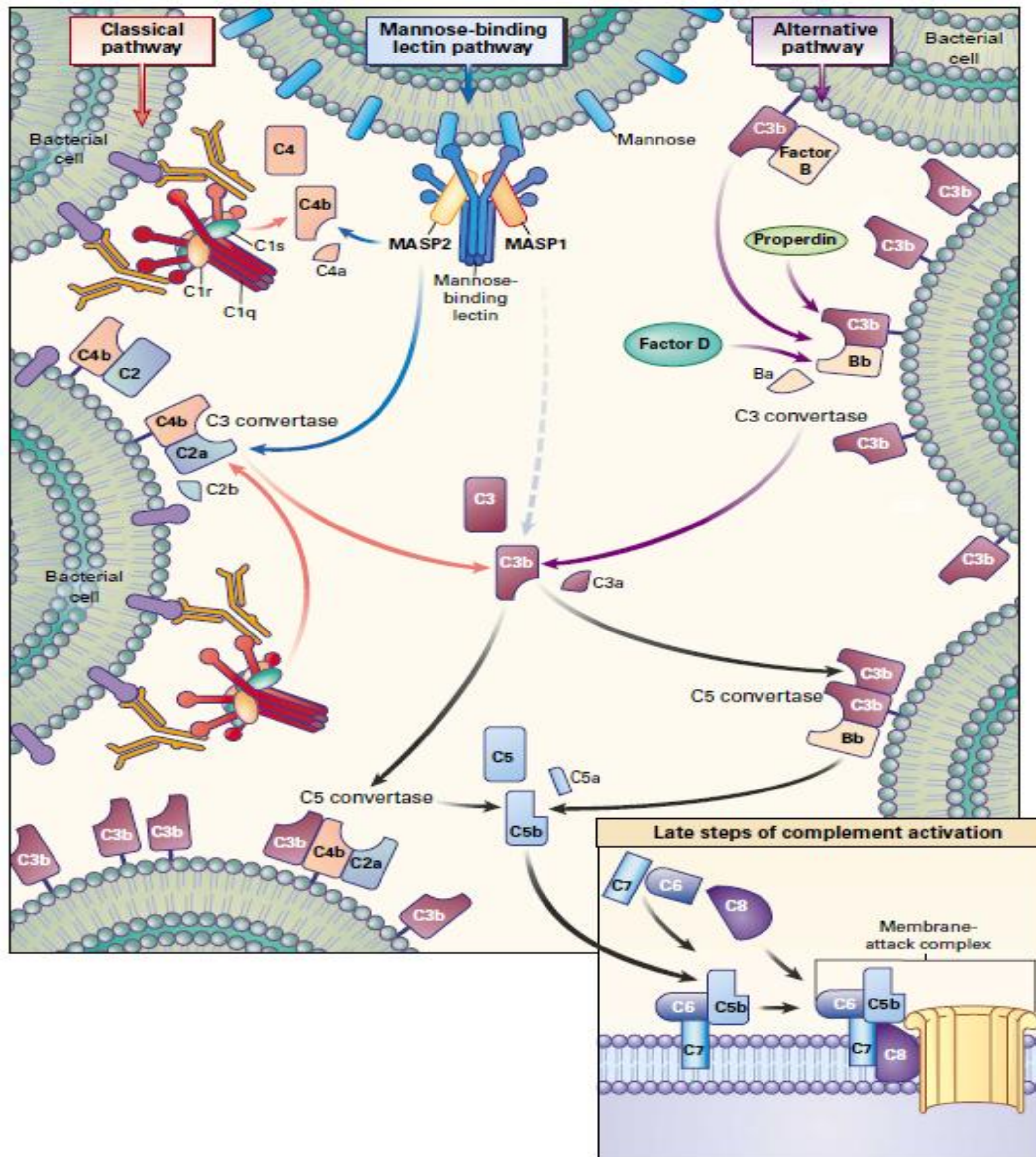

Figure 7. The Three Pathways of Complement: the Classical, Alternative, and Mannose-Binding Lectin Pathways. The three complement pathways come together at the point of cleavage of C3. The classical pathway is activated by the binding of the C1 complex to antibodies bound to an antigen on the surface of a pathogen. Initially, C1s cleaves C4, which binds covalently to the bacterial surface, and then cleavage of C2 leads to the formation of a C4b2a, C3 convertase of the classical pathway. Activation of mannose-binding lectin pathway is initiated by binding of mannose-binding lectin to the mannose groups on the surface of a bacterial cell. The alternative pathway is activated by the covalent binding of C3b to the cell-surface and is activated by low-grade cleavage of C3 in plasma. Binding of factor B to C3b forms a C3bB complex and factor D cleaves factor B bound to C3b to form alternative pathway C3 convertase complex, C3bBb, and

this complex was stabilized by properdin enzyme. The C3 convertase enzyme complex cleaves many molecules of C3 to C3b, and some of this C3b binds to the C4b and C3b in the convertase enzymes for forming C5 convertase enzymes of the classical and alternative pathways, respectively. This C3b acts as an acceptor site for C5, and initiates the formation of C5b-9 complex or membrane-attack complex (MAC). Source: - **Walport MJ,** (2001). Complement: First of Two Parts. *N Engl J Med,* Vol. **344:** (14); 1058-66.

2. Aim of the study

The overall aim of the thesis was to investigate complement in severe *Plasmodium falciparum* malaria infections.

The specific objectives of the different papers were as follows

Paper 1

In vitro investigation of complement activating entity from *Plasmodium falciparum* infected erythrocytes, and its identification as the digestive vacuole.

Contribution: P.D., S.D.H., and S. Bhakdi performed the laboratory experiments; M.B., K. Reifenberg, and C.O. performed the animal experiments; M.T. and C.O. performed the immunohistochemistry work; S. Bhakdi and S.C.B. conceived of the project; P.D., S. Bhakdi, K.L., and K. Reiss designed the research; P.D., S. Baumeister, K.L., R.U., K. Reiss, and S. Bhakdi analyzed the data; and P.D partially involved in the manuscript preparation, and S. Bhakdi wrote the manuscript.

Paper 2

Investigation into the consequences of selective opsonisation of the digestive vacuole on polymorphonuclear leukocytes.

Contribution: S. Bhakdi and S.C.B. conceived the project; S.Bhakdi, K.R., and P.D. designed the research; P.D. and S. Bhakdi performed the experiments; R.L. performed the electron microscopy; S. Bhakdi, P.D., S. Baumeister, K.L., R.U., and K.R. analyzed the data; and P.D partially involved in the manuscript preparation, and S. Bhakdi wrote the manuscript.

3. DISCUSSION

3.1 Discussion Paper 1

There is ample evidence that complement is consumed during malaria infections. The complement studies in humans infected with *Plasmodium vivax* demonstrated that complement was depressed in infected patients. The maximum amount of complement was found to be consumed between the time of peak fever and within 10 h after the fever (Neva et al. 1974). These findings were confirmed with experiments in rhesus monkeys infected with P*lasmodium coatneyi,* and proved that the decline in complement levels was coincided with onset of schizont rupture and the degree of parasitemia (Glew et al. 1975). Complement activation has also been demonstrated in experimental human malaria (Rosenberg et al. 1990). Recent clinical data shows that complement activation takes place in Gambian children with severe malaria infections (Helegbe et al. 2007). Experimental infection of mice with *plasmodium berghei* suggests the role of complement activation in cerebral malaria. The C5 deficiency protects mice from cerebral malaria (Patel et al. 2008) in which dysregulation of terminal complement sequence is an important contributor to the pathogenesis of experimental cerebral malaria (Ramos et al. 2011). Hemozoin, which is formed in the digestive vacuole (DV) during intra-erythrocytic stage of the parasite, has emerged as a possible trigger of inflammation. This assumption is primarily based on the fact that hematin considered to represent as a synthetic analogue of hemozoin (Pagola et al. 2000) which provokes inflammatory responses in macrophages (Jaramillo et al. 2004; Prato et al. 2005), and activates the alternate complement pathway (Pawluczkowycz et al. 2007). However, when the mature schizont ruptures, the malaria pigment or hemazoin is still encased by the DV membrane and it is the organelle rather than free haemozoin that naturally gain contact with the host environment (Bannister et al. 2000a; Abkarian et al. 2011).

Another long standing conundrum in malaria pathophysiology is reduced synthesis and increased consumption of most procoagulant factors. Increased prothrombin time and partial thromboplastin time is known to occur in fulminant malaria infections (Pukrittayakamee et al. 1989; Clemens et al. 1994). In severe malaria, a diminished level of factor XII and prekallikrein indicates the activation of intrinsic coagulation pathway (Clemens et al. 1994). High concentrations of plasma fibrinogen are common in both uncomplicated falciparum infections and cerebral malaria. Further, some reports do not support the role coagulation cascade in malaria pathogenesis because fibrin is not always found in autopsy studies (Francischetti 2008). Recent data describes that P*. falciparum*-parasitized red blood cells (pRBCs) induce tissue factor (TF) expression in endothelial cells and support the assembly of multimolecular coagulation

complexes. Autopsy studies on brain endothelium of *P. falciparum*-infected children, who had died from cerebral malaria, showed increased expression of TF (Francischetti et al. 2007).

Although complement consumption and blood coagulation defects during *P falciparum* infection have been recognized for decades, no single entity of parasite origin has yet been identified that might be implicated directly in triggering these events. Rupture of each *P. falciparum* infected erythrocyte is accompanied by release of 16-32 infectious merozoites along with a single DV into the circulation. We discovered that DVs, which are liberated in to the blood stream, has the capacity to dually activate complement and coagulation cascades.

The present study emanated from observations that rupture of parasitized red blood cell (pRBC) in active non-immune human serum consumed complement and resulted in binding of C3, and C5b-9 to the complement activating DV particle, with sparing of merozoites. Naturally liberated, purified DVs from the culture supernatants activate complement and coagulation in a dose dependent manner. Immunofluorescence staining and ELISA for C5b-9 indicated that C5b-9 is present in membrane bound form. Complement activation requires direct contact of DV membrane with serum. DVs that had lost their membrane by sonication lost their functional properties. Parasitophorous vacuole membrane enclosed merozoite structures (PEMS), in which DVS were remained encased with membrane, did not activate the complement. Isolated hemozoin crystals were unable to activate complement and coagulation. This was unexpected because synthetic hematin, which has identical chemical and physical properties, (Pagola et al. 2000) activates the alternative complement pathway albeit at very high concentrations (Pawluczkowycz et al. 2007).

Activation of alternative complement pathway is independent of antibody and activation can take place on foreign cell surfaces (Atkinson et al. 1991). Complement activating properties depends on the capacity of C3b to form covalent bond with reactive functional groups on the foreign cell surface and thereby creates the nidus for assembly of the C3 convertase complex. Complement regulatory molecules (Medof et al. 1984; Liszewski et al. 1996) protect host cells from complement attack, and lack of such regulatory mechanism on microbial cell surface allows complement activation (Atkinson et al. 1991). Furthermore, chemical composition of the cell surface also protects the cells from complement attack. Sialic acid and sulphated acid mucopolysaccharides are the chemical constituents on the cell surface which influence the competition between factor B and factor H for the binding site on C3b. The competition between factor B and factor H for binding site on C3b determines C3 convertase formation or decay of C3 convertase (Kazatchkine et al. 1979). Most microbial cells have decreased levels of complement regulatory molecules on their cell surface and that decreases affinity of factor H for cell bound C3b. Thus, factor B binds to cell surface bound C3b and facilitates alternative complement

activation and amplification on microbial surface. In some bacteria, sialic acid is the main chemical constituent on the cell surface of group B and C meningococci, Type 3 group B streptococci, and K1 *Escherichia coli* and these bacteria are resistant to alternative complement pathway activation (Fearon and Austen 1980).

Currently there is not much information available on the chemical composition and organisation of the DV membrane. In *Plasmodium falciparum,* the haemozoin crystal formation takes place within the neutral lipid droplets inside the DV (Pisciotta et al. 2007). The hemozoin crystals and the lipid bodies are encased within the DV membrane. When DVs were treated with phospholipase C, they lost their procoagulant activity but not the complement activating properties. It is likely that assembly of multimolecular complex of clotting enzymes is generally triggered by $Ca2^{+}$ bridged interactions with negatively charged phospholipid head groups. Activation of clotting cascade with DVs does not depend on any interaction with blood platelets and it was triggered in platelet-free plasma. DVs directly activate the thrombin generation with purified FXa and prothrombin and DVs replace phospholipid vesicles in the reaction mixture.

We further investigated whether isolated DVs from *P. falciparum* had the capacity to activate complement of other species. When DVs were injected into rat tail veins, complement was consumed within minutes after injection and all the animals became lethargic and behavioural changes were observed. The effects of DV infusions were transient, similar to that of lipopolysaccharide injection, which also induces systemic inflammatory responses and complement activation (Smedegård et al. 1989). Termination of the systemic inflammatory responses to DVs might be explained by their rapid clearance by phagocytic cells. Indeed, phagocytic cells were rarely seen in the bloodstream of the animals, and the uptake of all the DVs by tissue phagocytes was impressive.

If the major tenets of our hypothesis turn out to be correct, strategies to inhibit DV-dependent activation of complement and coagulation might have therapeutic potential. Low molecular weight dextran sulfate (LMW-DXS) is known to inhibit complement (Wuillemin et al. 1997) and coagulation pathways (Wuillemin et al. 1996; Anderson et al. 2001) at micromolar concentration, which does not induce bleeding complications and well tolerated in humans(Johansson et al. 2006; Schmidt et al. 2008). Indeed**,** LMW-DXS protected the rats from the harmful effects at very high doses of DV injection and caused no side effects in the animals. The LMW-DXS was applied at the same concentrations that have been used previously in transplantation models (Wuillemin et al. 1997). It has also been reported that LMW-DXS suppress merozoite re-invasion (Clark et al. 1997). So, the use of LMW-DXS may simultaneously fulfill dual beneficial functions.

3.2 Discussion Paper 2

Phagocytosis of microorganisms by polymorphonuclear granulocytes (PMN), monocytes and tissue macrophages is a major mechanism in the innate host defence against microbial infections. Phagocyosis is a two-step process implicating attachment and engulfment of phagocytic particle. Serum factors called opsonins enhance the rate of uptake of microorganism by binding specifically to the receptor on the surface of phagocytes on the one hand and to the microbes on the other. This type of phagocytosis is called opsonophagocytosis (Ofek and Sharon 1988). Two types of opsonins have been identified, the C3b and C3bi fragments of the third component of the complement (C3), and the antibodies, usually immunoglobulin G (IgG) molecules. Opsonophagocytosis can take place in three different modes. In the first, only antibody participates by binding to surface antigen on the microorganisms and to the corresponding Fc receptors on the phagocytes. In the second mode, only the C3b or C3bi fragment of complement binds to the activated microbial surface and to the phagocyte surface via specific complement receptors. The third one, antigen-antibody complex activates the complement of classical pathway and facilitates the deposition of C3b onto the microbial surface. The phagocytic particle which are now coated with both the opsonins bind to both receptors on the phagocytes and enhances much stronger binding than that with either antibody or the fragments of C3 alone (Ofek and Sharon 1988). Some microorganisms are phagocytosed by neutrophils in the absence of opsonins. This type of ingestion is facilitated by recognition of specific sugars on one or both the cells. This type phagocytosis is termed as *lectinophagocytosis* (Ofek and Sharon 1988). However, most microorganisms must be coated with opsonins for phagocytosis by neutrophils.

Phagocytic cells play a vital role in innate immune host defence against malaria. Circulating neutrophils, monocytes and tissue macrophages are involved in the clearance of malaria pigment (hemozoin), parasitized and nonparasitized RBCs that are ingested by the phagocytes (Vernes 1980; Facer and Brown 1981; Sun and Chakrabarti 1985; Laing and Wilson 1972). *In vitro* phagocytosis of schizont-infected RBCs by monocytes and macrophages were markedly increased in the presence of immune serum (Abdalla and Weatherall 1982; Celada et al. 1983; Celada et al. 1982; Abdalla 1987). The interaction of phagocytic CD36 with Pf EMP1 has also been implicated in the clearance of infected RBC by monocytes and macrophages in humans (Ayi et al. 2005) and also in rodent malaria (Su et al. 2002). There is scant information on the role of PMN in malaria infections. Malaria pigment is microscopically detectable in the circulating polymorphonuclear granulocytes (PMN) of malaria patients, and there is a positive correlation between the number of circulating pigment-containing PMN and severity of the disease (Nguyen et al. 1995; Lyke et al.

2003). Although, the malaria pigment is detectable in circulating PMN, the question how the pigment enters the cell has never been described.

Another long standing enigma in malaria pathophysiology is that children from African countries with fever – often suffer from invasive bacterial infections (Were et al. 2011; Bronzan et al. 2007). Accumulated evidence from sub-Saharan African countries, where malaria is endemic, demonstrates that non-typhoidal *Salmonellae* (NTS) infections are the most common bacterial infections associated with elevated paediatric morbidity and mortality (Mabey et al. 1987; Berkley et al. 1999; Bronzan et al. 2007; Were et al. 2011). Current hypotheses regarding the causes underlying this general state of immune suppression focus mainly on the functional disturbances of macrophages, dendritic cells and the adaptive immune system.

Rupture of each parasitized erythrocyte in *P. falciparum* malaria liberates one DV alongside with 16-32 infectious merozoite into the bloodstream which invade new RBC for further cycle (Bannister et al. 2000b; Abkarian et al. 2011). High parasitemia is thus inseparably associated with high loads of DVs at the sites of pRBC rupture. Arese et al group has been conducting studies with naturally released DVs, and mainly focused on their effects on monocyte functions (Schwarzer et al. 1992; Skorokhod et al. 2004; Prato et al. 2010). Isolated hemozoin pigment provoked a respiratory burst after ingestion and the production of respiratory burst was blunted upon subsequent provocation. Later on, Fiori et al showed that monocytes fed with mature infected erythrocytes have reduced microbial activities (Fiori et al. 1993). The fact that most DVs will probably be ingested not by tissue macrophages rather by the surrounding PMN has never been considered prior to the present work, an oversight that likely derives from several reasons. There is a tacit assumption that the malaria pigment itself is endowed with biological properties and synthetic hematin which is considered identical to natural malaria pigment hemozoin (Pagola et al. 2000; Bohle et al. 2002).

Binding of C3b complement marks DV particle for phagocytosis. When DVs were marked with C3b opsonin in active serum they were found to be rapidly phagocytosed by PMN. This raised the possibility that ingestion of DVs explains the major pathway leading to the presence of malaria pigment in PMN of malaria patients. However, sonicated DVs or purified hemozoin crystals incubated with PMN in the presence of complement did not induce any respiratory burst and were not engulfed by the PMN. We also could not find any phagocytosis of schizonts in non-immune serum. We propose that hemazoin pigment in PMN of malaria patients derive mainly from phagocytosis of C3b complement marked intact DVs following schizont rupture. Previously described electron microscopic pictures of both PMN and macrophages containing malarial pigment (Wickramasinghe et al. 1987) are highly suggestive for two reasons. First, although the cells had not phagocytosed intact schizonts, aggregated haemozoin crystals could be readily seen

in the cell cytoplasm. Such aggregates do not persist when the pigment is artificially liberated from the organelle *in vitro*. Second, the aggregated hemozoin crystals can be seen to be surrounded by membrane structures in the electron micrographs. These findings are fully in line with our hypothesis that phagocytosis of DVs underlies the appearance of malaria pigment in the cells.

In the next experiment we investigated whether DVs might be preferentially phagocytosed by PMN in the presence and absence of high-titered antibodies against *P. falciparum*. Schizonts were allowed to rupture in the presence of active instead of heat-inactivated serum so that complement activation would immediately occur upon erythrocyte rupture. Merozoite DNA was stained with Hoechst prior to addition of surface-stained PMN, rendering rapid fluorescent microscopic analysis feasible. These experiments revealed the striking fact that DVs but not merozoites were selectively marked by complement and phagocytosed in the presence of active, non-immune serum. In the presence of high-titered antibodies against *P. falciparum* phagocytosis of both DVs and merozoites were observed. However, preferential uptake of DVs still appeared to persist. Thus, despite the fact that as occurs *in vivo*, merozoites outnumbered DVs by an order of magnitude, PMN were never observed to contain merozoites only. If preferential uptake of DVs really takes place, this might impinge on each cell's capacity to ingest merozoites.

The oxidative or respiratory burst plays a vital role in the microbicidal machinery of mammalian phagocytes which degrades internalized particles and bacteria (Jandl et al. 1978; Lee et al. 2000). ROS generation by phagocytosed DVs might therefore be followed by a state of hyporesponsiveness. Experiments with murine malaria have been reported that merozoites were susceptible to the cytotoxic action of ROS. It was of interest to determine whether ROS production might serve a protective function by killing bystander parasites, therefore enriched late-stage schizonts were incubated with ten-fold excess of non-infected RBCs in the presence or absence of PMN, ROS produced by PMN after ingestion of DVs led to no reduction of merozoite invasion. We further investigated that the capacity to mount ROS production upon subsequent bacterial challenge was severely compromised following DV uptake by PMN. Challenging of PMN with bacteria after ingestion of DVs still has the capacity to phagocytose bacteria. However, PMN preloaded with DVs had reduced microbicidal activity.

Thus, having served its physiological purpose, the liberated DVs after schizont rupture appears to function as a decoy, and is exploited by the parasite to divert and perturb central elements of the innate immune system. The alternative complement pathway and intrinsic clotting are simultaneously activated by the DV surface (Dasari et al. 2012). The preferential phagocytosis of DVs by blood phagocytes initially may provide beneficial function to the host by restricting activation of complement and coagulation. But in the long run, as parasitemia increases, there will

be detraction of the phagocytes away from their true targets. This might lead to over activation of ancient enzyme cascades by DVs which provides inflammatory mediators to contribute to the development of severe malaria. We found that high-titered *P. falciparum* specific antibodies induce some phagocytosis of merozoites. Possibly because efficient engulfment of DVs by PMN is still prevalent, PMN ingested only a small amount of merozoites and the majority of parasites remained outside the cell. In accord with this observation, there were no substantive reductions in the rate of merozoite re-invasion. However, our findings accorded with earlier reports in which little (Kumaratilake et al. 1990) or no protective effects of specific antibodies against *P. falciparum* plus PMN could be observed *in vitro* (Kumaratilake and Ferrante 2000; Kumaratilake et al. 1990). Monocytes apparently synergized more efficiently with the antibodies (Bouharoun-Tayoun et al. 1990), but the significance of this finding remains unclear since the cells were employed at unphysiologically high numbers. Most recently, it has been reported that the presence of specific antibodies induced strong ROS responses in PMN upon incubation with a mix of merozoites and DVs was reported to correlate with better *in vivo* protection compared with antibodies that induced little ROS production (Joos et al. 2010).

DV uptake and cellular activation could cause PMN to remain mainly sequestered in the microcirculation. Then, the actual number of DV-laden phagocytes would probably be considerably higher than suggested by the mere numbers of circulating hemozoin-containing cells. Sequestered, activated PMN possibly augment pathological processes in the microcirculation. At the same time, their systemic overloading may gradually set the stage for septicemic complications to develop whenever bacterial pathogens chance to gain entry into the circulation. The leading roles played by *Salmonellae* and *Enterobacteriaceae* in African children may derive simply from the high endemic prevalence of these agents.

3.3 Conclusion

In conclusion, digestive vacuoles released into the blood stream after schizont rupture had the capacity to activate both complement and coagulation. The digestive vacuole is a membrane bound organelle packed with the malaria pigment hemozoin which gains the contact with the host environment. Sonicated DVs lost its complement-activating and procoagulant biologically properties *in vivo* and *in vitro.* Procoagulant property of DVs was destroyed by phospholipase-C treatment. Purified hemozoin was unable to activate complement and coagulation. Intravenous injection of DVs into the rats induced alternative pathway complement activation and provoked apathy and reduced nociceptive responses. Complement and coagulation properties were blocked by low-molecular weight dextran sulfate and protected animals from the deleterious

effects of DV infusion. In the presence of active non-immune serum, DVs but not the merozoites were selectively opsonised by C3b and rapidly phagocytosed by PMN. High titered antibodies against *Plasmodium falciparum* induced phagocytosis of both DVs and merozoites, but the preferential engulfment was DVs than merozoites. Late stage schizonts and purified hemazoin crystals were not phagocytosed by PMN. Isolated DVs rapidly phagocytosed by PMN and drove the cells in a state of functional exhaustion. Phagocytosed DVs induced ROS production in PMN, and the ROS production was diminished with subsequent challenging of PMN with *S. aureus.* DV phagocytosed PMN effectively engulfed *S. aureus,* but the micobicidal activity was compromised. Together, the studies indicate that the DV may represent a novel determinant of parasitic pathogenicity that can simultaneously compromise the coagulation and the innate immune system.

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APPENDIX

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RED CELLS, IRON, AND ERYTHROPOIESIS

Digestive vacuole of *Plasmodium falciparum* released during erythrocyte rupture dually activates complement and coagulation

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Severe *Plasmodium falciparum* malaria evolves through the interplay among capillary sequestration of parasitized erythrocytes, deregulated inflammatory responses, and hemostasis dysfunction. After rupture, each parasitized erythrocyte releases not only infective merozoites, but also the digestive vacuole (DV), a membrane-bounded organelle containing the malaria pigment hemozoin. In the present study, we report that the intact organelle, but not isolated hemozoin, dually activates the alternative complement and the intrinsic clotting pathway. Procoagulant activity is destroyed by phospholipase C treatment, indicating a critical role of phospholipid head groups exposed at the DV surface. Intravenous injection of DVs caused alternative pathway complement consumption and provoked apathy and reduced nociceptive responses in rats. Ultrasonication destroyed complement-activating and procoagulant properties in vitro and rendered the DVs biologically inactive in vivo. Low-molecular-weight dextran sulfate blocked activation of both complement and coagulation and protected animals from the harmful effects of DV infusion. We surmise that in chronic malaria, complement activation by and opsonization of the DV may serve a useful function in directing hemozoin to phagocytic cells for safe disposal. However, when the waste disposal system of the host is overburdened, DVs may transform into a trigger of pathology and therefore represent a potential therapeutic target in severe malaria. (*Blood*. 2012;119(18):4301-4310)

Introduction

Severe malaria evolves through the interplay among capillary sequestration of parasitized erythrocytes, a deregulated inflammatory response, and hemostasis dysfunction.[1-5] The responsible molecular mechanisms remain the subject of debate, and the identification of the triggering events still constitutes a major open challenge in science. Seminal findings on complement activation in malaria were published in the 1970s. Complement turnover was shown to be triggered in human patients suffering from severe malaria,[6] and experiments in monkeys demonstrated that complement consumption coincided with schizont rupture.[7] Clinical data now show that substantial complement activation occurs in human patients[8] and enhanced early complement activation in experimental human malaria has been demonstrated.[9] Experiments in a murine model of cerebral malaria also suggest a pathogenic role of complement activation. C5 deficiency protects mice against cerebral malaria,[10] in which dysregulation of the terminal complement sequence is apparently centrally involved[11]

Hemozoin, which is formed in the digestive vacuole (DV) of developing intra-erythrocytic parasites, has emerged as a possible trigger of inflammation. This assumption is primarily based on the fact that hematin, considered to represent the synthetic analog of hemozoin,[12] provokes inflammatory responses in macrophages[13,14] and activates the alternate complement pathway.[15] Also suggestive is the finding that malarial pigment colocalizes with fibrin in tissues.[16] However, when the erythrocytic schizonts rupture, the malarial pigment is still surrounded by the membrane of the DV and it is the organelle rather than free hemozoin that naturally gains contact with the host environment.[17,18]

We reported recently that when parasitized erythrocytes rupture in blood, the DVs are rapidly phagocytosed by polymorphonuclear granulocytes (PMNs).[19] Phagocytosis only occurred in active serum and complement factor C3 was found attached to the surface of the organelle. It followed that the DV might directly activate complement, so experiments were performed to explore this possibility, leading to the novel findings reported herein. We show that the DV is endowed with the capacity to dually activate the alternative complement and the intrinsic clotting pathway. It is possible that efficient opsonization of the DV is initially beneficial because it enables the host to rapidly dispose of the waste product; however, transition to a pathologic state may occur as the disposal system is overrun. Unreigned activation of complement and coagulation may then contribute to events underlying the pathogenesis of severe malaria.

Methods

Serum

Banked human blood, group A Rh^{+} RBCs, and group A human serum were continuously supplied by the Transfusion Center of the University Medical

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Center in Mainz, Germany. Aliquots of human serum were stored at −20°C until use. Serum was heat inactivated for 30 minutes at 56°C for use in routine parasite cultures. Active serum was used as indicated.

Abs and reagents

Rabbit anti–human C3c Ab was from DakoCytomation and mAb clone 979/143 against C5b-9 neoantigen (purified IgG, 10 mg/mL) was from this laboratory.[20] mAb 1E1 specific for $MSP1_{19}$ was a kind gift from A. Holder (Medical Research Council, London, United Kingdom).[21,22] Alexa Fluor 594–conjugated goat anti–mouse IgG, donkey anti–rabbit IgG, Alexa Fluor 488–conjugated goat anti–mouse IgG, and 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) were from Invitrogen. mAb against RBC band 3 protein, protease inhibitor L-trans-epoxy-succinyl-leucylamido- (4-guanidino)butane (E64), Hoechst 33342, and low-molecular-weight dextran sulfate (LMW-DXS; MW5000) were from Sigma-Aldrich.

Plasmodium falciparum culture and isolation of DVs and merozoites

P falciparum clone 3D7 was maintained as a synchronous continuous culture and DVs were isolated and banked in 50% glycerol at −20°C as described previously.[19] DVs were enumerated using a hematocytometer (CELL-DYN Ruby; Abbott; Version 2.0ML software). Hemozoin was isolated as described previously,[19] and heme was quantified by measuring absorbance at 405 nm as described previously.[15]

To isolate merozoites, cultures were tightly synchronized twice with a 4-hour lap using 5% sorbitol. Naturally liberated merozoites and DVs were isolated from the supernatants of enriched cultures. DVs were first selectively sedimented by centrifugation at 400*g* for 5 minutes, and the merozoites were then obtained by subsequent centrifugation at 2400*g* for 5 minutes in a tabletop centrifuge. Merozoites were washed 3 times with RPMI and stored frozen at −20°C.

Isolation of PEMSs

Enriched, late-stage parasitized RBC (pRBC) cultures were suspended to 0.2% hematocrit in culture medium containing 10% inactivated serum in the presence of 15μM E64 for approximately 8 hours as described previously.[23] The culture was centrifuged at 250*g* for 5 minutes to remove intact cells, at 1600*g* for 5 minutes to remove intact late schizonts, and then at 2500*g* for 10 minutes to pellet the parasitophorous vacuole membrane-enclosed merozoite structures (PEMSs). The pellet was washed twice with RPMI and twice with PBS and used for the complement consumption experiments.

Complement consumption experiments

To measure consumption during pRBC rupture, enriched cultures of late-stage pRBCs were suspended in Veronal-buffered saline with 0.25mM Ca^{2+} and 0.8mM Mg^{2+} (Virion/Sirion) containing 20% active normal human serum (NHS) at 50% hematocrit. Cells were incubated at 37°C. At hourly intervals, small aliquots were collected and analyzed for hemolysis and complement consumption, which was detected by hemolytic assays using sensitized sheep erythrocytes and expressed as the percentage relative consumption as described previously.[24] Background absorption derived from lysed, parasitized cells were determined in parallel controls using heat-inactivated serum and subtracted from absorption values of the consumption assay. Two-dimensional immunoelectrophoretic analysis of C3 conversion was performed as described previously.[24] In 2 experiments, 10mM EGTA/2mM Mg^{2+} was added to prevent classic pathway activation, and complement turnover was assessed by C3 immunoelectrophoresis. Classic pathway of the serum used was in the normal range of 70-80 hemolytic units/mL.

For the measurement of consumption with isolated DVs, DVs were incubated with 10% or 20% NHS in the presence or absence of 10mM EGTA/2mM Mg^{2+}. C3 conversion was analyzed in 20% NHS after 20 minutes, and consumption of hemolytic activity with either sensitized sheep erythrocytes or rabbit erythrocytes in 10% serum after 60 minutes at 37°C.

Staining procedures

Hoechst 33342 was used to stain DNA. BCECF-AM (10μM) was added to pRBC cultures or to isolated DVs for 30 minutes. Cells and DVs were then washed 3 times and fluorescence microscopy was performed immediately on air-dried, unfixed smears.

Staining of merozoites and DVs after schizont rupture

Tightly synchronized cultures containing 8%-10% late-stage pRBCs were enriched and diluted to 0.2% hematocrit in active serum without the addition of fresh RBCs to prevent reinvasion of merozoites. Cultures were closely monitored for hemolysis and, immediately after the majority of schizonts ruptured, were centrifuged at 150*g* for 5 minutes. The supernatants were centrifuged at 1900*g* for 10 minutes in a Sorvall RC2B centrifuge to sediment the merozoites and DVs. Pellets were resuspended in RPMI medium and centrifuged twice at 400*g* for 1 minute in a tabletop centrifuge to remove the RBCs. Merozoites and DVs were then sedimented at 2400*g* for 5 minutes. Pellets were resuspended in RPMI medium and thin smears were prepared on glass slides, air-dried, and fixed with methanol for 10 seconds or in 2% paraformaldehyde for 10 minutes at room temperature and stained with anti-C3c or anti–C5b-9 as described in the next paragraph.

Staining of isolated DVs

DVs were incubated with 10% active human serum for 1 hour at 37°C or with 10% inactive serum as a control. DVs were washed with PBS and thin smears were made on glass slides. After fixation, the slides were washed with PBS and then blocked in 3% BSA for 1 hour at room temperature. The slides were washed and incubated with rabbit polyclonal C3c Ab (1:250 dilution), monoclonal anti–human C5b-9 (1:250 dilution), monoclonal anti–human band 3 (1:1000 dilution), or monoclonal anti-MSP1 (1:100 dilution) in blocking buffer for 1 hour at room temperature. Nonimmune rabbit serum and isotype-matched mouse IgG were used as respective controls. After incubation, the slides were washed and then incubated with Alexa Fluor 594–conjugated goat anti–mouse IgG, donkey anti–rabbit IgG, or Alexa Fluor 488–conjugated goat anti–mouse IgG (Invitrogen), all at a 1:500 dilution for 1 hour at room temperature. Nuclei were stained with Hoechst 33342 for 5 minutes at room temperature. Samples were viewed in a fluorescence microscope (Axiovert 200M; Carl Zeiss; AxioVision Version 4.7 software) or an Axioskop 2 microscope (Carl Zeiss) at a 1000× magnification. Images were obtained using AxioVision software.

Western blotting

RBC ghosts were prepared by lysis of RBCs with ice-cold 5mM sodium phosphate buffer, pH 8.0. RBC ghosts, isolated merozoites, and DVs were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with mouse anti-MSP1 (1:200) and mouse anti–band 3 Abs (1:5000). HRP-conjugated goat anti–rabbit Ab (New England Biolabs), goat anti–mouse or goat anti–human Abs (both Santa Cruz Biotechnology) were used at a dilution of 1:10 000 for detection, and bands were visualized by enhanced chemiluminescence (Roche).

Flow cytometry

DVs were incubated in 10% NHS for 1 hour at 37°C and washed 3 times with PBS. After incubation in 3% BSA/PBS for 30 minutes, DVs were stained for C5b-9 as described in "Staining of isolated DVs" and analyzed in a FACS Scanflow cytometer (BD Biosciences). Controls consisted of identical DV preparations treated with heat-inactivated serum.

Determination of clotting times in recalcified plasma

Citrated blood was centrifuged and the cell-free plasma was diluted with 1 volume of saline. DVs were incubated with plasma for 1 minute at 37°C, and reactions were initiated with the addition of 10mM $CaCl^{2+}$. Clotting times were measured in a coagulometer (KC10A micro; Amelung).

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Prothrombinase assay

Assessment of thrombin formation was performed in a 2-step amidolytic substrate assay.[25] The DVs were preincubated in a solution containing 150mM NaCl, 50mM Tris (pH 7.4), and 0.1% BSA with 1nM factor Va, 6.2 pM factor Xa, and 2mM $CaCl_2$ at 25°C for 5 minutes. The reaction was initiated by the addition of 1μM prothrombin and incubated for 5 minutes at 25°C. The reaction was stopped by the addition of EDTA to a 10mM final concentration. The DVs were removed by centrifugation, and thrombin formation was assessed at 405 nm immediately after the addition of the chromogenic substrate S-2238 (0.1mM; Chromogenix Instrumentation Laboratory) in a kinetic microplate spectrophotometer (Multiskan RC; Labsystems) with Genesis Life Version 3.0 full mode software.

Inhibition assays with LMW-DXS

LMW-DXS (MW5000) was used at the given concentrations in complement consumption and coagulation assays. For in vivo experiments, 6 mg of LMW-DXS in PBS was injected intraperitoneally 45 minutes before injection of 5×10^9 DVs.

Animal experiments

Male Sprague-Dawley outbred rats [Crl:CD(SP)] were purchased from Charles River Laboratories and introduced into experiments at a body weight of 60-70 g. IV injections (300-350 μL) were performed after catheterization (24 G) of the lateral tail vein. All experimental protocols were approved by the responsible authority (Landesuntersuchungsamt Koblenz, approval number 23 177-07/G 10-1-034) and conducted according to European Union guidelines for the care and use of laboratory animals. Three video clips showing the behavioral reactions of the animals can be found in supplemental Videos (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

The hot-plate test

The hot-plate test was adapted from the method of Woolfe and McDonald.[26] In brief, a rat is introduced into an open-ended plexiglas cylinder (18-cm diameter) to confine the animal to the heated surface of the hot-plate apparatus. The plate is adjusted to 56°C. When the pain threshold is reached, the rat starts to react by licking the fore or hind paws. Normal reaction times are in the range of 10 seconds. Reaction times are recorded with a maximum cutoff of 30 seconds to avoid tissue damage.

Statistical analysis

The assumptions for normality and equal variance were verified with SigmaStat Version 3.1 software (SYSSTAT). Depending on the experiment, either repeated measures ANOVA or 1-way ANOVA was followed by the Bonferroni *t* test. All results represent means ± SD of at least 3 independent experiments, if not indicated otherwise. $P < .05$ was considered to be statistically significant.

Results

Complement activation occurs on the surface of DVs but not on merozoites

In vitro culture of *P falciparum* was undertaken conventionally and late-stage pRBCs were enriched (Figure 1A Giemsa stain). BCECF-AM, a membrane-diffusible acetylated carboxyfluorescein derivative, becomes entrapped intracellularly after its cleavage by cytoplasmic esterases that are absent in RBCs but present in nucleated cells and DVs. Entrapment occurs only when an intact biologic membrane is present to limit its egress from the respective compartment. We found that merozoites and DVs could both be stained and were then clearly distinguishable from each other (Figure 1B). As was to be expected, hemozoin characterized the DVs, whereas Hoechst 33342 stained merozoites but not the DVs (Figure 1C). DVs and merozoites could therefore be easily distinguished. More importantly, intactness of the DV membrane, simply probed with the fluorescent dye, would subsequently emerge as the decisive element underlying the singular biologic properties of the DVs reported herein.

Naturally liberated DVs were isolated from supernatants of hemolyzed pRBCs. As expected from a previous study,[27] they presented as platelet-sized particles that could be enumerated in a hematocytometer (Figure 1D). Staining with BCECF-AM could still be undertaken after their isolation and storage at −20°C in glycerol (Figure 1E), attesting to the remarkable stability of their membranes. Staining for DNA was negative (Figure 1F). To exclude potential contaminations of the DV preparation with either erythrocyte or merozoite fragments, the presence of the RBC anion transporter (band 3) and the merozoite surface protein 1 (MSP1) was analyzed. The band 3 protein was not detectable by immunofluorescence on the DV surface (Figure 1G). Instead, staining could only be observed on erythrocytes that were artificially added as controls (Figure 1H). This finding was confirmed by immunoblot analysis demonstrating the lack of band 3 on DVs and purified merozoites (Figure 1I-J). The presence of MSP1 was examined by Western blots of purified merozoites and DVs. The latter carry the C-terminal 19-kDa fragment of MSP1 but not the full-length protein,[28] which was readily detectable on merozoites but virtually absent in the DV preparations (Figure 1K).

To determine whether complement activation would occur after schizont rupture, late-stage pRBCs were allowed to lyse in the presence of active, nonimmune serum, and complement activity, C3 turnover, and pRBC hemolysis were recorded (Figure 2A). No changes occurred before schizont rupture, but the onset of hemolysis was accompanied by C3 turnover and a decrease in complement activity. These findings are in agreement with the early observations of Glew et al that complement consumption coincided with schizont rupture in infected monkeys.[7]

To identify the activation pathway, schizont rupture was analyzed in the presence of 10mM EGTA/2mM Mg^{2+}, which prevents classic pathway activation. As shown in Figure 2A, complement activation indeed occurred and could thus be attributed to the alternative pathway (Figure 2A bottom panels). At the same time, these experiments showed that Ca^{2+} was dispensable at the final stage of parasite development and was released from the RBCs. Because the alternative complement sequence is not triggered by soluble molecules, merozoites and DVs were examined for the presence of assembled C5b-9, the prime marker of membrane-associated complement activation.[20,29] Positive stainings were observed on the DVs but never on merozoites (Figure 2B). The DV membrane was thus identified as the critical site of complement activation. To exclude any additional requirement of soluble activators, experiments were conducted with isolated, washed DVs. Figure 3A shows a dose-response experiment with matching data for complement consumption and C3 turnover in human serum. Decreases in total complement activity with concomitant C3 turnover were already provoked by low numbers (approximately 10^7/mL) of DVs. Activation occurred in the presence or absence of EGTA/Mg^{2+}. The depicted panels are from an experiment with EGTA/Mg. When the fluid phase SC5b-9 was measured in the supernatants, a bell-shaped dose-response curve was observed that indicated a shift to the membrane-bound state as the number of DVs increased. A representative example is shown in Figure 3B. All DVs stained positively for C3 (Figure 3C) and C5b-9 (Figure 3D). The latter finding was also confirmed by FACS

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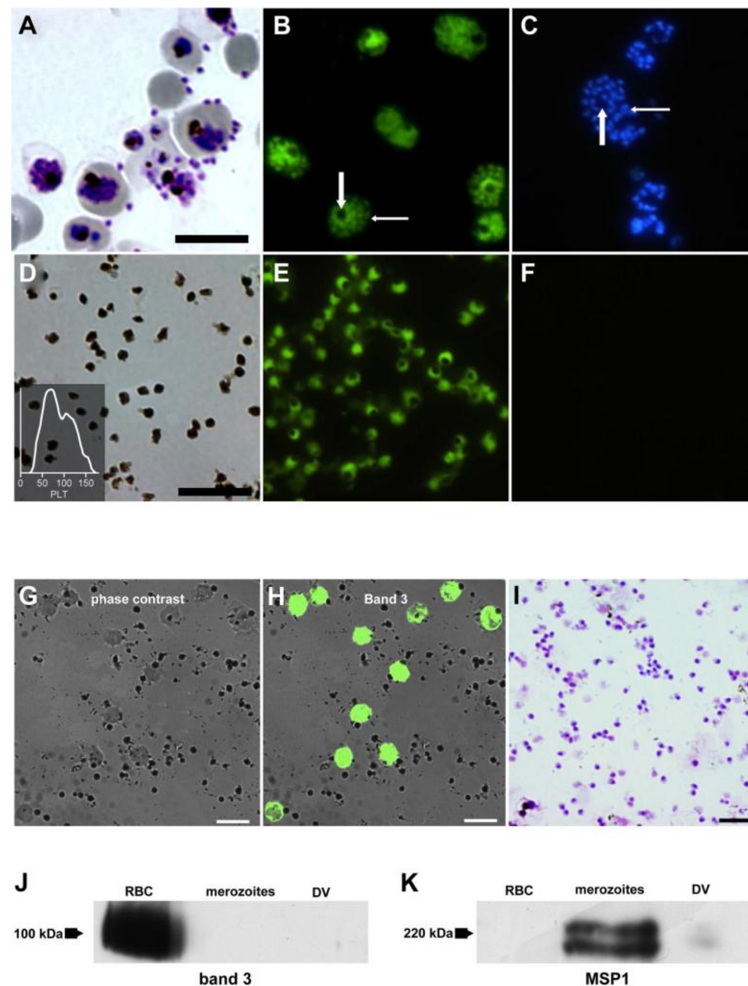

Figure 1. Differential staining of merozoites and DVs. (A) Giemsa stain of late-stage pRBCs undergoing schizont rupture. (B) Vital stain of the same culture with BCECF-AM showing intense staining of DVs (large arrow) alongside staining of merozoites (small arrow). (C) Same culture stained with Hoechst 33342, which detects merozoites (small arrow) but spares the DVs (large arrow). (D) Giemsa stain of isolated DVs. Insert shows the recording of analysis in a hemocytometer with DVs presenting as particles with the size of platelets (PLT). (E) Staining of isolated DVs with BCECF. (F) No staining with Hoechst 33342. (G) Phase-contrast image of DVs to which RBCs were added. (H) Same smear fluorescently stained for RBC band 3 protein. (I) Giemsa stain of purified merozoite preparation used in Western blots. (J) Western blots of RBC membranes, merozoites, and DVs probed with Abs against RBC band 3 protein and MSP1 (K). DV preparations contained very scant contaminations with MSP1. Arrows depict the positions of the respective molecular weight markers. Scale bars indicate 10 μm.

analysis, which demonstrated impressively that all DVs stained positive for the terminal complement complex (Figure 3D bottom panel).

Contact of the intact DV membrane with plasma is required for complement activation

To discern whether direct contact of the DVs with plasma was required for complement activation, intact parasitophorous vacuoles and purified hemozoin were compared for their complement-activating capacity with DVs prepared from naturally ruptured pRBCs or from trophozoites. Parasitophorous vacuoles were prepared by permitting late-stage parasitized RBCs to lyse in the presence of the protease inhibitor E64.[23] When the released PEMSs (Figure 4A) were tested, they were found to be devoid of complement-activating properties (Figure 4D). Control experiments showed that E64 itself did not suppress complement activation by isolated DVs. To determine whether activation required the presence of an intact DV membrane, DVs were isolated from hemolysis supernatants or from trophozoite-stage infected RBCs.[27,30] The latter were prepared by saponin-lysis of late-stage infected RBCs and, as known from previous studies,[27,30] such preparations contained some contaminating RBC-membrane debris (Figure 4B). Both preparations had similar complement-activating properties that were lost after sonication, and this finding was correlated with the detectability of C3 and C5b-9 exclusively on the intact DV surface but never on any surrounding membrane debris (data not shown). Centrifugation of the sonicates through Percoll led to retrieval of purified dispersed hemozoin crystals (Figure 4C), which were also devoid of complement-activating properties when tested at concentrations of 10-1000 μg of heme/mL (Figure 4D). Therefore, activation of complement required direct contact of intact DV membranes with serum and could not be

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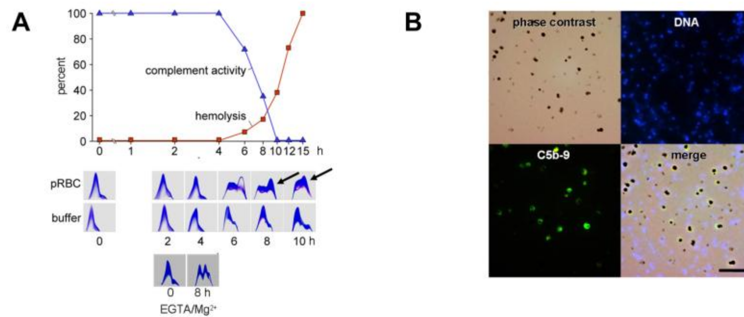

Figure 2. Complement activation occurs to completion on the surface of DVs but not on merozoites. (A) Late-stage pRBCs were cultured and allowed to rupture in the presence of 10% active human serum. Complement activity and hemolysis were recorded over time and found to be inversely correlated. Two-dimensional immunoelectrophoresis confirmed that C3 turnover occurred at the onset of hemolysis concomitant to the fall in complement activity (6 hours). Buffer controls (in the absence of pRBCs) are shown in the second row of panels. The bottom 2 plates show C3-immunoelectrophoresis at 0 and 8 hours from an experiment conducted with pRBCs in the presence of 10mM EGTA/2mM Mg^{2+}. First-dimension electrophoresis is shown left to right and second-dimension immunoelectrophoresis bottom to top. (B) Synchronized late-stage pRBCs were allowed to rupture in active human serum, whereafter unlysed cells were pelleted and merozoites and DVs were harvested from the supernatants and stained for DNA or C5b-9. Top left is phase-contrast microscopy; top right, Hoechst 33342 DNA stain; bottom left, complement C5b-9 complex; bottom right, merge. Note selective staining of DVs for C5b-9.

mimicked by isolated hemozoin, which was also found to be devoid of procoagulant activity (data not shown). The fact that unfractionated, sonicated lysates had no activity reiterated that the activating effects could not have derived from protein or DNA contaminants in the DV preparations.

DVs activate the intrinsic clotting pathway

Several mechanisms involving the extrinsic clotting pathway have mainly been discussed as the cause of hemostasis dysfunction in malaria patients.[3-5] However, intrinsic pathway activation also appears to be involved[31] and late-stage pRBCs reportedly support the assembly of multimeric coagulation complexes.[32] In the present study, we have demonstrated that the DV also has direct procoagulant activity.

We tested the intrinsic clotting pathway routinely by determination of activated partial prothrombin time, in which an empiric mixture of activators is added to citrated plasma and clotting times after recalcification are read. Long clotting times (> 400-1000 seconds) were observed if the activators were omitted. When DVs were used instead of the activators, dose-dependent shortening of clotting times was observed (Figure 5A). These results were corroborated in prothrombinase assays using isolated factors FVa, FII, and FXa. In the conventional test, phospholipids are added to provide platforms for Ca^{2+}-dependent assembly of the prothrombinase complex FVa/FXa. In the present study, the phospholipids were omitted and replaced by DVs, which dose-dependently provoked thrombin formation (Figure 5B). Therefore, the DVs could directly assemble the key convertase of the clotting pathway and, as with complement activation, this required only low numbers of DVs corresponding to less than 1% hematocrit. Prothrombinase assembly is generally mediated via Ca^{2+}-bridged interactions with phospholipid head groups. When DVs were

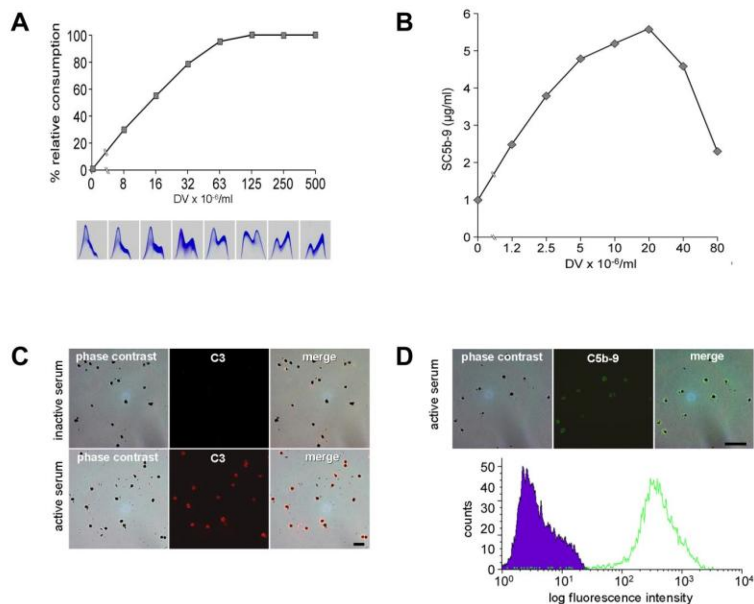

Figure 3. Isolated DVs activate the alternative complement pathway. (A) Addition of isolated DVs to serum in the presence of EGTA/Mg dose-dependently provoked complement consumption (as assessed with rabbit erythrocytes) and C3 turnover. (B) Concentration of fluid-phase SC5b-9 in serum spiked with increasing numbers of isolated DVs. (C) Staining of C3 on isolated DVs after incubation with serum. Top row shows the controls, which were incubated in inactive serum; bottom row, cells incubated in active serum. (D) Detection of C5b-9 on DVs after incubation with active human serum. Corresponding flow cytometric analysis shows staining of all DVs in the sample. Representative results of 4 independent experiments are shown.

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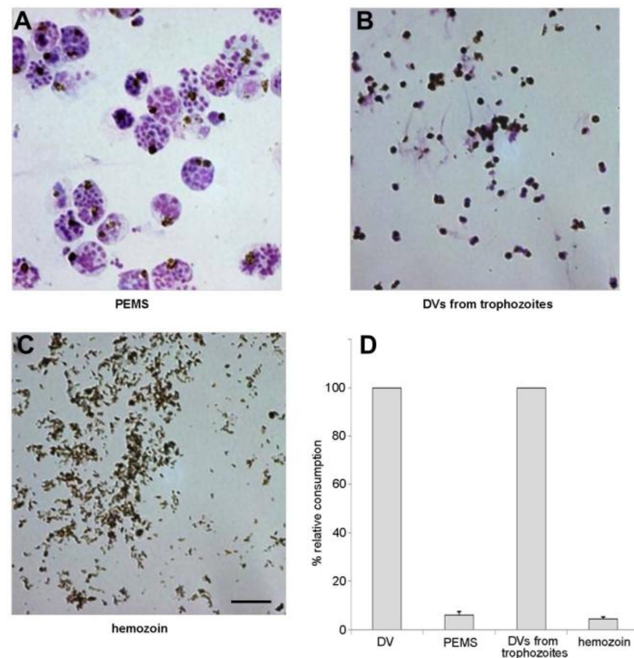

Figure 4. Preparations of PEMSs, intact DVs, and free hemozoin. (A) Merozoites and DVs encased in the parasitophorous vacuole from pRBCs after rupture in the presence of the protease inhibitor E64, which inhibits lysis of the parasitophorous vacuole membrane but not lysis of the erythrocyte membrane. (B) DVs isolated from trophozoites. (C) Hemozoin crystals isolated from sonicated DVs by centrifugation in Percoll. Scale bar indicates 5 μm. (D) Complement consumption tests were performed with materials in panels A through C using 10% NHS. DVs were used as a positive control and similar concentrations of heme were used throughout. Neither PEMSs nor isolated hemozoin had complement-activating capacity. One representative assay of 3 independent experiments is shown, with SD from triplicate determinations.

treated with phospholipase C, procoagulant activity was indeed destroyed (Figure 5C), whereas complement-activating capacity remained intact (not shown).

LMW-DXS suppresses the activation of complement and coagulation by the DV

LMW-DXS blocks both the complement[33] and coagulation cascade[34,35] at micromolar concentrations that, in contrast to heparin, do not cause bleeding complications and are well tolerated in humans.[36,37] If DV-induced activation of complement and clotting should indeed contribute to malaria pathogenesis, the existence of a well-established inhibitor could be of interest. Therefore, we tested the effect of LMW-DXS on DV effects. LMW-DXS at concentrations of 10-100 μg/mL effectively inhibited DV-dependent activation of the intrinsic clotting pathway (Figure 6A) and abrogated the assembly of prothrombinase complexes (Figure 6B). In agreement with a previous study,[36] somewhat higher concentrations were required to block complement activation, but these effects were also apparent at a 100 μg/mL concentration (Figure 6C).

DVs activate the alternative complement pathway and produce clinical symptoms in rats

Animal experiments were performed to obtain an indication of whether the DVs also activated complement in vivo and might contribute to the development of clinical symptoms. These experiments were performed in rats because alternative complement pathway activity is extremely low in mice. Alternative pathway activity was determined using rabbit erythrocytes as targets. The first group of animals received 4-5 $\times$ 10^9 DVs injected into the tail vein. Nociceptive responses were determined with the hot-plate test and behavioral reactions were filmed. The acute onset of clinical symptoms was observed in all cases, commencing 1-2 minutes after injection. Animals became lethargic, exhibited diminished reactions to tactic and acoustic stimuli, and nociceptive responses were retarded (Figure 7A and supplemental Video 1). Alternative complement pathway activity was reduced by $> 85\%$ in all animals ($n = 5$). The second group of animals received the same batch and dose of DVs but that had been sonicated before application. Alternative complement pathway activity remained unchanged in the serum of these animals, which also developed no clinical symptoms ($n = 3$; Figure 7A and supplemental Video 2). The third group of animals received 6 mg of LMW-DXS IP, which led to a $> 65\%$ reduction in alternative pathway complement activity in plasma after 45-60 minutes. Remarkably, all animals ($n = 4$) were fully protected from the detrimental effects of DV infusion (Figure 7A and supplemental Video 3).

Rapid cellular uptake of DVs by mononuclear cells in rats after IV injection

To trace the fate of the DVs, rats were killed after 4-6 hours. DVs and PMNs were rarely observed in blood smears (not shown). Paraffin-embedded sections of spleen and lung were investigated microscopically. Staining with H&E revealed intracellular accumulation of DVs in the marginal zone of the spleen containing abundant macrophages (Figure 7B left panel). This pattern became very impressive after polarization at a lower magnification (Figure 7B right panel); likewise, intravascular accumulation of mononuclear cells with myriad intracellular DVs could be observed in the lungs (Figure 7C). These data demonstrated that free DVs are rapidly taken up by PMNs.

Discussion

Although complement activation and coagulation defects during *P falciparum* malaria have been recognized for decades, no single

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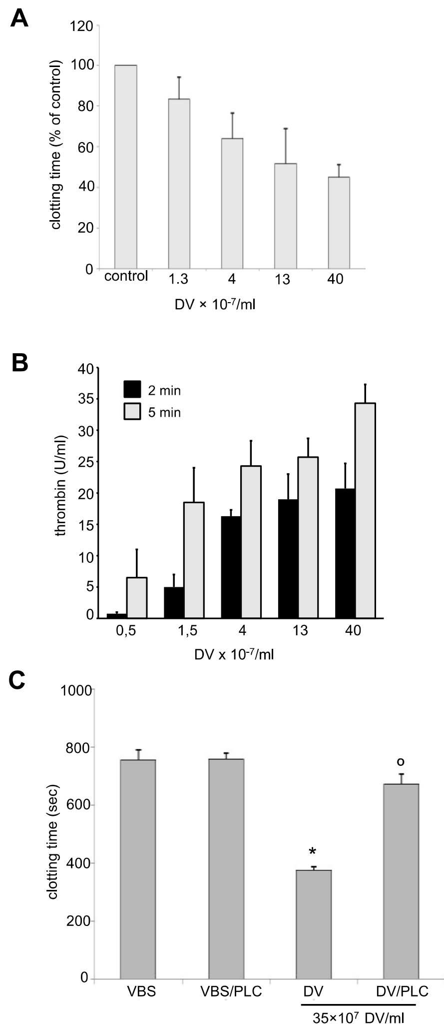

Figure 5. DVs directly activate the intrinsic clotting pathway. (A) Clotting time after recalcification of 50% plasma was accelerated significantly in the presence of DVs. Clotting times of the respective buffer controls were taken as the 100% reference in each experiment. Data are expressed as means ± SEM of 5 independent experiments. (B) Isolated DVs dose-dependently enhanced thrombin generation in the prothrombinase assay (n = 4 ± SEM). Controls without DVs did not induce any thrombin generation and are not shown. (C) Procoagulant activity of DVs is sensitive to phospholipase C treatment. Clotting times of 50% citrated plasma were determined after recalcification in the presence of Veronal-buffered saline, Veronal-buffered saline plus PLC, DVs, or DVs after PLC treatment. Clotting time was significantly accelerated in the presence of DVs (*$P < .05$ vs control). This effect was abolished after PLC treatment (°$P < .05$). Data are expressed as means ± SD of 3 independent experiments.

entity of parasite origin has yet been identified that might be involved directly in triggering these events. Rupture of each *P falciparum* parasitized erythrocyte is accompanied by release of one DV into the circulation. However, whereas the effects of DV uptake on macrophage function are under study, no significance has yet been attached to their presence in the circulation. The present study is the first to reveal the DV's capacity to dually activate complement and coagulation. In severe malaria, parasitemia levels of several percent develop corresponding to $\geq 10^8$ cells/mL of blood, and capillary sequestration further heightens the local load of DVs. Activation of both complement and coagulation became detectable at concentrations of approximately 10^7 DVs/mL and displayed simple dose dependency with no prozone effects (Figure 3A and Figure 5A-B). Therefore, increases in the load of DVs as occurs at sites of pRBC sequestration would be expected to simply augment activation of both enzyme cascades. Provocation of clinical symptoms will naturally depend on myriad local factors of

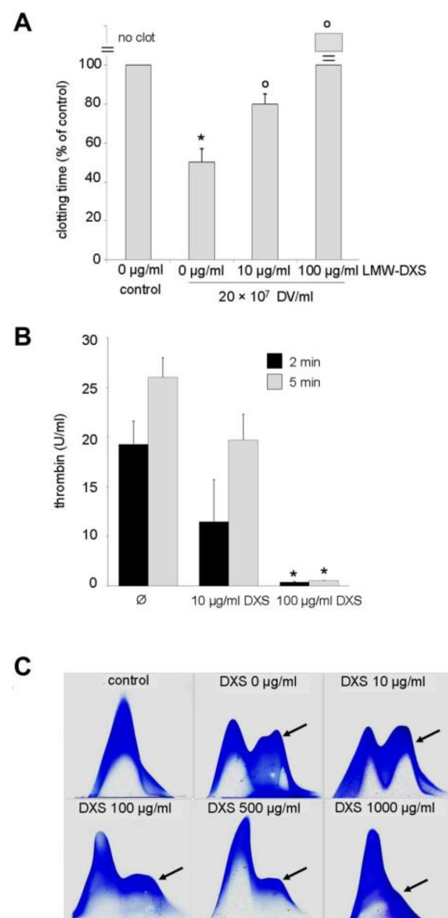

Figure 6. LMW-DXS abrogates procoagulant and complement triggering action of DVs. (A) Clotting times were determined after recalcification of 50% citrated plasma in the absence (control) or presence of DVs and LMW-DXS at the depicted concentrations. DVs provoked a significant reduction in clotting time (*$P < .05$ vs control), which was significantly reversed by 10 µg/mL of LMW-DXS (°$P < .05$). LMW-DXS (100 µg/mL) completely prevented clot formation despite the presence of DVs. Data are expressed as means ± SD of 3 independent experiments. (B) LMW-DXS abrogates prothrombinase assembly on DVs. Prothrombinase assays were performed in the presence of 5×10^7 DVs/mL and in the absence or presence of LMW-DXS at the given concentrations, and thrombin generation was determined after 2 and 5 minutes (n = 3 ± SD). DXS (100 µg/mL) abolished prothrombinase assembly (*$P < .05$ vs control). (C) Inhibition of DV-dependent C3 turnover by 100-1000 µg/mL of LMW-DXS. In the control (top left panel), 20% NHS was incubated for 30 minutes at 37°C. DVs/mL (10^8) were added to 20% NHS in the absence or presence of LMW-DXS at the depicted concentrations. Inhibition of C3 turnover (arrows) was observed at 100 µg/mL of LMW-DXS. Results shown are representative of 3 independent experiments.

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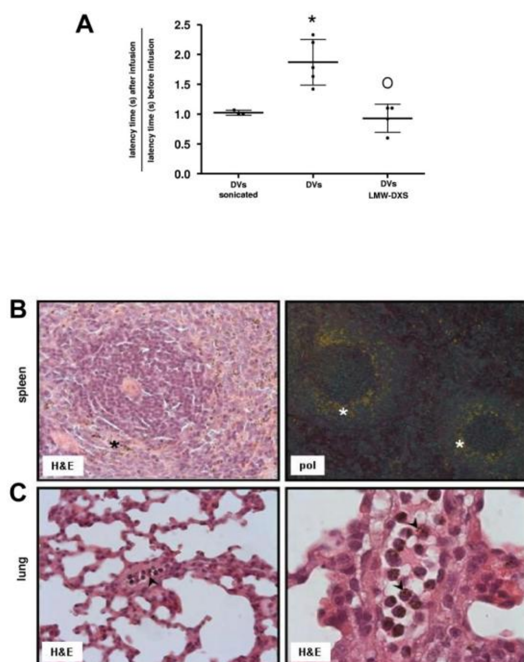

Figure 7. Effect of IV injection of isolated DVs in rats. (A) Reduction of nociceptive responses. The hot-plate test was used, in which rats were placed in jars warmed to 56°C and latency periods until licking of the paws were measured. Results are depicted as the quotient of reaction times measured after DV infusion to reaction times determined before infusion in each animal. One group of animals received a bolus injection of 5×10^9 DVs (n = 5). The second group received an injection of 5×10^9 sonicated DVs (n = 3). The third group received 6 mg of LMW-DXS IP 45 minutes before injection of 5×10^9 DVs (n = 4). All experiments were performed with the same banked DV pool. DVs provoked a significant increase in latency time (*$P < .05$ vs control), which was significantly decreased in the presence of LMW-DXS (°$P < .05$). Bars represent the mean values with SD. (B-C) Rapid cellular uptake of DVs by mononuclear cells. Paraffin-embedded sections of spleen (B) and lung (C) were stained with H&E. (B) Intracellular accumulation of DVs in the marginal zone (asterisk) containing abundant macrophages (left panel; magnification 252×). This pattern is illustrated in the right panel after polarization at lower magnification (63×). (C) Intravascular accumulation of PMNs (left panel, arrowhead; magnification 252×) containing intracellular DVs (right panel, arrowhead; magnification 630×).

the microenvironment such as the presence or absence of regulatory factors such as tissue factor and thrombomodulin.[1-5] Transactivation events occurring among the coagulation system, complement, platelets, and blood cells may then pave the way to devastating disease.

The present study originated from the observation that rupture of pRBCs in active serum led to C3 conversion and to binding of C3b and C5b-9 to the DVs, with conspicuous sparing of merozoites. The results were reproduced with isolated DVs, and attachment of C5b-9 indicated its presence in membrane-bound form. Direct contact between the DV membrane and serum was required for activation to take place, and PEMSs, in which DVs remained encased within the parasitophorous vacuole membrane, were without effect. DVs isolated via saponin lysis of late-stage infected RBCs also activated complement. These preparations contained contaminating membrane material that did not stain positively for C3 or C5b-9. The findings do not entirely exclude the possibility that other membranes or organelles may also have complement-activating properties. However, these remain to be identified. Quite remarkably, isolated hemozoin activated neither complement nor coagulation. This was somewhat unexpected because hematin, which is considered to represent the synthetic analog of hemozoin, activates the alternative complement pathway, albeit at very high concentrations.[15] The possibility that the biologic properties of hematin and hemozoin may not be identical merits close attention in future studies.

Disruption of the DV membrane destroyed both complement-activating and procoagulant properties. Therefore, our findings led to the question of what particular membrane characteristics enabled the DV but not the merozoite to activate both cascades. Information on the composition and organization of the DV membrane is currently not available. Such analyses are impeded by the fact that entities other than hemozoin are encased within the DV, including lipid bodies of poorly defined composition.[38] However, we found that procoagulant activity was selectively destroyed by phospholipase C treatment and must thus be borne by phospholipid head groups. This finding is not surprising because multimolecular assembly of clotting enzymes is generally promoted by Ca^{2+}-bridged interactions with negatively charged phospholipid head groups. It is very likely that, like other activating surfaces, exposed phospholipid head groups play essential roles. Further studies are needed to identify the responsible moieties, but the key recognition remains that an intact membrane is required for the DV to unfold both its complement-activating and procoagulant properties. The latter are intrinsically borne and not dependent on any interaction with platelets. Clotting was triggered in platelet-free plasma, and thrombin could be generated directly by incubating purified DVs with FXa and prothrombin.

Why complement activation in nonimmune serum occurs exclusively on the DV remains to to be clarified. Glycophosphatidylinositol-anchored proteins, including complement inhibitors,[39,40] have been shown to shuttle from the RBC to the parasitophorous vacuole membrane,[41-44] and the possibility is being examined whether they are further recruited to the merozoite surface to shield the parasite from complement attack. Regardless, the DV might serve as a decoy for central host defense elements. Indeed, we have found that complement activation marks the DV for selective phagocytosis by neutrophil granulocytes, whereas merozoites are left free to reinvade cells. This situation was found to persist in the presence of serum from malaria patients.[19] It is possible that alternative pathway activation plays a first role in mediating selective opsonization of DVs in nonimmune serum. As specific Abs appear, classic pathway activation may be triggered on both the DVs and merozoites. The density of activated complement components will consequently remain higher on the DV, thereby sustaining its preferential phagocytosis.

When DVs were injected into rat tail veins, complement consumption occurred within minutes and the animals became lethargic and behavioral responses were impaired. The clinical symptoms possibly derived from systemic activation of endothelial and phagocytic cells with the release of inflammatory molecules and mediators, which would be expected to occur after triggering of the clotting[5] and alternative complement pathway.[45] The effects of bolus DV infusions were transient, reminiscent of lipopolysaccharide injection, which also provokes systemic inflammatory responses and complement activation.[46] Termination of the reaction to DVs might be explained by their rapid clearance by phagocytic cells. Indeed, PMNs were rarely seen in the bloodstream of the animals, and the massive uptake of DVs by tissue phagocytes was impressive. At the onset of malarial infections, the low load of DVs is perhaps first cleared by PMNs; then, as the clearance capacity is overrun, tissue macrophages may come into play. Malaria pigment is present in these cells in human patients,[16,47] and our finding that

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isolated DVs reside in tissue macrophages within hours after IV application also agrees with this concept. Dysfunction of these cells occurring after DV uptake has been reported in several studies,[14,48-50] and likely also contributes to the pathogenesis of disease.

A major challenge facing the DV-complement activation theory is the fact that high parasite loads develop in many patients, particularly in Africa and the Pacific, without provoking severe symptoms. Only 2 speculations for this have been advanced at this point: (1) the dynamics of DV release and removal may be important and the efficacy of phagocyte uptake of DVs is possibly subject to wide interindividual variations, and (2) genetic deficiencies in late complement components occur worldwide with varying and sometimes surprisingly high frequency.[51] Heterozygotes are not prone to suffer from bacterial infections because serum complement activity is only lowered, but perhaps these individuals are protected against the effects of complement overactivation that lead to severe malaria.

If the major tenets of our hypothesis turn out to be correct, strategies to inhibit DV-dependent activation of complement and coagulation might have therapeutic potential. Activation of both cascades was found to be inhibited by LMW-DXS. In contrast to heparin, LMW-DXS does not cause bleeding complications and is well tolerated in humans. Indeed, the agent caused no side effects in rats but protected the animals from the harmful effects of very high doses of DV infusion. The agent was applied at the same concentrations that have been used previously in transplantation models.[36] LMW-DXS has also been reported to suppress merozoite re-invasion[52] and may therefore simultaneously fulfill dual beneficial functions.

It is intriguing that, having served its physiologic purpose in the lifecycle of the parasite, the DV should be endowed with the capacity to activate the 2 archaic enzyme cascade systems of the blood into which it is cast. Perhaps these events initially serve a protective function by enabling the infected host to rapidly remove alien waste material. However, pathologic consequences may evolve when the disposal machinery suffers overload, with these events then contributing to the evolution of severe malaria. Future studies should determine whether a novel determinant of parasite pathogenicity has been discovered that might also be targeted for therapy in patients suffering from what remains one of the most prevalent life-threatening diseases in the world.

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Authorship

Contribution: P.D., S.D.H., A.F., A-L.Z., and S. Bhakdi performed the laboratory experiments; M.B., K. Reifenberg, and C.O. performed the animal experiments; M.T. and C.O. performed the immunohistochemistry work; S. Bhakdi and S.C.B. conceived of the project; P.D., S. Bhakdi, K.L., and K. Reiss designed the research; P.D., S. Baumeister, K.L., R.U., K. Reiss, and S. Bhakdi analyzed the data; and S. Bhakdi wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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PHAGOCYTES, GRANULOCYTES, AND MYELOPOIESIS

Digestive vacuoles of *Plasmodium falciparum* are selectively phagocytosed by and impair killing function of polymorphonuclear leukocytes

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Sequestration of parasitized erythrocytes and dysregulation of the coagulation and complement system are hallmarks of severe *Plasmodium falciparum* malaria. A link between these events emerged through the discovery that the parasite digestive vacuole (DV), which is released together with infective merozoites into the bloodstream, dually activates the intrinsic clotting and alternative complement pathway. Complement attack occurs exclusively on the membrane of the DVs, and the question followed whether DVs might be marked for uptake by polymorphonuclear granulocytes (PMNs). We report that DVs are indeed rapidly phagocytosed by PMNs after schizont rupture in active human serum. Uptake of malaria pigment requires an intact DV membrane and does not occur when the pigment is extracted from the organelle. Merozoites are not opsonized and escape phagocytosis in nonimmune serum. Antimalarial Abs mediate some uptake of the parasites, but to an extent that is not sufficient to markedly reduce reinvasion rates. Phagocytosis of DVs induces a vigorous respiratory burst that drives the cells into a state of functional exhaustion, blunting the production of reactive oxygen species (ROS) and microbicidal activity upon challenge with bacterial pathogens. Systemic overloading of PMNs with DVs may contribute to the enhanced susceptibility of patients with severe malaria toward invasive bacterial infections. (*Blood.* 2011; 118(18):4946-4956)

Introduction

Severe malaria develops as a consequence of capillaric sequestration of parasitized RBCs (pRBCs),[1-3] activation of complement[4-6] and coagulation,[7-9] and increases in vascular permeability, which together can lead to microcirculatory disturbances with comatous death as the ultimate outcome.[1,7,10,11] One unresolved puzzle relates to the fact that children with severe malaria frequently suffer from septicemia due to bacteria that otherwise play no major role in this potentially fatal affliction.[12-14] Approximately 50% of these infections are caused by nontyphoidal *Salmonellae* and other Enterobacteriae, indicating a gut origin. Current hypotheses regarding the causes underlying this general state of immune compromise focus mainly on disturbances in the function of macrophages, dendritic cells, and the adaptive immune system.[15]

During its developmental cycle in the RBC, the malaria parasite ingests hemoglobin and packages the waste product hemozoin, which is also known as malaria pigment, in an organelle designated the digestive vacuole (DV).[16] Copious numbers of DVs are released into the bloodstream in patients with severe *Plasmodium falciparum* malaria, yet their biologic properties and fates have been only minimally studied. Hematin is widely regarded to be equivalent to hemozoin,[17,18] and most investigators have focused on the effects of this artificial molecule on the immune system.[19-25] However, hematin is not packaged within a membrane-bound organelle and any biologic activity deriving from the DV membrane would therefore be missed. In a different study, we have shown that the DV membrane, but not hemozoin itself, is endowed with the unique capacity to dually activate the alternative complement and the intrinsic clotting pathway (P.D., S. Heber, S. Baumeister, K.L., K.R., S.C.B., and S. Bhakdi, unpublished data, September 2011). Overactivation of these ancient enzyme cascades would be expected to trigger and sustain pathobiological events that coincide with severe malaria. The discovery that DVs activate complement prompted investigations into the fate of this organelle. In particular, we strove to determine whether complement would mark the DV for uptake by polymorphonuclear granulocytes (PMNs). If so, this would satisfactorily account for the long-known fact that malaria pigment can regularly be observed in circulating PMNs of patients with severe malaria.[26,27] By extrapolation, it would also provide a further explanation as to why hemozoin is found in tissue macrophages of animals after infection.[28]

Several novel observations were made in the present study. First, DVs are opsonized and rapidly phagocytosed by PMNs after schizont rupture in active human serum. Second, the uptake of DVs induces a respiratory burst in PMNs, but the generated reactive oxygen species (ROS) fail to suppress the infective capacity of invading merozoites. Third, firing of ROS on ingested DVs drives PMNs into a state of functional exhaustion. Their ability to phagocytose bacteria prevails, but their capacity to mount a respiratory burst is reduced and microbicidal activity is compromised. It is proposed that these events might be linked to the development of septicemic episodes in patients with severe malaria.

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Methods

Cell preparation

P falciparum culture, isolation of digestive vacuoles and hemozoin, and staining procedures for DNA and C3 were undertaken as described previously (P.D., S.H., S. Baumeister, K.L., K.R., S.C.B., and S. Bhakdi, unpublished data, September 2011) and as detailed in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Human PMNs were isolated from heparinized blood of healthy volunteers using conventional procedures[29] and were kept in PBS on ice until use. Surface labeling of PMNs was performed with PE-conjugated polyclonal rabbit anti–human CD16 IgG (BD Biosciences) following the manufacturer's recommendation. After labeling, 50 μL of Ab solution was added to 0.5 mL of cells (10^7 PMNs/mL) for 20 minutes at room temperature, and the cells were subsequently washed twice with PBS. By microscopic assessment, it was ascertained that surface labeling had no effect on phagocytic function. Erythrocytes, banked human blood, and human sera, all group O, Rh^+, were kindly provided by Roland Conradi and Walter Hitzler (Blood Transfusion Center of the University of Mainz, Mainz, Germany). C8-deficient human serum was prepared as described previously.[30] Polyclonal rabbit anti–human C3 IgG was obtained from Dakocytomation.

PMNs phagocytosis of DVs

PMNs suspended at 5×10^6 cells/mL in 10% active or heat-inactivated serum were challenged with 3-5 DVs/cell and phagocytosis was analyzed by microscopy of Giemsa-stained smears. To obtain quantitative data, DVs were fluorescently labeled by 20 minutes of incubation with 7.5 μg/mL CellMask Deep Red plasma membrane stain (Invitrogen), washed twice, and added to the PMNs. After the given times at 37°C, 50 μL of the reaction mixture was removed, diluted in 500 μL of veronal buffered saline (VBS), and analyzed in a FACSScan flow cytometer (BD Biosciences).

Detection of ROS generation

Single-cell observations were conducted using PMNs loaded with 0.1μM 2′,7′-dichlorodihydrofluorescein diacetate (Sigma-Aldrich) for 30 minutes at 37°C, and washed 4 times with PBS. Next, 0.2 mL of cell suspensions (3×10^6/mL in VBS with 10% active serum) were transferred to Eppendorf tubes and DVs/PMNs at a ratio of 2:5 were added. Incubation was conducted at 37°C. Smears were prepared every 2 minutes and viewed in an Axioskop 2 microscope (Carl Zeiss) at a 1000× magnification. Images were obtained using AxioVision software.

Luminol-based quantification of ROS

Experiments were performed in microtiter plates using a thermostated luminometer (MicroLumat LB96P; Berthold Technologies). Each well contained 0.2 mL of PMNs (3×10^6/mL in VBS) with 10% active or inactive serum. Luminol (Sigma-Aldrich) was added to a final concentration of 125μM to each well. DVs were applied at a ratio of 3:5 DVs/PMNs and chemiluminescence was measured every 4 minutes. In bacterial challenge experiments, luminol was re-added after 60 minutes, a ratio of 10:15 *Staphylococcus aureus*/PMNs was applied, and measurements were continued for another 60 minutes.

Assessment of uptake of DVs and merozoites by PMNs after rupture of pRBCs

Synchronized enriched cultures containing 60%-70% late-stage pRBCs were diluted to 0.2% hematocrit in RPMI 1640 medium and maintained at 37°C. Giemsa-stained smears were prepared every 30 minutes. When the first schizont rupture was observed, cultures were continued for another 30 minutes, during which time > 75% of the parasitized cells lysed. The culture was then centrifuged in a Beckman Coulter Allegra 6KR centrifuge at 200*g* for 5 minutes to sediment unlysed cells. The supernatant was centrifuged at 3000*g* for 10 minutes in a Sorvall RC2B centrifuge to pellet the merozoites and DVs. The pellet was resuspended in RPMI and centrifuged once again at low speed (400*g*) for 1 minute to remove any residual unlysed cells. The supernatant was then centrifuged at 17 000*g* for 2 minutes, washed 3 times, and stained with Hoechst 33342. Isolated DVs and merozoites were added to Eppendorf tubes, each containing 3×10^6/mL of PMNs (prelabeled with PE-conjugated polyclonal rabbit anti–human CD16 IgG) in 0.2 mL of VBS with 10% active serum (3-5 DVs and 50-100 merozoites/PMNs). After 30 minutes at 37°C, thin smears were prepared and air dried, and Z-stacked images were taken in a fluorescence microscope (Axiovert 200M; Carl Zeiss) using AxioVision Version 4.7 software.

Infection experiments

Synchronized late-stage pRBCs were enriched to 55%-70% and suspended at 0.4% hematocrit in RPMI with 20% active human serum; 0.5 mL was applied per well in a 24-well cell culture plate (Greiner Bio-One). To this, 0.1 mL of 10% noninfected RBCs ± 10^6 PMNs were added, and the total volume brought to 1 mL with RPMI to give a final ratio of PMNs:pRBCs: RBCs of ~ 1:10:100. After 24 hours, 0.2 mL of the culture was stained with 100μM hydroethidine for 30 minutes at 37°C, and infection rates were quantified by flow cytometry.[31] Slides were prepared and stained with Giemsa for microscopy.

Ab protection experiments

Assessment of Ab protection was undertaken using a 10-fold higher number of PMNs and the ^{3}H-hypoxanthine incorporation assay.[32] For this assay, 5×10^6 schizonts and 5×10^7 noninfected RBCs were suspended in 0.2 mL total volume in 96-well flat-bottom microtiter suspense culture plates (Greiner Bio-One) and supplemented as follows: (1) 20% active serum, (2) 20% active serum + 10^7 PMNs, (3) 20% active serum + 0.2 mg of IgG, and (4) 20% active serum + 0.2 mg of IgG and 10^7 PMNs. Experiments were performed in duplicate. Plates were incubated for 10-12 hours at 37°C, after which time the medium was exchanged and 1μCi ^{3}H-hypoxanthine (Perkin Elmer) was added per well. After another 12 hours, genomic DNA was isolated using the DNeasy Blood and Tissue Kit (QIAGEN) and hypoxanthine incorporation was measured in a liquid scintillation counter (LS 6000 TA; Beckman Coulter).

Abs

Sera were obtained from Thai patients with acute *P falciparum* malaria, which was confirmed by microscopic examination of Giemsa-stained blood films on admission. The sera were tested for Abs to malarial antigens with the indirect fluorescent Ab test using methanol-fixed films of *P falciparum*–infected RBCs. Five patients with high titers (> 1:625) had parasitemia ranging from 4.5%-21%; 4 patients had low indirect fluorescent Ab titers (< 1:25) and parasitemia ranging from 0.5%-1.1%. Pools of the high- and low-titered sera were prepared. Experiments were performed with both the individual sera and the 2 serum pools. IgG was purified using Spintrap columns (GE Healthcare Life Sciences) and concentrated using Vivaspin 6 Centricon tubes (Sartorius) to their original volume. The IgG samples were dialyzed against RPMI 1640, the protein concentrations were measured using Bradford reagent and the Bio-Rad protein assay, and stored at −20°C until use.

Phagocytosis and bacterial killing assays

Experiments with *S aureus* were performed with cell-rich plasma obtained after dextran sedimentation of heparinized blood samples from healthy volunteers.[33] DVs were added to 0.9 mL of cell-rich plasma to give a ratio of 3:5 DVs/PMNs and incubated at 37°C for 1 hour. Thereafter, 0.1 mL of *S aureus* in PBS was added to give a ratio of 10:20 bacteria/PMNs. At given time points, 0.1-mL samples were withdrawn and admixed with 0.1 mL of a 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) solution. After 5 minutes at 37°C, 0.8 mL of water was added and incubation continued for 3 minutes at 37°C. This treatment ensured complete liberation of bacteria from the cells under retention of their viability.[33] Finally, 20-μL samples were diluted 1:200 in saline and 20 μL of this solution was plated in duplicate for colony counting.

Experiments conducted with a clinical isolate of *Salmonella typhimurium* (obtained from the Diagnostics Laboratory of the Department of

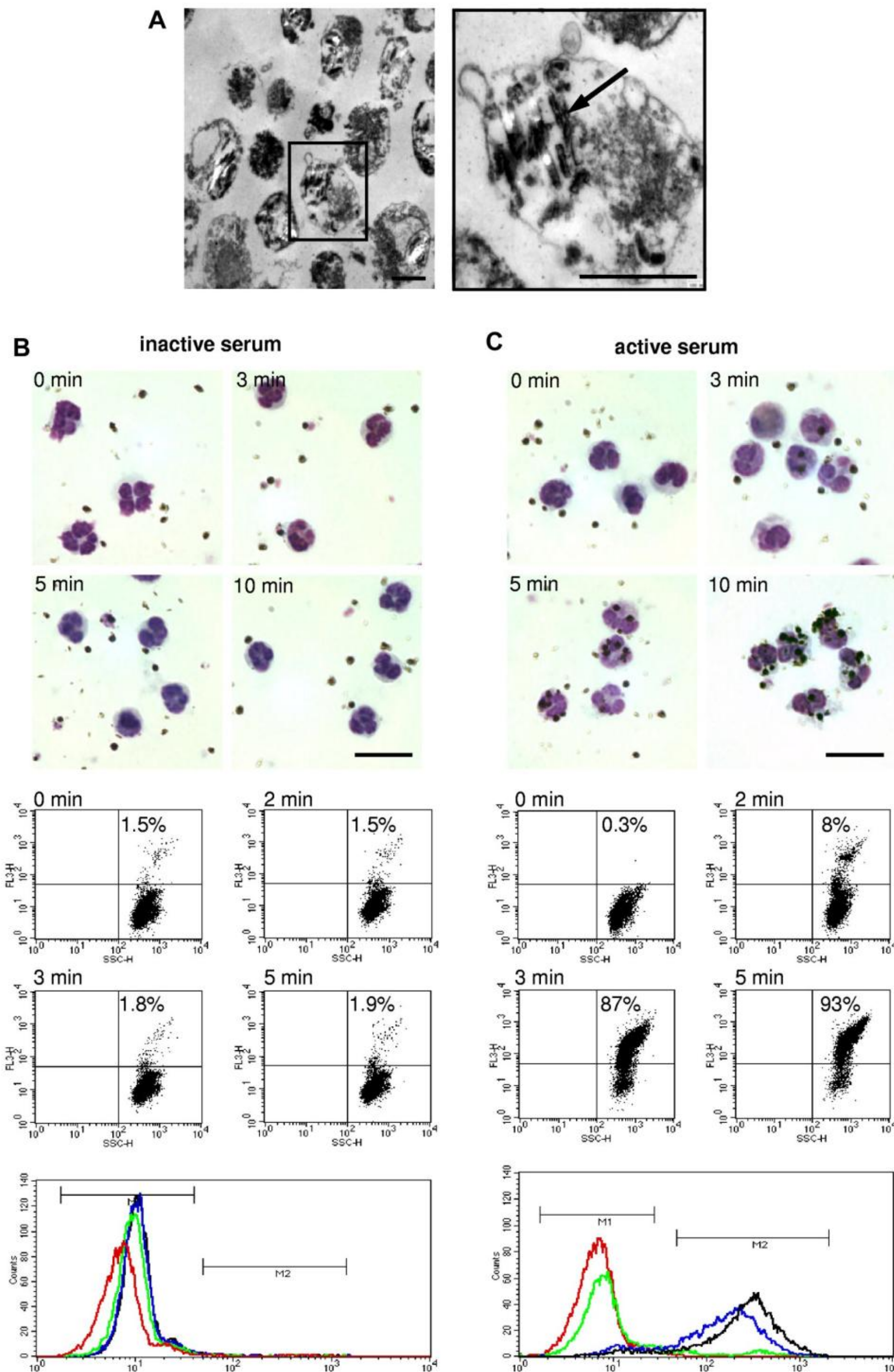
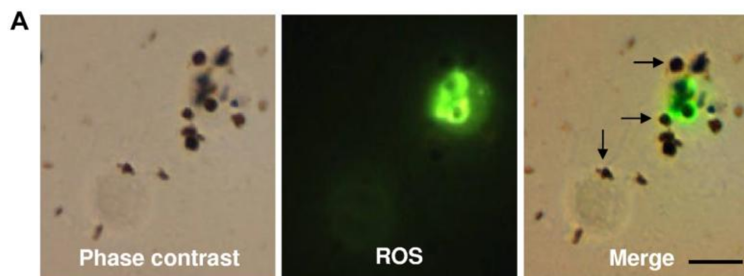


Figure 1. Phagocytosis of isolated DVs by PMNs. (A) Electron microscopic appearance of DVs naturally liberated from late-stage parasitized RBCs showing ~ 1- to 2-μM vesicles with varying content of hemozoin (arrow) bound by a limiting membrane. Scale bars indicate 1 μm. (B) DVs were incubated with PMNs prelabeled with PE-conjugated anti-CD16 IgG in 10% inactive serum. Microscopic Giemsa smears were prepared at the depicted times and flow cytometric analyses of phagocytosis were performed by gating PMNs and assessing the number of red fluorescent cells. Histograms of the corresponding dot plots are shown in the bottom panel (red: 0 minutes; green: 2 minutes; blue: 3 minutes; and black: 5 minutes). M1 is the region set for nonfluorescent cells and M2 for fluorescent cells. The numbers counted in the M2 region are shown in the dot plots. (C) Same experiment performed in active serum. The results of flow cytometric analyses are shown at 2 and 3 minutes to illustrate the abrupt onset of phagocytosis occurring in active serum. The findings in inactive serum remained stationary over the period of observation of 30 minutes. One representative experiment of 3 independent experiments is shown. Scale bars indicate 20 μm.



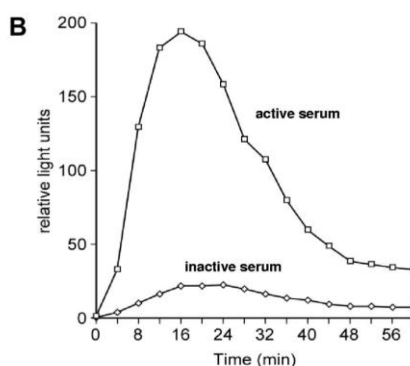


Figure 2. Phagocytosis of DVs induces ROS production in PMNs. (A) PMNs were laden with dichlorol-fluorescein to visualize ROS generation at the single-cell level. Phagocytosis of DVs was accompanied by triggering of the respiratory burst, and ROS generation was seen to surround intracellular DVs. ROS generation was not observed when DVs were just attached to the cells (arrows). Scale bar indicates 10 μm. (B) Luminol-based chemiluminescence assay for ROS generation in PMNs during phagocytosis of DVs revealed a bell-shaped response curve covering a time span of 30-40 minutes. PMNs were challenged with 3-5 DVs/cell and the chemiluminescence response was recorded in the presence of 10% active or heat-inactivated human serum. One representative experiment of 3 independent experiments is shown.

Medical Microbiology and Hygiene, University of Mainz, Mainz, Germany) were performed using isolated PMNs and C8-deficient serum that had been prepared as described previously.[30] PMNs were suspended at 2×10^6 cells/mL in 50% C8-deficient serum/VBS and experiments were conducted as described above.

Transmission electron microscopy

DVs were centrifuged, fixed with 2.5% glutaraldehyde in PBS at 4°C overnight, washed in PBS, post-fixed in 2% OsO_4, dehydrated in ethanol, and embedded in Araldite (Sigma-Aldrich). Ultrathin sections were mounted on Formvar-coated grids and double stained with a saturated solution of uranyl acetate in 70% methanol and lead citrate. The grids were examined with a Zeiss EM 900 transmission electron microscope equipped with a digital camera system.

Statistical analysis

The assumptions for normality and equal variance were verified with SigmaStat 3.1 software (Erkrath; SYSSTAT). The Holm-Sidak test was used for comparisons against a control group. Results represent means ± SEM of at least 3 independent experiments or the means of at least triplicates of 1 experiment ± SD as indicated. $P < .05$ was considered statistically significant.

Results

Rapid complement–dependent phagocytosis of intact DVs by PMNs

DVs were isolated from supernatants of hemolyzed pRBCs and, in accordance with descriptions in the literature,[34,35] were found to comprise a population of dispersed, 1- to 2-μM sized vesicles with various contents of hemozoin crystals and amorphous material (Figure 1A). No merozoites or RBCs membrane debris could be discerned in the electron micrographs.

DVs and PMNs were admixed at a ratio of 5:1 in 10% active or 10% inactive human serum and phagocytosis was followed microscopically and by flow cytometry. For the latter analyses, DVs were fluorescently labeled, the PMNs were gated based on side and forward scatter and the uptake of fluorescently labeled DVs was assessed. No phagocytosis of the DVs by PMNs occurred in inactive serum within the 30-minute period of observation, as was apparent from the Giemsa-stained smears and from the corresponding absence of a red fluorescent shift of the cells (Figure 1B). In contrast, phagocytosis in active serum could be readily observed microscopically, commencing within 3 minutes and essentially reaching completion at 5-8 minutes. Correspondingly, PMNs abruptly started to assume red fluorescence within 2-3 minutes and, in agreement with the microscopic observations, > 90% of the cells were laden with DVs after just 5 minutes (Figure 1C).

PMNs were loaded with dichlorofluorescein, which allowed visualization of ROS production in single cells. In agreement with the classic response during phagocytosis, a burst of ROS could always be observed surrounding a DV during and immediately after its uptake (Figure 2A). The single-cell observations were borne out by luminol-based quantification of ROS generation, which revealed a bell-shaped chemiluminescence response covering a time span of ~ 40 minutes that was not seen when heat-inactivated serum was used (Figure 2B).

Merozoites escape phagocytosis by PMNs in nonimmune serum

Late-stage synchronized pRBCs were cultured at 0.2% hematocrit in medium containing 10% active, nonimmune human serum until schizont rupture occurred, and merozoites and DVs were harvested

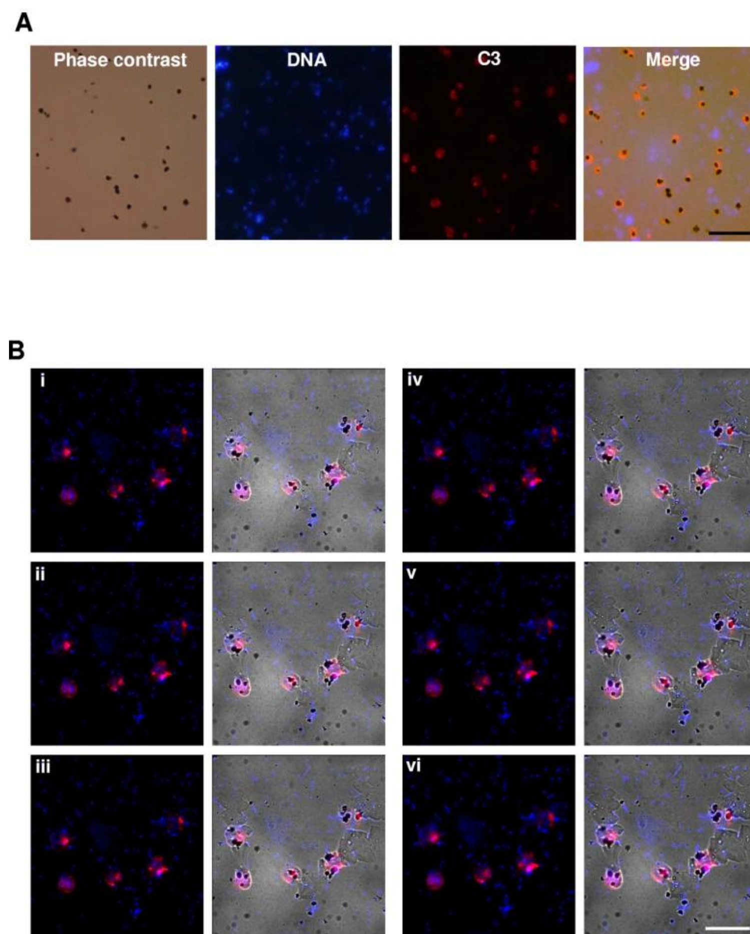


Figure 3. Selective opsonization and phagocytosis of DVs after pRBC rupture. (A) Immediately after lysis of late-stage pRBCs, merozoites and DVs were harvested and stained with polyclonal Abs directed against native and activated C3 (C3b) and DNA. C3 immunoreactivity was exclusively restricted to DV membranes. Scale bar indicates 20 μm. (B) DNA staining was used to identify merozoites and PE-surface-labeled PMNs were added to the mixture with DVs. Stacked images (i-vi: top to bottom) were prepared that revealed extracellular localization of merozoites. Left rows: fluorescence; right rows: merged fluorescence and phase-contrast images. The micrographs shown are representative of 3 independent experiments. Scale bar indicates 20 μm.

and stained for DNA and bound C3 (Figure 3A). The polyclonal Abs used recognized native and activated C3 (C3b). Hemozoin contained within the DVs was visualized in the phase-contrast image. Merozoites were not seen here, but showed up in the blue fluorescence image generated by the DNA stain. Immunofluorescence staining revealed the presence of C3 exclusively on the DVs, as was strikingly apparent in merged images, in which the red C3 fluorescent stain is seen colocalizing with DVs, but was invariably absent on merozoites. Weaker stainings were also obtained using an Ab against C3d, which reacts with the remnant molecule after C3b is cleaved and removed (data not shown). This experiment was performed 3 times with serum from different donors. Merged images were randomly taken and 100 hemozoin particles were evaluated for the presence of C3. In all 3 experiments, C3 colocalized with > 85% of the DVs. DVs liberated just before harvest would have had insufficient time to become sufficiently coated with C3b. In striking contrast to these findings, not a single merozoite was ever observed to carry C3 deposits.

It followed that selective phagocytosis might occur as a natural consequence of intravascular schizont rupture. Therefore, in the next experiment, merozoites and DVs were isolated as above and DNA staining was undertaken to stain the parasites. PE-surface-labeled PMNs (1 PMN:3-5 DVs: 50-100 merozoites) were then added together with 10% NHS, and stacked fluorescence microscopic images were prepared after 30 minutes. These revealed virtually exclusive uptake of DVs, and merozoites were rarely seen to colocalize with the DVs in the central plane of the cells (Figure 3Biii and 3Biv).

It has been reported that PMNs may phagocytose late-stage parasitized RBCs.[36] Such cells were stained with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), which enabled intracellular parasites to be visualized (P.D., S. Heber, S. Baumeister, K.L., K.R., S.C.B., and S. Bhakdi, unpublished data, September 2011), and incubated with PE-labeled PMNs in the presence of active serum for 30 minutes. However, erythrophagocytosis could never be observed (Figure 4A).

Isolated hemozoin lacking the encasing DV-membrane is not phagocytosed

Malaria pigment is microscopically detectable in circulating PMNs of patients with malaria, and the relative number of pigment-containing PMNs provide a prognostic criterion for disease outcome.[26,27] The question of how the pigment enters the cell has never been addressed. A tacit assumption is that DVs are labile structures from which hemozoin is released and then phagocytosed. However, we have found that the DV membrane is remarkably stable, requiring extreme measures to effect its disruption in vitro (P.D., S. Heber, S. Baumeister, K.L., K.R., S.C.B., and S. Bhakdi, unpublished data, September 2011). Once freed of the encasing membrane, the pigment disperses into small, crystalline unit

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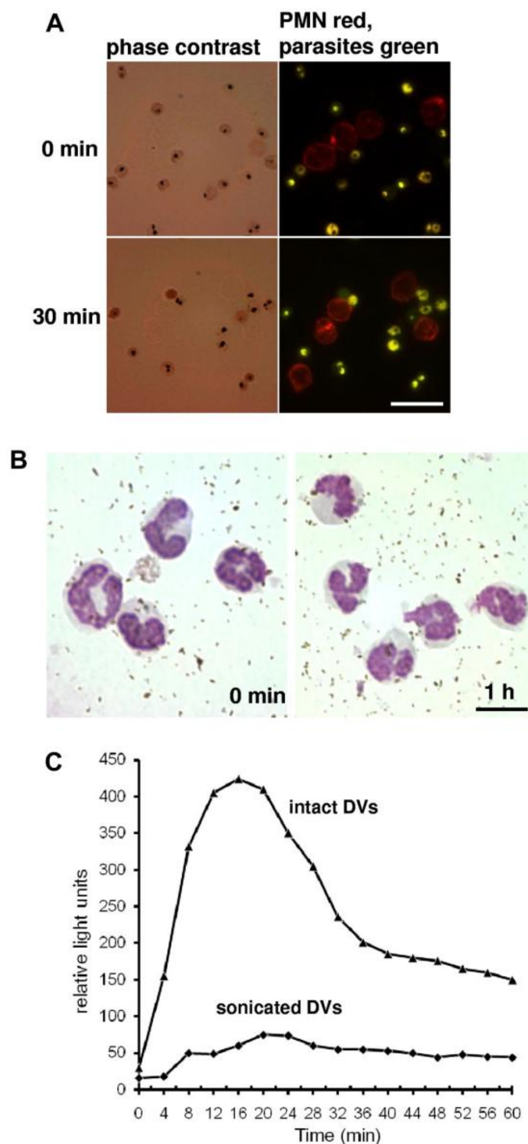

Figure 4. PMNs do not phagocytose late-stage pRBCs or hemozoin in nonimmune serum. (A) pRBCs were stained with BCECF-AM, a nonfluorescent acetoxymethylester that is enzymatically hydrolyzed to fluorescent BCECF, to visualize parasites and intracellular DVs, and incubated with PE-labeled PMNs in active human serum. No erythrophagocytosis could be discerned after 30 minutes. Scale bar indicates 20 μm. (B) DVs were disrupted by sonication and hemozoin was isolated from a Percoll gradient and incubated with PMNs in active serum. No evidence for phagocytic uptake and no appearance of cells with characteristic malaria pigment could be discerned in Giemsa-stained smears. Scale bar indicates 10 μm. (C) Luminol-based chemiluminescence assay were performed in PMNs and active serum upon incubation with intact DVs or with sonicated DVs (hemozoin). Representative results are shown from 1 of 3 similar experiments.

structures devoid of complement-activating properties and morphologically distinct from the compact deposits visible within intact DVs (P.D., S. Heber, S. Baumeister, K.L., K.R., S.C.B., and S. Bhakdi, unpublished data, September 2011). This agrees with published data showing that the intravesicular malaria pigment consists of multiple aggregates of hemozoin crystals.[17] It is these aggregates and not the dispersed crystals that present as malaria pigment in circulating leukocytes.[26,27] In the next experiment, DVs were disrupted by sonication or by detergent lysis, and hemozoin was purified by centrifugation through Percoll. The isolated hemozoin was incubated in 10% NHS with PMNs for 1 hour. No microscopic evidence for attraction of PMNs to and uptake of isolated hemozoin could be obtained, and cells presenting with the characteristic malaria pigment were not observed (Figure 4B). In agreement with these observations, chemiluminescence assays revealed that sonication of DVs immediately destroyed their ROS-inducing property (Figure 4C). The same was found when isolated hemozoin was applied to the cells (not shown).

DV-induced ROS-production in PMNs does not suppress merozoite reinvasion

Merozoites have been reported to be susceptible to the cytotoxic action of ROS.[37] This conclusion was based on experiments with murine malaria merozoites and may not be extrapolatable to *P falciparum*. Nevertheless, it was of interest to determine whether ROS generation might indirectly serve a protective function because of bystander killing of the parasites. In the next experiment, a 10-fold excess of noninfected RBCs was added to enriched, late-stage pRBCs and cultures were maintained for 24 hours in the presence or absence of PMNs, which were added in supraphysiological numbers (~ 1:100 RBCs) so that no effects would be missed. Figure 5Ai depicts the control without PMNs, showing the presence of freshly infected cells with ring-stage parasites. When PMNs were present in the culture, these were seen to have phagocytosed the DVs (Figure 5Aii). However, ring forms could still be discerned and quantification of infected cells by flow cytometric analyses of DNA-stained samples generated superimposable curves (Figure 5Aiii left peaks, uninfected nonfluorescing cells; right peaks, infected cells). Therefore, ROS generation accompanying phagocytic uptake of DVs by PMNs led to no reduction in the infective capacity of merozoites. This experiment was performed 3 times with PMNs from different donors with the same result.

At this juncture, it was of interest to determine whether the failure of PMNs to prevent parasite reinvasion might be rectified in the presence of specific Abs. Experiments were undertaken with IgG from 5 patients with high Ab titers, from a pool of high-titered sera from 10 patients, and from 5 patients with low Ab titers.

Phagocytosis experiments were performed as in the experiment shown in Figure 3, and reinvasion was quantified by measuring DNA incorporation of ^{3}H-hypoxanthine. No effects of low-titered Abs could be discerned in any experiments. However, in the presence of high-titered Abs, merozoites were observed to be phagocytosed along with comparable numbers of DVs (Figure 5B). Inspection of 100 PMNs revealed that, although the merozoites outnumbered DVs by an order of magnitude in the incubation mixture, a phagocyte was never seen harboring merozoites alone. Therefore, it appeared that preferential uptake of DVs persisted even in the presence of the Abs. This might have reduced each cell's capacity to phagocytose merozoites, allowing the majority to escape phagocytosis. Indeed, only small Ab-mediated reductions of parasite reinvasion could be detected in the ^{3}H-hypoxanthine incorporation assays, even though the PMNs were present in 10-fold higher numbers in these experiments (Figure 5C).

The capacity to produce ROS is blunted in DV-laden PMNs

ROS generation is subject to multiple pathways of feedback regulation,[38,39] so the possibility that ingestion of DVs might lead to impaired respiratory burst on subsequent bacterial challenge emerged. PMNs in 10% serum were incubated for 60 minutes in the presence or absence of 2-4 DVs per cell and subsequently

challenged with *S aureus*. Figure 6A shows the chemiluminescence recordings observed. In control PMNs, *S aureus* challenge provoked a sustained generation of ROS. DVs induced the initial bell-shaped chemiluminescence response, but thereafter, the second burst of ROS provoked by *S aureus* was blunted. Identical results were found with PMNs from 4 different donors.

DV uptake reduces microbicidal activity of PMNs

These findings prompted phagocytosis and killing assays. Cell-rich plasma obtained after dextran sedimentation of erythrocytes from heparinized blood was spiked with 2-5 DV/PMN and incubated for 60 minutes at 37°C. The PMNs were then challenged with 10-20 *S aureus* per cell. Microscopy revealed that the capacity of DV-loaded PMNs to subsequently ingest the bacteria remained unimpaired, and most of the bacteria were phagocytosed as in the controls after 15-20 minutes (Figure 6B). However, colony counting undertaken after detergent solubilization of the PMNs led to the striking finding that the capacity of DV-laden cells to kill ingested bacteria was significantly compromised (Figure 6C). ROS reduction did not directly impact oxygen-independent microbicidal mechanisms, so the killing function was reduced but not entirely abrogated.

Five to 10% of African children with severe malaria suffer from sepsis episodes, half of which are caused by enteric nontyphoid *Salmonellae*.[12-14] Phagocytic killing assays were therefore also performed with a clinical isolate of *S typhimurium*. Enteric *Salmonellae* display widely varying degrees of serum complement resistance. This unpredictable potential confounder was eliminated by conducting assays with isolated PMNs in the presence of 50% C8-deficient human serum. Killing could then be attributed solely to the microbicidal function of the neutrophils. As found with *S aureus*, a significant reduction of bactericidal capacity was observed for *S typhimurium* in PMNs that had been laden with DVs (20-minute kill in controls: 48% $\pm$ 5% and in DV-laden cells: 20% $\pm$ 2%; n = 4, $^*P < .001$).

Discussion

Lysis of each parasitized erythrocyte in *P falciparum* malaria liberates one DV along with infectious merozoites into the bloodstream.[16,40] High parasitemia is therefore inseparably associated with high loads of DVs at the sites of RBC rupture. It is all the more remarkable that, whereas seminal work on the biogenesis and biochemical events occurring within the DV is ongoing,[34,35,41-43] only one group of investigators has been conducting studies with naturally released DVs, and these are devoted to their effects on monocyte functions.[44-48] The first study was conducted with unpurified malarial pigment obtained by hypotonic lysis of infected erythrocytes. In that study, evidence was presented that the pigment was phagocytosed by human macrophages, and that this provoked a respiratory burst that was blunted upon subsequent provocation. Bactericidal assays were not performed, but it was surmised that this immune suppression might bear general clinical relevance when malarial pigment reached the macrophages in the spleen and other organs.[44] The fact that most DVs will probably be ingested not by tissue macrophages but rather by the surrounding PMNs has never been considered before the present work, an oversight that likely derives from several reasons. There is a tendency to assume that the malaria pigment itself is endowed with biologic properties, and that synthetic hematin, which is considered equivalent to natural malaria pigment,[17,18] is readily available. The popularity of hematin as a research tool is understandable, but its use could not have led to present discoveries because free pigment lacks all of properties described herein. In this context, it is not common knowledge that the DV is released as an intact, enveloped organelle and it has not been recognized that the DV membrane is remarkably stable. Therefore, a widespread assumption is that malaria pigment itself rapidly contacts the host environment. Consequently, the possibility has been missed that the DV membrane may fulfill biologic functions that are entirely distinct from those of isolated hematin or hemozoin.

The starting point of the present investigation was our recent discovery that the DV membrane is endowed with the capacity to dually activate the alternative complement and intrinsic clotting pathways (P.D., S. Heber, S. Baumeister, K.L., K.R., S.C.B., and S. Bhakdi, unpublished data, September 2011). Binding of complement marks a particle for phagocytosis, and experiments described herein naturally followed. Isolated DVs were indeed found to be rapidly phagocytosed in a complement-dependent fashion, which raised the possibility that engulfment of DVs represents the major pathway leading to the presence of malaria pigment in PMNs of patients. The latter is a widespread finding, attesting to the generality of the phenomenon. Previous concepts have envisaged phagocytosis of hemozoin crystals or schizonts to be responsible, but no evidence for either could be obtained in this study. Sonicated DVs or isolated hemozoin did not induce a respiratory burst and were not detectably taken up by the PMNs. We also could not observe any phagocytosis of late-stage parasitized RBCs in nonimmune serum. The possibility that specific Abs might alter the latter situation, as suggested in an early study,[36] is not excluded. However, PMNs containing ingested parasitized RBCs are seldom seen in clinical samples, so we propose at this stage that malaria pigment in the PMNs of patients derives mainly from phagocytosis of intact DVs after their extrusion into the blood. Electron micrographs of both PMNs and macrophages bearing malarial pigment have been published.[49] The findings are highly suggestive for 2 reasons. First, although the cells had not phagocytosed parasitized erythrocytes, aggregates of hemozoin crystals could be seen in the cell cytoplasm. Such aggregates do not persist when the pigment is artificially liberated from the organelle in vitro. Second, the aggregates can be seen to be surrounded by membrane structures in the electron micrographs. These findings are fully in agreement with our hypothesis that phagocytosis of DVs underlies the appearance of malaria pigment in the cells.

Whether DVs might be preferentially phagocytosed was investigated in the presence and absence of high-titered Abs against *P falciparum*. Synchronized late-stage pRBCs were used, enabling the time point of schizont rupture to be closely monitored so that the notoriously short-lived merozoites could be retrieved together with the DVs. Culture was performed in active instead of heat-inactivated serum so that complement activation would immediately occur upon erythrocyte rupture. Staining of merozoite DNA was undertaken before the addition of surface-labeled PMNs, rendering rapid fluorescent microscopic analyses feasible. These experiments revealed the striking fact that DVs but not merozoites were selectively opsonized and phagocytosed by PMNs in the presence of active, nonimmune serum. In the presence of high-titered Abs, phagocytosis of merozoites was also observed. However, preferential uptake of DVs still appeared to persist. Therefore, although merozoites outnumbered DVs by an order of magnitude, as also occurs in vivo, PMNs were never observed to contain merozoites only. If preferential uptake of DVs really takes place, this might impinge on each cell's capacity to ingest merozoites.

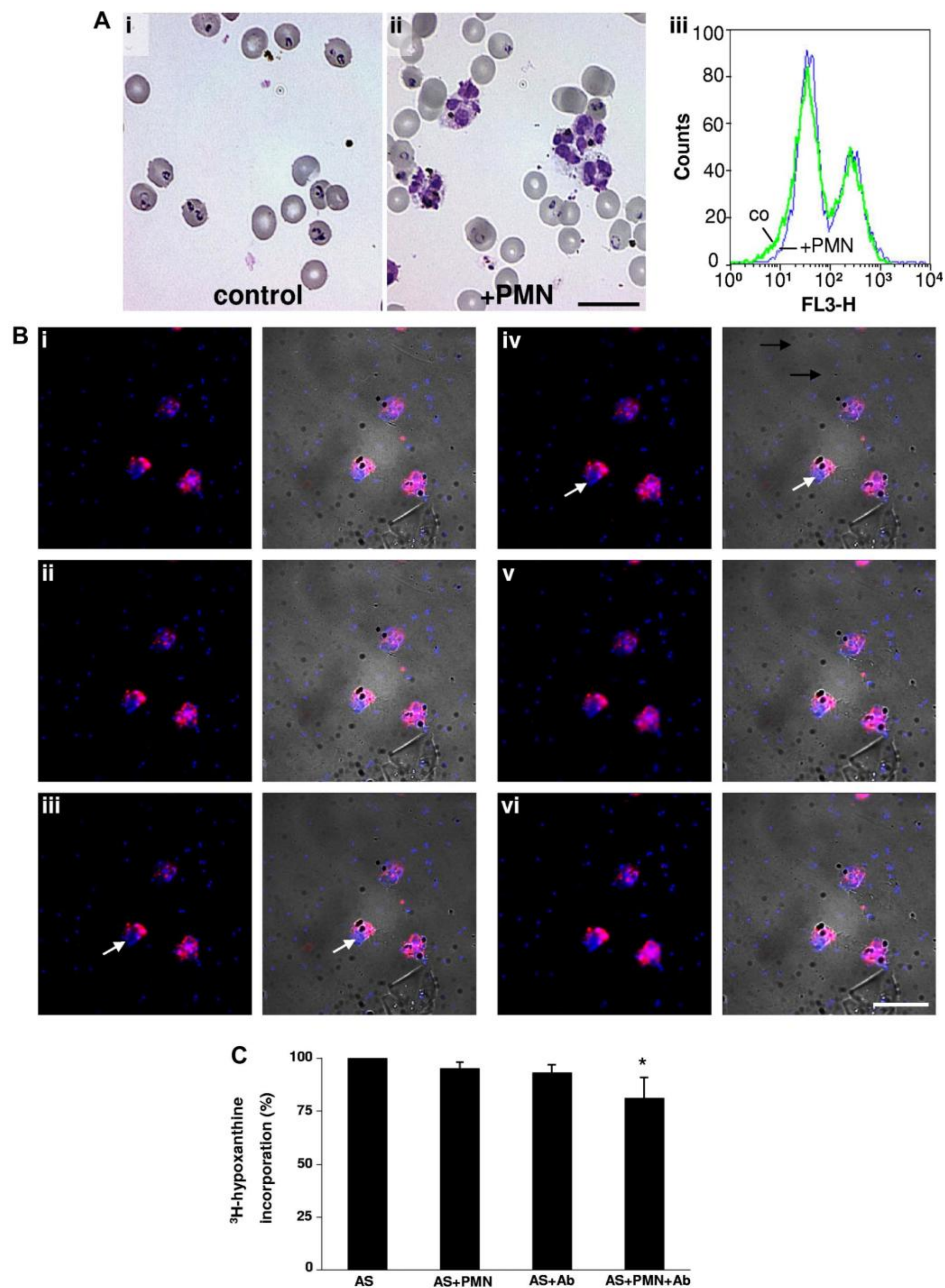

Figure 5. Lack of protective effect of PMNs on merozoite reinvasion. (A) Late-stage pRBCs were allowed to rupture and new infection was allowed to proceed in active human serum in the absence (i) or presence (ii) of PMNs. Twenty-four hours later, ring forms could be seen microscopically in both cases. Infected erythrocytes were detected by fluorescent staining of parasite DNA with hydroethidium-bromide (iii). Flow cytometric analyses revealed identical infection rates in the presence and absence of PMNs (right peaks: fluorescing infected cells; left peaks: nonfluorescing, noninfected cells). (B) Detection of merozoite phagocytosis mediated by Abs against *P falciparum.* The experiment shown in Figure 3B was repeated in the presence of 1 mg/mL of IgG isolated from a pool of 5 sera containing high-titered Abs against *P falciparum.* Stacked images (i-vi, top to bottom) now revealed that merozoites (blue) had been phagocytosed alongside with the DVs. Left rows: fluorescence; right rows: merged fluorescence and phase-contrast images. Arrows: phagocytosed merozoites colocalizing with DVs in the central plane (iii-iv) of a cell. Similar results were obtained with IgG from 5 individual sera with high-titered Abs against *P falciparum.* Scale bar indicates 20 μm. (C) Paucity of protective effects of PMNs and specific Abs upon parasite reinvasion. The ^{3}H-hypoxanthine DNA-incorporation assay was used and values obtained in active serum alone were defined as 100%. Results obtained with 5 high-titered Abs are shown. ^{3}H-hypoxanthine incorporation was assessed in active serum (AS) in the presence of either PMNs or IgG (Ab) or in the presence of PMNs and IgG. A small but significant reduction in ^{3}H-hypoxanthine incorporation was observed in the presence of Abs plus PMNs compared with the control ($n = 5$; $^*P < .001$).

ROS generation, the central element in the microbicidal machinery of mammalian phagocytes, is subject to feedback regulation via multiple pathways.[38,39] Induction of the respiratory burst by phagocytosed DVs might therefore be followed by a state of hyporesponsiveness. Indeed, the capacity to mount a respiratory burst on subsequent bacterial challenge was severely compromised after DV uptake. PMNs preloaded with DVs were still fully capable of phagocytosing bacteria. However, their microbicidal capacity was reduced. This was shown using the classic target *S aureus* and a clinical isolate of *S typhimurium*. The latter was used because noninvasive *Salmonella* are the leading cause of septicemia in African children with severe malaria, and these experiments now offer a possible explanation for this finding.

Having served its physiologic purpose, the liberated DV appears to function as a decoy, and is used by the parasite to divert and derange central elements of the innate immune system. The intrinsic clotting and alternative pathway simultaneously become activated on its surface. Both pathways generate potent mediator molecules that may trigger and sustain microcirculatory disturbances and vascular leakage, which contribute to the clinical syndromes of malaria. DVs from other *Plasmodium* species are probably endowed with similar properties. Complement activation has been shown in monkeys infected with *P coatneyi*[50] and in mice infected with *P berghei*.[51] High levels of parasitemia exceeding 10% are found almost exclusively in *P falciparum* infections, and capillaric sequestration of pRBCs will further heighten the local load of DVs. Other than the living parasite, the organelle is then preferentially taken up by blood phagocytes. This may initially provide benefit to the host by restricting activation of complement and coagulation. Differences in PMN-dependent clearing capacity may explain why high parasitemia is sometimes tolerated and vice versa. However, the price for this indirectly beneficial PMN function may ultimately be high. As parasitemia increases, so will the detraction of the cells away from their true targets. We found that high-titer-specific Abs invoked some phagocytosis of merozoites. This finding would appear to confirm earlier studies in which Abs from patients reportedly promoted phagocytosis of merozoites by neutrophils.[52,53] However, merozoite preparations described in those studies were retrieved as a dark band from Percoll gradients and thus may have contained appreciable numbers of DVs. We are not aware of any previous study in which phagocytosis of merozoites and DVs has been cleanly differentiated, such as is illustrated herein in Figures 3 and 5.

Possibly because efficient ingestion of DVs still prevailed, PMNs contained relatively small numbers of merozoites and the majority of parasites remained outside of the cells. In agreement with this observation, substantive reductions in the rates of reinvasion could also not be discerned. These findings are preliminary because they were performed with only a small number of antisera and one laboratory strain of *P falciparum.* Homologous Abs might more efficiently redirect PMN phagocytosis toward merozoites to afford some protection. However, our findings agree with earlier studies in which little[32] or no[54,55] protective effects of specific Abs plus PMNs could be observed in vitro. Monocytes apparently synergized more efficiently with the Abs,[55] but the significance of this finding remains unclear because the cells were used at unphysiologically high numbers. Most recently, the presence of Abs provoking strong ROS responses in PMNs upon incubation with a mixture of merozoites and DVs was reported to be correlated with better in vivo protection compared with Abs that induced little ROS production.[56] Whether ROS generation is correlated with uptake of merozoites requires further investigation. These open questions notwithstanding, it appears quite clear that in

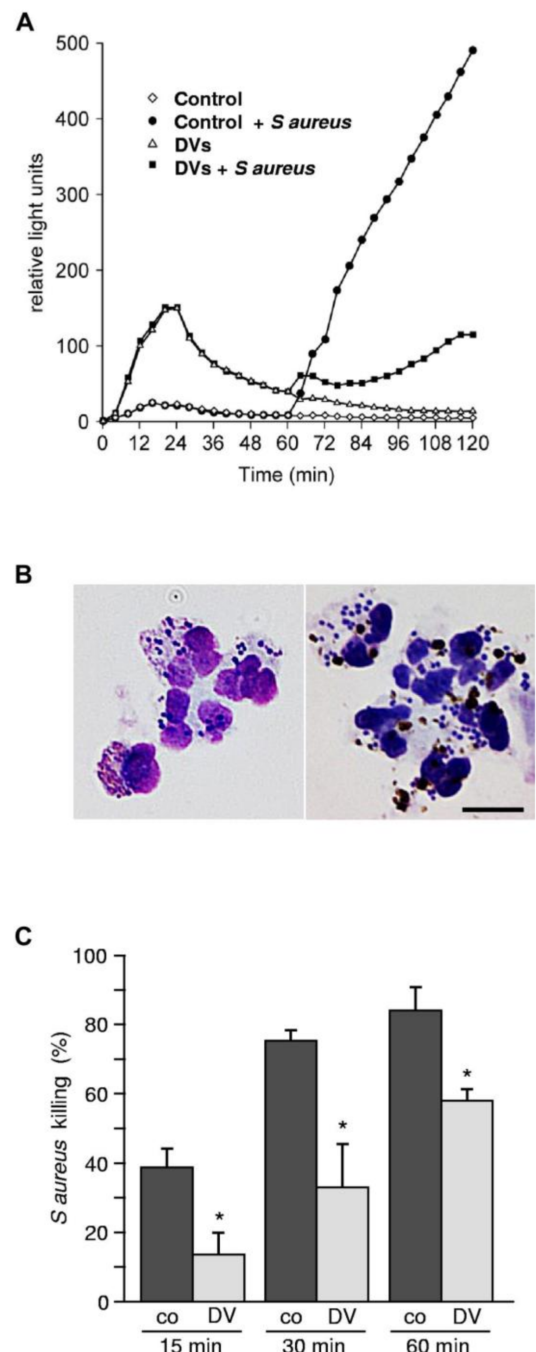

Figure 6. Phagocytosis of DVs leads to impairment of PMNs-function. (A) ROS generation was detected using the luminol-based chemiluminescence assay. Control PMNs incubated for 60 minutes in medium and subsequently challenged with *S aureus* mounted a vigorous response. Incubation of cells with DVs induced an initial bell-shaped response, but ROS generation upon subsequent bacterial challenge was conspicuously blunted. (B) Microscopic examination undertaken 20 minutes after *S aureus* challenge revealed effective phagocytic uptake of the bacteria in both cases (left: control PMNs; right: PMNs preloaded with DVs). Scale bar indicates 10 μm. (C) Assessment of bacterial killing revealed marked impairment of bactericidal activity in DV-laden cells. One representative experiment of 4 independent experiments is shown. Results are expressed as means of triplicates ± SD; *$P < .001$ compared with control.

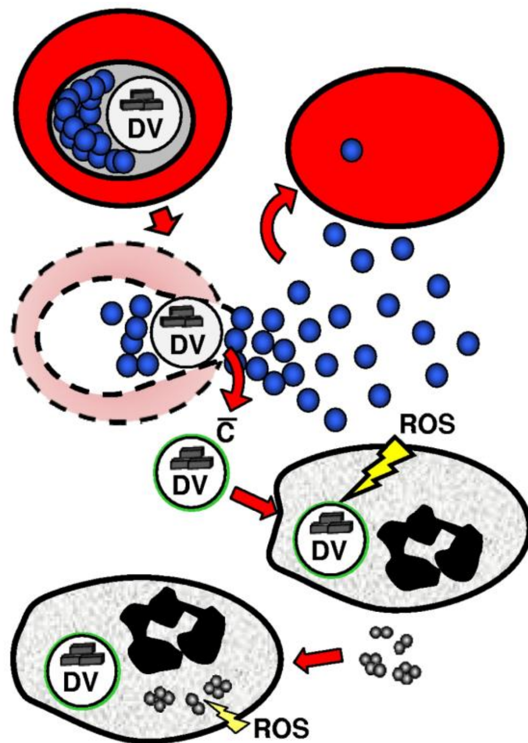

Figure 7. Schematic presentation of the concept of immune decoy by the DV. Rupture of a parasitized cell liberates one DV along with 32 merozoites. In nonimmune serum, complement is activated only on the DV, so exclusive uptake of the vesicle follows, leaving merozoites free to invade new cells. ROS generation in response to DV uptake is unable to harm the merozoites, but instead drives the cells into a state of functional exhaustion so that efficient killing of subsequently engulfed bacteria is no longer ensured.

vivo protection mediated by anti-merozoite Abs can generally not be very efficient, because patients in endemic regions often have circulating Abs that can neither inhibit infection nor totally suppress disease progression.

DV uptake and cellular activation could cause PMNs to remain mainly sequestered in the microcirculation. If that is the case, then the actual number of DV-laden phagocytes would probably be considerably higher than suggested by the mere numbers of circulating, hemozoin-containing cells. Sequestered, activated PMNs possibly augment pathologic processes in the microcirculation. At the same time, their systemic overloading may gradually set the stage for septicemic complications to develop whenever bacterial pathogens chance to gain entry into the circulation (Figure 7). The leading roles played by *Salmonellae* and *Enterobacteriaceae* in African children may derive simply from the high endemic prevalence of these agents. Should the principle tenets of this investigation turn out to be correct, the DV would emerge as a major, multifaceted determinant of parasitic pathogenicity. Clinically oriented studies are called for to test this hypothesis.

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Authorship

Contribution: S. Bhakdi and S.C.B. conceived the project; S. Bhakdi, K.R., and P.D. designed the research; P.D. and S. Bhakdi performed the experiments; R.L. performed the electron microscopy; S. Bhakdi, P.D., S. Baumeister, K.L., R.U., and K.R. analyzed the data; and S. Bhakdi wrote the manuscript.

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REVIEW

Pathogenesis of malaria revisited

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Abstract *Plasmodium falciparum* malaria claims 1 million lives around the globe every year. Parasitemia can reach remarkably high levels. The developing parasite digests hemoglobin and converts the waste product to hemozoin *alias* malaria pigment. These processes occur in a vesicular compartment named the digestive vacuole (DV). Each parasitized cell releases one DV upon rupture. Myriads of DVs thus gain entry into the blood, but whether they trigger pathobiological events has never been investigated. We recently discovered that the DV membrane simultaneously activates the two major enzyme cascades in blood, complement and coagulation. Activation of both is known to occur in patients with severe malaria, so discovery of the common trigger has large consequences. The DV membrane but not the merozoite has the capacity to spontaneously activate the alternative complement and intrinsic clotting pathway. Ejection of merozoites and the DV into the bloodstream, therefore, results in selective opsonization and phagocytosis of the DV, leaving merozoites free to invade new cells. The DV membrane furthermore has the capacity to assemble prothrombinase, the key convertase of the intrinsic clotting pathway. The dual capacity of the DV to activate both complement and coagulation can be suppressed by low-molecular-weight dextran sulfate. This agent protects experimental animals from the detrimental consequences, resulting from intravenous application of purified DVs. Phagocytosis of DVs not only deploys PMN away from merozoites, but also drives the cells into a state of functional exhaustion. This may be one reason for the enhanced susceptibility of patients with severe malaria toward systemic bacterial infections. Together, these findings indicate that the DV may represent a hitherto unrecognized, important determinant of parasite pathogenicity.

Keywords Malaria · Digestive vacuole · Complement · Coagulation · Immunopathogenesis

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Preamble

Severe falciparum malaria evolves through interplay between capillaric sequestration of parasitized erythrocytes, a deregulated inflammatory response and hemostasis dysfunction [1–5]. Vascular leakage can occur in all organs. In the brain, vascular occlusion resulting from erythrocyte sludging and fibrin formation provokes hypoxia and acidosis, which may culminate in cerebral malaria. Due to the multi-facetted nature of pathological events, the clinical manifestation of severe malaria can be very varied.

Interaction of PRBC with the endothelium is mediated by parasite proteins that are exported to the RBC membrane and become exposed at its surface. Why sequestration of the cells should then provoke inflammatory processes and activate the coagulation system remains an enigma. No single entity of parasite origin has been identified that might serve as a common trigger. Identification of any such entity requires its isolation, the demonstration that it invokes effects that are observed in vivo, the delineation of underlying mechanisms, and the demonstration that counteracting these events ameliorates disease. As with other systemic inflammatory diseases, it is generally assumed that derangement of host mediator systems

Appendix

invokes pathology. Cytokines have been a focus on most discussions, while other mediators have received less attention. This pertains in particular to the complement system.

Complement and malaria: the unknown connection

Seminal findings on complement activation in malaria were published in the 1970s. Complement turnover was shown to be triggered in human patients suffering from severe malaria [6], and experiments in monkeys demonstrated that complement consumption coincides with schizont rupture [7]. Since then, clinical data have accumulated to show that substantial complement activation occurs in human patients [8]. Complement activation has also been demonstrated at the earliest stages of experimental human malaria [9]. Finally, experiments in a murine model of cerebral malaria suggest a pathogenic role of complement activation [10, 11]. Yet, no study has ever been devoted to the identification of a complement activator that might be released during rupture of the parasitized cell.

We discovered that when parasitized RBC rupture in autologous serum, the alternative complement pathway is immediately activated [12] (Appendix 4). This pathway generally becomes activated on particulate surfaces [13], and a directed search led to the identification of the digestive vacuole as the triggering element.

The digestive vacuole of *Plasmodium falciparum*

During its developmental cycle in the red blood cell, the malaria parasite ingests hemoglobin and packages the waste product hemozoin *alias* malaria pigment in an organelle designated the digestive vacuole (DV) [14]. Vesicles containing hemoglobin bud from the parasitophorous vacuole membrane (PVM) are endocytosed by the parasite. The PVM is subsequently degraded. The DVs, now bounded by a single membrane derived from the parasite, fuse with each other to create a large vacuole in which hemoglobin is degraded, and heme is polymerized to form hemozoin crystals. At the end of the developmental cycle, the DV remains a separate, distinct entity devoid of contact with the newly formed merozoites. Upon completion of the developmental cycle, the PRBC rupture releasing one DV along with the infective merozoites into the bloodstream. High parasitemia is thus synonymous with high loads of DVs at the site of RBC rupture. Yet, whereas seminal work on the biogenesis and biochemical events occurring within the DV is ongoing, only one group of investigators has been studying the biological properties of the DV following its release from the RBC, and these are devoted exclusively to its effects on monocyte functions [15–17].

There are several reasons for this. First, it is widely assumed that the malaria pigment itself is biologically active. The synthetic product hematin is reportedly similar in structure to hemozoin [18]. It is not common knowledge that the DV is released as an intact organelle nor that its membrane should be particularly stable. Therefore, it is widely assumed that malaria pigment itself rapidly contacts the host environment. On this background, most researchers elect to test the effects of the readily available and chemically defined hematin.

However, we found that activation of both complement and clotting was triggered not by hemozoin, but by its encasing membrane (Fig. 1). The DV membrane was furthermore found to directly assemble the key convertase of the intrinsic clotting pathway, promoting fibrin formation in platelet-free plasma (Fig. 2) [12].

Merozoites did not share this property and did not become opsonized. The question why complement should be activated on the membrane of the DV but not on merozoites remains to be resolved, but the finding directly leads to the hypothesis that rupture of PRBC in blood should lead to selective uptake by neutrophil granulocytes. This would explain an old finding in the field.

Uptake of malarial pigment in PMN

Malarial pigment is microscopically detectable in circulating PMN of patients, and the relative number of pigment-containing PMN provides a prognostic criterion for disease outcome [19]. The question of how the pigment enters PMN has never been stringently addressed. A tacit assumption is that hemozoin is first released from the DV and then phagocytosed. However, we found this not to be the case.

When parasitized cells lysed in active serum, complement attack occurred exclusively on the membrane of the DV and not on merozoites. This was followed by rapid phagocytosis of the DVs by PMN. Uptake required an intact DV membrane and did not occur when the pigment was extracted from the organelle. Merozoites were not opsonized and escaped phagocytosis. Anti-malarial antibodies mediated some uptake of merozoites, but to an extent that was not sufficient to markedly reduce invasion rates. Phagocytosis of DVs caused PMN activation, driving the cells into a state of functional exhaustion and reducing their microbicidial activity [20]. Severe malaria is known to predispose patients toward invasive bacterial infections [21, 22], and we now propose that systemic overloading of PMN with DVs may contribute to this state (Fig. 3).

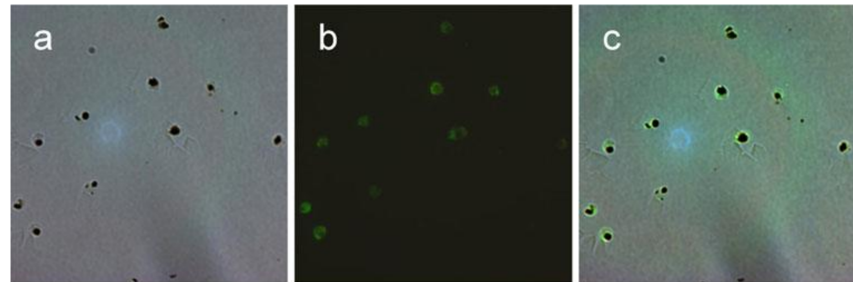

Fig. 1 Detection of C5b-9 on DVs following incubation with active serum. **a** Phase contrast; **b** C5b-9 immunofluorescence; **c** merge of both. Corresponding flow analysis shows staining of all DVs in the sample. Representative results of 4 independent experiments are shown (taken from Dasari et al. [12], Appendix 4)

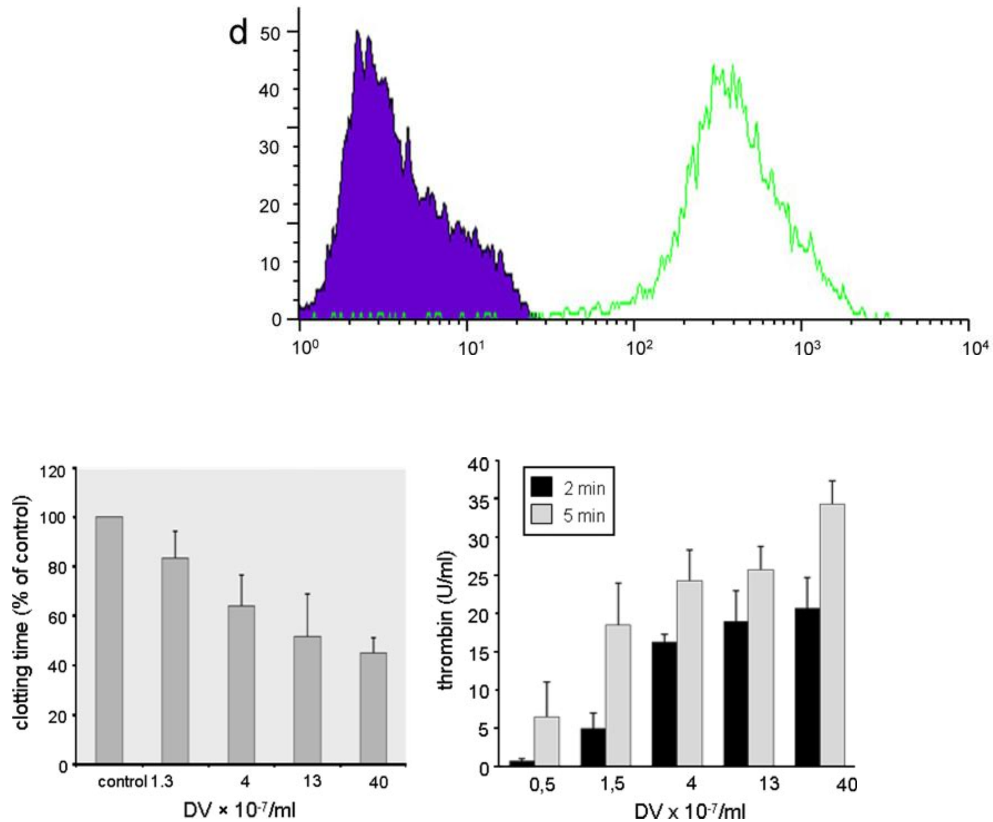

Fig. 2 DVs directly activate the intrinsic clotting pathway. *Left* clotting time after recalcification of 50 % plasma was significantly accelerated in the presence of DVs. Clotting times of the respective buffer controls were taken as the 100 % reference in each experiment. Data are expressed as mean ± SEM of five independent experiments. *Right* isolated DVs dose-dependently enhanced thrombin generation in the prothrombinase assay ($N = 4 \pm$ SEM). Controls without DVs did not induce any thrombin generation and are not shown (taken from Dasari et al. [12])

The DV and pathogenesis of malaria: an emerging hypothesis

A picture begins to emerge in which the DV, having fulfilled its physiological function, is cast into the bloodstream, and there becomes a decoy for key effectors of the innate immune system. Activation of the complement system deploys PMN away from their true targets. Merozoites outnumber DVs by over an order of magnitude. Preferential phagocytosis of the DV conceivably promotes escape of the parasites. At the same time, activation of complement and clotting could well contribute to detrimental events underlying severe malaria. The increasing burden of DVs may furthermore undermine the phagocytic

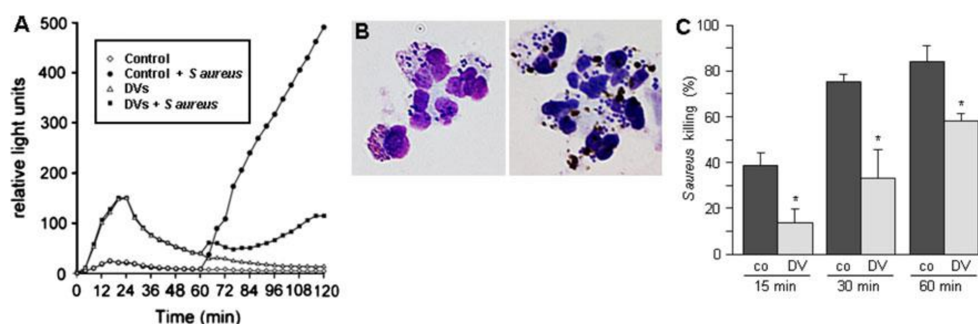

Fig. 3 Phagocytosis of DVs leads to impairment of PMN function. **a** ROS generation was detected using the luminol-based chemiluminescence assay. Control PMN incubated for 60 min in medium and subsequently challenged with *S. aureus* mounted a vigorous response. Incubation of cells with DVs induced an initial bell-shaped response, but ROS generation upon subsequent bacterial challenge was conspicuously blunted. **b** Microscopic examination undertaken 20 min after *S. aureus* challenge revealed effective phagocytic uptake of the bacteria in both cases (*left* control PMN; *right* PMN preloaded with DVs). **c** Assessment of bacterial killing revealed marked impairment of bactericidal activity in DV-laden cells. One representative of four independent experiments is shown. Results are expressed as means of triplicates ± SD. *$P < 0.001$ compared with control (co) (taken from Dasari et al. [20])

defense system, resulting in functional neutropenia with all its consequences (Fig. 4) [20].

Prospects of reducing complement and clotting activation in severe malaria

If DV-induced activation of complement and clotting should contribute to malaria pathogenesis, the existence of a potential inhibitor could be of high interest. Low-molecular-weight dextran sulfate (LMW-DXS) blocks both the complement and the coagulation cascade at micromolar concentrations that, in contrast to heparin, do not causing bleeding complications and, as demonstrated in volunteer experiments, are perfectly tolerated in humans (relevant literature cited in 14). The effect of LMW-DXS on the DVs was, therefore, investigated. It was found that LMW-DXS at therapeutically realistic concentrations effectively inhibited DV-dependent activation of the intrinsic clotting pathway and abrogated assembly of prothrombinase complexes and also diminished complement activation. In the first animal experiments, injection of DVs into rats caused alternative pathway complement consumption and provoked apathy and reduced nociceptive responses in the animals. LMW-DXS blocked the activation of both complement and coagulation and protected animals from the harmful effects of DV infusion [12].

Antibody-mediated protection against malaria: quo vadis

Arguments for a key role of specific antibodies in providing immune protection against malaria are quite compelling, and they provide the conceptual basis for all past and current efforts to develop a vaccine. The only vaccine candidate that is currently undergoing phase 3 clinical trial is sporozoite-based: antibodies protect by suppressing hepatocyte invasion by the parasites. Suppression will need to be very effective: should just a few sporozoites escape, the specific antibodies would no longer be able to inhibit the intraerythrocytic multiplication cycles that follow. First results of the clinical trial have been published [23]. Their reception has been guarded [24].

Intuitively, a merozoite-based vaccine would seem to hold at least as much promise. MSP1 has been at the center of discussion, and humanized monoclonal antibodies against this protein have been produced [25–27]. For want of animal models, the protective efficacy of these antibodies has been tested only in vitro. In these assays, reinvasion rates of RBC are quantified in cell culture in the presence and absence of the specific antibodies [25, 26]. Reductions were seen, but required very high antibody concentrations [25, 26]. The findings were analogous to what had been previously observed with polyclonal human antibodies, which also only partially suppress reinvasion in vitro [28, 29]. Because of these findings, the prospects of immune-targeting MSP1 are not generally viewed with optimism.

However, a crucial point is possibly being missed. The general assumption is that anti-MSP antibodies protect by inhibiting merozoite invasion [25, 26], or by promoting Fc-dependent phagocytosis by monocyte/macrophages [27]. But perhaps their critical function is to trigger classical complement pathway activation on the merozoites, so that the complement inhibition barrier is overcome and phagocytosis by PMN is promoted. (This is the principle of protection conferred by antibodies against Streptococcus

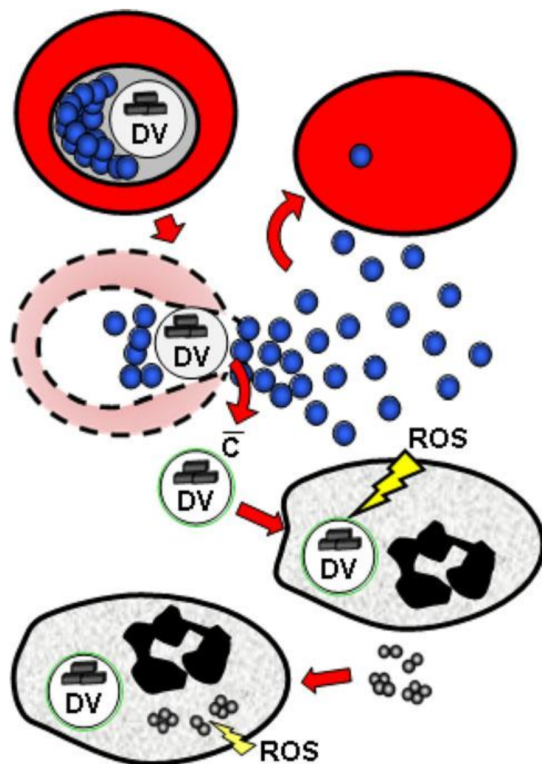

Fig. 4 Schematic presentation of the concept of immune decoy by the DV. Rupture of a parasitized cell liberates the DV along with 32 merozoites. In nonimmune serum, complement is activated only on the DV, so exclusive uptake of the vesicle follows, leaving merozoites free to invade new cells. ROS generation in response to DV uptake is unable to harm the merozoites, but instead drives the cells into a state of functional exhaustion, so that efficient killing of subsequently engulfed bacteria is no longer assured (taken from Dasari et al. [20])

pneumoniae). Then, their protective efficacy could only be correctly assessed in the presence of active serum and PMN. Such assays have never been performed.

Concluding remarks

The collective findings indicate that the DV represents an important novel determinant of parasite pathogenicity and opens a new research area in the field of malaria. A number of intriguing questions await resolution. At sites of RBC sequestration, vast numbers of DVs will be released whose clearance may be retarded due to the relatively low numbers of circulating PMN and absence of tissue macrophages. Situations will thus arise wherein myriads of particles bearing highly active complement convertases will contact naïve bystander blood cells. Theoretically, it could be imagined that this might lead to bystander deposition of activated complement components on the cells, which in turn might have functional consequences. Testing of this hypothesis may prove rewarding.

What is the cause of selective complement activation on the DV? The organelle forms through endocytic invaginations of the merozoite, and its membrane is thus an inside out version of the parasite. Lipidic surfaces lacking complement inhibitors are probably exposed at the surface of the DV, which is why the convertases assemble with such efficacy. It will be of high interest to examine whether the merozoite is protected because GPI-anchored inhibitors shuttle from the RBC to the parasite surface, or because the coat created by merozoite surface proteins prevents access of complement to the membrane.

Systematic immunohistological studies are urgently called for. It would be of high interest to determine whether complement deposits can be seen colocalizing with malarial pigment in tissues of patients with severe malaria. Such studies will provide direct evidence for or against the hypotheses that we have raised.

New therapeutic approaches may be worth investigating. The protective capacity of humanized anti-merozoite antibodies may hitherto have been underrated and can be reassessed with an improved reinvasion assay in the presence of active serum and neutrophil granulocytes. Independently, the therapeutic potential of low-molecular-weight dextran sulfate needs to be assessed in an appropriate model of infection.

It is to be hoped that further investigations in this area will advance our understanding on the complex pathogenesis of malaria, to the benefit of patients suffering from this disease that has plagued mankind throughout the centuries.

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ERKLÄRUNG

ich versichere, dass ich meine Dissertation

Biological properties of the digestive vacuole of Plasmodium falciparum: Activation of complement and coagulation

selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

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(Ort/Datum) (Prasad Dasari)