Selbstmörderischer Erythrozytentod in der Malaria

Suicidal Erythrocyte Death in Malaria

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ABBREVIATIONS

AA	Arachidonic acid
AIF	Apoptosis inducing factor
ANOVA	Analysis of Variance between groups
ATP	Adenosine 5´ triphosphate
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CFSE	5,6-carboxylfluorescein diacetate succinimidyl ester
Cl^-	Chloride ion
CO_2	Carbon dioxide
COX	Cyclooxygenase
CSP	Circum sporozoite protein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EIPA	5-(N-ethyl-N-isopropyl) amiloride
FACS	Fluorescence activated cell sorter
FL-1	Florescence channel 1
FSC	Forward scatter
g	gram
G6PD	Glucose-6-phosphate dehydrogenase
Gardos channel	Calcium activated potassium channel
GSH	Glutathione
h	Hour
Hb	Hemoglobin
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
K^+	Potassium ion
KCl	Potassium chloride
L-NAME	*Nω*-Nitro-L-arginine methyl ester
MCV	Mean corpuscular volume
mg	Milligram
$MgSO_4$	Magnesium sulphate

min	Minute
ml	Milliliter
mM	Millimolar (mmol/L)
MSP	Merozoite surface protein
Na^+	Sodium ion
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nM	Nanomolar
NO_3	Nitrate
NPP	New Permeability Pathways
NSC	Nonselective Cation Channel
PAF	Platelet activating factor
$Pb(NO_3)_2$	Lead nitrate
PBS	Phosphate buffered saline
PCD	Programmed cell death
PGE_2	Prostaglandin E_2
PLA_2	Phospholipase A_2
ppm	Parts per million
PS	Phosphatidylserine
RBCs	Red Blood Cells
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RTC	Reticulocytes
SEM	Standard error mean
TNF	Tumor necrosis factor
TRAP	Thrombospondin-related adhesive protein
µM	Micromolar
WBC	White blood cells

SUMMARY

Malaria is one of the most devastating diseases with lethal outcome in more than 1 million humans per year. The course of the disease is not only a function of the pathogen but is heavily influenced by properties of the host. Mechanisms possibly conferring protection against a severe course of malaria include suicidal death of the infected cell. A particular form of suicidal erythrocyte death is eryptosis, which is characterized by Ca^{2+}-entry with subsequent activation of Ca^{2+}-sensitive K^+ channels, KCl exit and erythrocyte shrinkage, Ca^{2+}-sensitive scrambling of phospholipids resulting in the breakdown of cell membrane phosphatidylserine asymmetry and phosphatidylserine exposure at the cell surface, as well as Ca^{2+} dependent activation of calpain, proteolytic degradation of cytoskeletal proteins and cell membrane blebbing. Eryptosis is triggered by different well-known pro apoptotic stressors, namely hyperosmotic shock, oxidative stress and energy depletion, which all activate Ca^{2+} -permeable nonselective cation channels allowing Ca^{2+} entry into the erythrocyte. Eryptosis is enhanced in several inherited erythrocyte disorders such as, phosphate depletion, hemolytic uremic syndrome, sepsis, and Wilson disease. As macrophages are equipped with receptors specific for phosphatidylserine, cells exposing phosphatidylserine at their surface will be rapidly recognized, engulfed, degraded and thus cleared from circulating blood

Infection of erythrocytes with *Plasmodium falciparum* has been shown to trigger eryptosis, at least in part due to activation of host cell channels via oxidation of the cell membrane. Accelerated death of infected erythrocytes has been suggested to delay the development of parasitemia and protect against a severe course of the disease. Erythrocyte death could be triggered by induction of eryptosis. Triggers of eryptosis include hemolysin, PGE_2, platelet activating factor, heavy metals like mercury, iron deficiency, L-NAME, $Pb(NO_3)_2$ or chlorpromazine. The present studies has been performed to explore whether the addition of L-NAME or $Pb(NO_3)_2$ or chlorpromazine to the drinking water may modify the course of malaria and survival of *Plasmodium berghei* -infected mice and also to explore whether iron deficiency influences the course of malaria.

As a result, iron deficiency increased the phosphatidylserine exposure in *P. falciparum* infected human erythrocytes, an effect significantly more marked in iron deficiency erythrocytes than compared to control erythrocytes. Moreover, iron deficiency impairs *in vitro* intraerythrocytic growth and infection of erythrocytes. In mice, iron deficient erythrocytes are more rapidly cleared from circulating blood, an effect increased by infection with *P. berghei*. Parasitemia in *P. berghei* infected mice was significantly decreased (from 54% to 33% of circulating erythrocytes, 20 days after infection) and mouse survival significantly enhanced (from 0% to 20%, 30 days after infection) in iron deficient mice.

L-NAME (≥10 μM) increased phosphatidylserine exposure of *P. falciparum* infected human erythrocytes, an effect significantly more marked than in noninfected human erythrocytes. In parallel, parasitemia in *P. berghei* infected mice was significantly decreased (from 50% to 18% of circulating erythrocytes 20 days after infection) by addition of L-NAME (1mg/ml) to the drinking water. According to CFSE labelling, L-NAME treated infected erythrocytes disappeared more rapidly from circulating blood than nontreated erythrocytes

$Pb(NO_3)_2$ (≥10 μM) increased phosphatidylserine exposure of *P. falciparum* infected erythrocytes, an effect significantly more marked than in noninfected cells. We further show that $Pb(NO_3)_2$ treated erythrocytes are more rapidly cleared from circulating blood than nontreated erythrocytes. Parasitemia in *P. berghei* infected mice was significantly decreased (from 50% to 18% of circulating erythrocytes 20 days after infection) and mouse survival significantly enhanced (from 0% to 38% 30 days after infection) by addition of 100 μM $Pb(NO_3)_2$ (20 ppm) to the drinking water. The treatment did not significantly decrease erythrocyte number and hematocrit in noninfected mice and in infected animals mainly triggered the disappearance of *P. berghei* harbouring erythrocytes.

Chlorpromazine (≥10 μM) during *in vitro* infection of human erythrocytes increased phosphatidylserine exposure and decreased forward scatter. Chlorpromazine did not significantly alter intraerythrocytic DNA/RNA amplification but significantly (≥5 μM)

decreased *in vitro* parasitemia. Erythrocytes from chlorpromazine treated mice were more rapidly cleared from circulating blood than nontreated erythrocytes. Parasitemia in *P. berghei* infected mice was significantly decreased (from 50% to 28% of circulating erythrocytes 22 days after infection) and mouse survival significantly enhanced (from 0% to 80% 30 days after infection) upon addition of 1mM chlorpromazine to the drinking water from the first day of infection.

In conclusion, iron deficiency, L-NAME, $Pb(NO_3)_2$ or chlorpromazine enhances the susceptibility of eythrocytes to eryptosis. As a result, erythrocytes undergo accelerated eryptosis following infection with *Plasmodium.* The accelerated eryptosis precedes the full intraerythrocytic maturation of the pathogen and thus blunts the increase of parasitemia. The observations support the view that accelerated suicidal death of infected erythrocytes is a host mechanism to counteract infection with the intracellular pathogen. The observations may not only serve to understand the mechanisms of host defence against the malaria pathogen but open new perspectives for pharmacological treatment of this devastating disease.

ZUSAMMENFASSUNG

Malaria ist eine verheerende tödliche Krankheit, die jährlich über eine Million Menschen das Leben kostet. Der Verlauf dieser Krankheit ist nicht nur vom Erreger abhängig, sondern wird auch von bestimmten Merkmalen des Erkrankten stark beeinflusst. Möglicherweise verleihen gewisse Mechanismen Schutz vor einem schweren Verlauf der Malaria und der Selbstzerstörung der infizierten Zelle. Eine bestimmte Form des Erythrozytenselbstmordes ist die so genannte Eryptose, welche durch einen Ca^{2+}-Einstrom mit anschließender Aktivierung eines Ca^{2+}-sensitiven K^{+}-Kanals, KCL-Ausstrom und Zellschrumpfung und Ca^{2+}-abhängige Aktivierung von Phospholipiden, charakterisiert ist. Die Aktivierung von Phospholipiden hat zur Folge, dass die Anordnung der Membranphospholipide zusammenbricht und Phosphatidylserine auf der Membranaußenseite präsentiert werden. Zusätzlich kommt es zu einer Ca^{2+}-abhängigen Aktivierung von Calpain, zum proteolytischem Abbau von Cytoskelettproteinen und zur Membranauflösung. Eryptose wird ausgelöst von verschiedenen wohlbekannten apoptotischen Stimuli, zu denen der hyperosmotische Schock, oxidativer Stress und Energiemangel gehören. Diese aktivieren jeweils einen unselektiven Ca^{2+}-permeablen Kationen-Kanal durch den Ca^{2+} in die Zelle einströmen kann. Eryptose ist beispielsweise verstärkt nachzuweisen bei verschiedenen angeborenen Erythrozytendefekten, Phosphatmangel, HUS (hämolytisch urämisches Syndrom), Sepsis und Morbus Wilson. Da Makrophagen mit speziellen Phosphatidylrezeptoren ausgestattet sind, können sie Zellen, welche Phosphatidylserin auf ihrer Oberfläche präsentieren, erkennen, aufnehmen, abbauen und auf diese Weise aus dem zirkulierenden Blut entfernen.

Es konnte gezeigt werden, dass eine Infektion mit *Plasmodium falciparum* Eryptose auslösen kann. Teilweise erfolgt dies durch die Aktivierung von Kanälen der infizierten Zelle durch Oxidation der Zellmembran. Beschleunigter Zelltod infizierter Erythrozyten geht einher mit einer Verzögerung der Entwicklung einer Parasitämie und schützt vor einem schweren Verlauf der Krankheit. Erythrozytenzelltod könnte durch die Induktion der Eryptose ausgelöst werden. Faktoren, die Eryptose verursachen, sind Hämolysin, PGE_2, PAF (Plättchen aktivierender Faktor), Schwermetalle wie Quecksilber und Blei ($Pb(NO_3)_2$), Eisenmangel, L-NAME oder Chlorpromazine. Die vorliegende

Studie wurde durchgeführt um erklären zu können, ob die Zugabe von L-NAME, $Pb(NO_3)_2$ oder Chlorpromazine zum Trinkwasser den Verlauf der Malaria und das Überleben von mit *Plasmodien berghei*-infizierten Mäusen beeinflusst und ob Eisenmangel die Schwere des Verlaufs der Krankheit beeinflussen kann.
Eisenmangel erhöhte tatsächlich die Phosphatidylserinexposition bei mit *P. falciparum* infizierte Erythrozyten. Der Effekt, der bei Eisenmangelerythrozyten gemessen werden konnte, war signifikant erhöht im Vergleich zu den Kontrollzellen. Darüber hinaus beeinträchtigt Eisenmangel in vitro intraerythrozytäres Wachstum und Infektion der Erythrozyten. In Mäusen werden Eisenmangelerythrozyten schneller aus dem zirkulierenden Blut entfernt, ein Effekt, der durch eine Infektion mit P. berghei noch verstärkt wird. Die Parasitämie bei mit *P. berghei* infizierten Mäusen nahm signifikant ab (von 54% auf 33% der zirkulierenden Erythrozyten, 20 Tage nach Infektion) und die Überlebenszeit der Eisenmangel-Mäuse war im Vergleich zur Kontrollgruppe signifikant verlängert (von 0% auf 20%, 30 Tage nach Infektion).

L-NAME ($\geq$ 10µM) erhöhte die Phosphatidylserinexposition von *P. falciparum* infizierter menschlicher Erythrozyten signifikant im Vergleich zu nichtinfizierten. Gleichzeitig war die Parasitämie in P. berghei infizierten Mäusen durch Zugabe von L-NAME (1mg/ml) zum Trinkwasser signifikant vermindert (von 50% auf 18% der zirkulierenden Erythrozyten 20 Tage nach Infektion). Dazu übereinstimmend zeigten Versuche mit CFSE-Markierung, dass L-NAME behandelte infizierte Erythrozyten schneller aus dem zirkulierenden Blut beseitigt werden als unbehandelte Erythrozyten.

$Pb(NO_3)_2$ ($\geq$ 10 µM) erhöhte im Vergleich zu nichtinfizierten Zellen die Phosphatidylserinexposition P. falciparum infizierter Erythrozyten signifikant. Des Weiteren konnten wir zeigen, dass mit $Pb(NO_3)_2$ behandelte Erythrozyten schneller aus dem peripheren Blut beseitigt werden als unbehandelte. Die Parasitämie bei *P. berghei* infizierten Mäusen wurde (von 50% auf 18% der zirkulierenden Erythrozyten, 20 Tage nach Infektion) durch den Zusatz von 100µM $Pb(NO_3)_2$ (20ppm) im Trinkwasser signifikant vermindert, ebenso verlängerte sich die Überlebenszeit der Mäuse (von 0% auf 38% 30 Tage nach Infektion) signifikant. Durch die Behandlung verringerten sich

Hämatokrit und Erythrozytenzahl in beiden Gruppen nicht signifikant, sie führte hauptsächlich zur Beseitigung der *P. berghei*-haltigen Erythrozyten.

Chlorpromazine (≥10 µM) erhöhte die Phosphatidylexposition während der in vitro Infektion menschlicher Erythrozyten und senkte den Forward Scatter. Chlorpromazine hatte keinen signifikanten Einfluss auf die intraerythrozytäre DNS/RNS-Vervielfältigung, erniedrigte jedoch die in vitro gemessene Parasitämie signifikant (≥5 µM). Die roten Blutkörperchen Chlorpromazine behandelter Mäuse wurden schneller aus dem zirkulierenden Blut beseitigt als die von unbehandelten. Die Parasitämie *P. berghei* infizierter Mäuse nahm signifikant ab (von 50% auf 28% der zirkulierenden Zellen 22 Tage nach Infektion) nach Zugabe von 1mM Chlorpromazine zum Trinkwasser vom ersten Tag an der Infektion.

Zusammengefasst erhöhen Eisenmangel, L-NAME, $Pb(NO_3)_2$ oder Chlorpromazine die Empfindlichkeit der Erythrozyten gegenüber der Eryptose. Wir kamen zu dem Ergebnis, dass Erythrozyten nach Infektion mit Plasmodien beschleunigt eryptotisch werden. Die forcierte Eryptose erschwert die komplette intraerythrozytäre Reifung des Erregers und verhindert so ein Ansteigen der Parasitämie. Die Beobachtungen unterstützen die Ansicht, beschleunigter selbstinduzierter Zelltod infizierter Erythrozyten könnte ein Mechanismus des Wirts sein, der Infektion mit dem intrazellulären Erreger entgegenzuwirken. Sie könnten nicht nur dazu dienen, die Abwehrmechanismen des Wirts gegen den Malariaerreger zu verstehen, sondern auch neue Perspektiven für die pharmakologische Behandlung dieser verheerenden Krankheit bieten.

I. INTRODUCTION

I. Malaria

Malaria is one of the most important diseases of the developing world. It claims more than one million deaths per year. The term malaria (from the Italian mala "bad" and aria "air") was used by the Italians to describe the cause of intermittent fevers associated with exposure to marsh air or miasma. About 40% of the world's population is at risk of malaria. It continues to be a huge social, economical and health problem, particularly in the tropical countries [Sachs and Malaney 2002]. The prevalence of malaria has caused global concern and eradication of malaria has become a tough challenge for the scientist's world wide. Malaria was wide spread over many regions of the world. The figure 1 shows the world wide distribution of malaria.

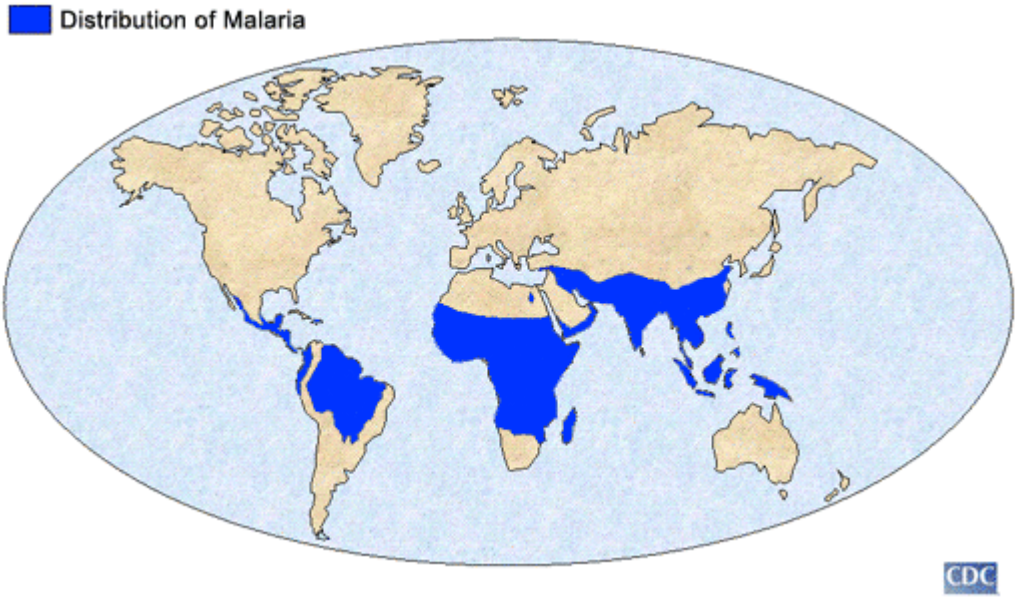

Fig. 1: World wide distribution of malaria 2005 (www.who.int)

I. 1. History, transmission and life cycle of the malaria parasite

I. 1. 1. History

Mentions of malaria can be found in the ancient Roman, Chinese, Indian and Egyptian manuscripts. Hippocrates was probably the first malariologist. By 400 B.C. he described the various malaria fevers of man [Grmek 1988]. In 1696, Morton presented the first detailed description of the clinical picture of malaria and its treatment with cinchona. Charles Louis Alphonse Laveran, a French physician identified the malaria parasite [Sherman 2005] and it was Sir Ronald Ross who showed that malaria is transmitted by mosquitoes and described the life cycle of malaria [Ross 1897 a,b].

I. 1. 2. Transmission of malaria

Malaria is caused by intracellular parasites of the genus *Plasmodium* that are spread from person to person through the bites of infected female *Anopheles* mosquito which is the vector for human malaria. Nearly 60 species of this mosquito have been identified as vectors for malaria, and their distribution varies depending upon the geographic regions. There are four identified species of the *Plasmodium* parasite causing human malaria namely, *Plasmodium vivax, P. falciparum, P. ovale* and *P. malariae*. *P. falciparum* is wide spread in Africa and is the most dangerous causing fatal cerebral malaria and resulting in high mortality. When a mosquito bites an infected individual, it aspirates the gametocytes, the sexual forms of the parasite, along with blood. These gametocytes continue the sexual phase of the cycle and develop into sporozoites that migrate through the salivary glands of the infested mosquito. When the female mosquito bites the man for a blood meal, it inoculates the sporozoites into human blood stream, thus spreading the infection.

I. 1. 3. Life cycle of *Plasmodium*

I. 1. 3. 1. Asexual Phase in Human host

Sporozoites are injected by the mosquito into the subcutaneous tissue and travel to the liver either directly or through lymphatic channels. They reach the liver in 30-40 minutes by brisk motility conferred by circum sporozoite protein (CSP). Approximately 8-15 sporozoites are injected and hence only a few hepatocytes are infected, therefore this stage of the infection causes no symptoms (rarely however, a prodromal illness characterised by vague aches and pains, headache, nausea etc. may be present). Recent

evidence indicates that sporozoites pass through several hepatocytes before invasion [Fujioka and Aikawa 2002]. The co-receptor on sporozoites for invasion involves, in part, the thrombospondin domains on the circum sporozoite protein and on thrombospondin-related adhesive protein (TRAP) [Robson et al., 1998]. These domains bind specifically to heparin sulfate proteoglycans on hepatocytes in the region in apposition to sinusoidal endothelium and kuppfer cells. Within the hepatocyte, each sporozoite divides into 10000-30000 merozoites. This stage is called exo-erythrocytic or pre-erythrocytic schizogony. At the completion of this phase, thousands of extra erythrocytic merozoites are released from each liver cell [Sherman 1998]. The time taken for the completion of the tissue phase is variable, depending on the infecting species (8 - 25 days for *P. falciparum,* 8 - 27 days for *P. vivax,* 9 - 17 days for *P. ovale,* and 15 - 30 days for *P. malariae*) and this interval is called as the pre-patent period. In case of *P. vivax* and *P. ovale*, some sporozoites may go into hibernation - the cryptobiotic phase in which they are called as hypnozoites. They can lie dormant for months or years and on reactivation they cause clinical relapse. At the completion of the pre-erythrocytic schizogony, the mature schizonts rupture the liver cells and escape into the blood, wherein they infect red blood cells. These infective forms are called merozoites and they continue their growth and multiplication within the red blood cells.

I. 1. 3. 2. The asexual phase in the mosquito

The merozoites released from the liver cells attach onto the red blood cell membrane and by a process of invagination, enter the red cell. Within the red blood cell, the asexual division starts and the parasites develop through the stages of rings, trophozoites, early schizonts and mature schizonts, each schizont develops into 8-32 new merozoites. These merozoites are released by the lysis of the red blood cell and immediately invade uninfected red cells. This repetitive cycle of invasion - multiplication - release - invasion continues. The growth and multiplication cycle within the RBCs (erythrocytic schizogony) takes about 48 hours for one cycle (72 hours in case of *P. malariae).* A small proportion of the merozoites in the red blood cells undergo transformation into male and female gametocytes. Mature gametocytes appear in the peripheral blood after a variable period and enter the mosquito when it bites an infected individual [Sinden and Smalley 1979].

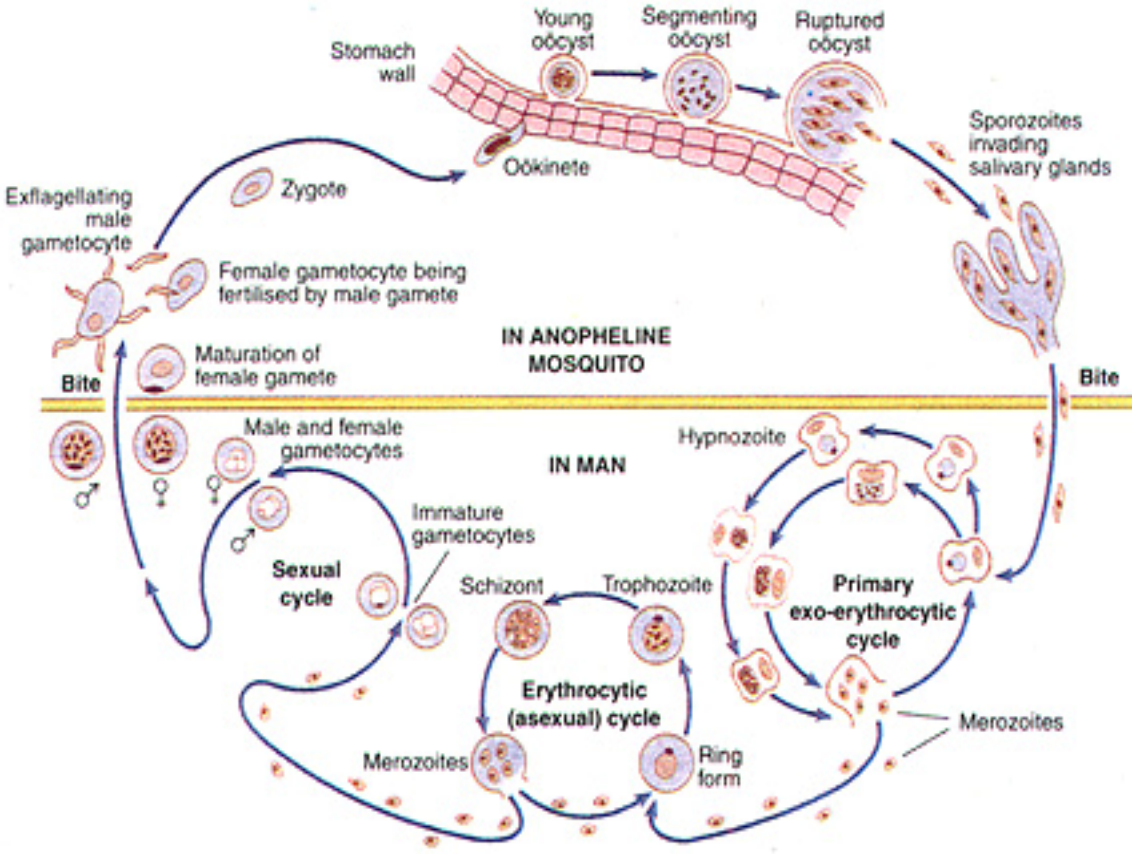

Fig. 2: Life cycle of *Plasmodium*

I. 1. 3. 3. The sexual phase in the mosquito: Sporogony

The gametocytes continue their development in the mosquito. The male and female gametes fuse and form a zygote. This transforms into an ookinete which penetrates the gut wall and becomes an oocyst. The oocyst divides asexually into numerous sporozoites which reach the salivary gland of the mosquito. On biting a man, these sporozoites are inoculated into human blood stream. The sporogony in the mosquito takes about 10 - 20 days depending on the species and thereafter the mosquito remains infective for 1 - 2 months. A diagrammatic representation of the life cycle of *Plasmodium* is shown in Fig. 2.

I. 2. Invasion into the erythrocyte

The sequence of invasion is probably similar for all *Plasmodium* spp. The merozoite first attaches to red blood cells. After the attachment to the red cell, the merozoite reorients itself so that apposition of apical end occurs. This is followed by localized invagination and interiorization of the merozoite. The entire process of invasion is completed in 30 seconds. In *P. falciparum*, erythrocyte binding antigen 175 and merozoite surface protein 1, 2 with sialoglycoproteins have been identified as the ligands [Soldati et al., 2004] and in *P. vivax*, duffy antigen on RBC is the site of binding.

After invasion into RBC the parasite depends on the host cell for its nutrition. The parasite has complex metabolic processes. It utilizes amino acids from hemoglobin and detoxifies heme. The toxic heme is in turn detoxified by heme polymerase and sequestrated as hemozoin (malaria pigment) [Egan et al., 2002]. It has been found that the parasites increase the permeability of RBC to get nutrients, yet maintain the RBC structure for 48 hours, allowing the immature parasite to survive. At the end, RBC ruptures and each schizont releases 6-36 merozoites

I. 3. Clinical manifestation and treatment of malaria

Malaria is a febrile illness characterised by fever and related symptoms. All the clinical features of malaria are caused by the erythrocytic schizogony in the blood. The growing parasite progressively consumes and degrades intracellular proteins, principally hemoglobin, resulting in formation of the 'malarial pigment' and hemolysis of the infected red cell. This also alters the transport properties of the red cell membrane, and the red cell becomes more spherical and less deformable. The rupture of red blood cells by merozoites releases certain factors and toxins (such as red cell membrane lipid, glycosyl phosphatidyl inositol anchor of a parasite membrane protein), which could directly induce the release of cytokines such as TNF and interleukin-1 from macrophages, resulting in chills and high grade fever [Sherman 1998]. This occurs corresponding to the erythrocytic cycle. Typical features include three stages viz. cold stage, hot stage and sweating stage. The febrile episode starts with shaking chills, that lasts from 15 minutes to 1 hour (the cold stage), followed by high grade fever, even reaching above 106^0 F, which lasts 2 to 6 hours (the hot stage). This is followed by profuse sweating and the fever gradually subsides over 2-4 hours. These typical features are seen after the infection gets established for about a week.

The febrile paroxysms are usually accompanied by head aches, vomiting, delirium, anxiety and restlessness. As the disease gets established, the patient starts getting relapse of symptoms at regular intervals of 48-72 hours. In *P. vivax* malaria, the young red blood cells are predominantly infected, while in *P. falciparum* malaria, red blood cells of all ages are affected. Thus the infective load and severity of infection are more in case of *P. falciparum* malaria.

I. 4. Erythrocyte cell death

I. 4. 1. Apoptosis of nucleated cells

Abundant, defective or potentially harmful nucleated cells are disposed by apoptosis or programmed cell death (PCD) [Bergamo et al., 2004; Brand et al., 2003; Green and Reed 1998; Long et al., 2003; Sturm et al., 2004; Wenzel and Daniel 2004] which is a biologically regulated process of self-destruction. Apoptotic cell death is distinguished from necrotic cell death in the morphological terms described below. Additionally, necrotic cell death is a passive process due to an acute cellular injury, where as apoptotic cell death is an active process that requires participation of the dying cell and changes in cellular biochemistry. Apoptosis allows the elimination of the cells without the release of intracellular materials into the extra cellular space and hence it usually does not cause inflammation [Gulbins et al., 2000] whereas necrosis leads to cell disintegration and the induction of unspecific and/or specific immune response. Apoptosis can be characterized by a series of stereotyped changes affecting the nucleus, cytoplasm and plasma membrane. It leads to the dismantling of the dying cell and to its rapid ingestion by macrophages or other neighbouring cells [Bratosin et al., 2001]. Hallmarks of apoptosis include nuclear condensation, DNA fragmentation, mitochondrial depolarization, cell shrinkage and breakdown of phosphatidylserine asymmetry of the plasma membrane [Green and Reed 1998, Gulbins et al., 2000]. In mammalian cells, there are two major apoptotic pathways that usually operate together and amplify each other. One involves the proteolytic activation of a family of aspartate-directed cysteine proteinases, the effector caspases. Another pathway involves mitochondrial outer membrane permeabilization. This permeabilization leads to the release of mitochondrial pro-apoptotic proteins into the cytosol. These proteins might either induce caspase activation, such as cytochrome *c* and Smac/Diablo, or might trigger caspase-independent effector pathways such as apoptosis-

inducing factor (AIF) [Bratosin et al., 2001]. Most, if not all, pro-apoptotic stimuli appear to require a mitochondrion-dependent step. Therefore, mitochondria have been proposed to play a central role in PCD [Bratosin et al., 2001, Green and Reed 1998].

I. 4. 2. Apoptosis of erythrocytes (Eryptosis)

Erythrocytes are devoid of nuclei and mitochondria and thus lack crucial elements in the machinery of apoptosis. However, similar to other cell types, erythrocytes have to be eliminated when they are defective or after their physiological life span of 120 days [Bratosin et al., 2001] in case of human beings. Due to the lack of key organelles involved in the process of apoptosis it was considered that erythrocytes are unable to undergo apoptosis and hence have to be eliminated by mechanisms other than apoptosis. It has been observed that erythrocyte senescence is associated with cell shrinkage, plasma membrane microvesiculation, a progressive shape change from a discocyte to a spherocyte, cytoskeleton alterations associated with protein (spectrin) degradation, and loss of plasma membrane phospholipid asymmetry leading to the externalization of phosphatidylserine in the erythrocyte membrane [Bratosin et al., 2001; Lang et al., 2005c]. The exposure of phosphatidylserine and further eat-me-signals at the cell surface triggers, and the decrease of cell volume facilitates, the engulfment of the dying cells by phagocytes [Boas et al., 1998; Eda and Sherman 2002]. *In vitro* storage of erythrocytes leads to the gradual accumulation of these modifications, and *ex vivo*, a very small subpopulation of human erythrocytes with a senescent phenotype can be isolated from the peripheral blood [Boas et al., 1998]. These modifications associated with erythrocyte senescence share striking similarities with some cytoplasmic features of apoptosis in nucleated cells. Recent observations have shown that PCD is not limited to nucleated cells but could similarly affect erythrocytes [Barvitenko et al., 2005; Bosman and Willekens 2005; Rice and Alfrey 2005]. Human erythrocytes activate non-selective cation (NSC) channels upon various stimuli which induce apoptosis-like death of mature erythrocytes. Hence, the term “eryptosis” was coined recently (Lang et al., 2005c) to describe erythrocyte cell death characterised by cell shrinkage and breakdown of phospatidyl symmetry, both typical features of apoptosis in nucleated cells [Lang et al., 2003 a,b,c,d].

I. 4. 3. Mechanisms of erythrocyte cell death or eryptosis

There are two major mechanisms of erpytosis that have been illustrated so far. There are calcium and lipid (ceramide)-mediated mechanisms. The role of cation channels have been identified in eryptosis. Usually erythrocyte cell membranes are tight and show little channel activity. Moreover, the erythrocytes are predominantly permeable to Cl^- [Bernhardt and Ellory 2003]. Osmotic cell shrinkage, however, activates non-selective cation channels in the erythrocyte cell membrane [Huber et al., 2001]. The same channels are activated by oxidative stress [Duranton et al., 2002]. Energy depletion impairs the replenishment of GSH and thus weakens the antioxidative defense of the erythrocytes [Bilmen et al., 2001; Mavelli et al 1984]. Accordingly, energy depletion similarly activates the cation channels [Lang et al 2002b]. The channels are not only permeable to monovalent cations but as well to Ca^{2+} [Duranton et al., 2002; Kaestner et al., 2000; Lang et al., 2003a]. Accordingly, exposure to osmotic shock or oxidative stress triggers erythrocyte Ca^{2+} uptake [Lang et al., 2003c]. Erythrocyte "apoptosis" can be induced by increase of intracellular [Ca^{2+}], e.g. by exposition of erythrocytes to the ionophore ionomycin. This fact suggests that the cation channel play an important role in erythrocyte "apoptosis" [Lang et al.,2003a].

The influx of intracellular Ca^{2+} stimulates the erythrocyte scramblase [Zhou et al., 2002], thus leading to the breakdown of phosphatidylserine asymmetry [Lang et al., 2003a]. The phosphatidylserine exposure following osmotic shock is blunted by amiloride [Lang et al., 2003d] and ethylisopropylamiloride (EIPA) [Lang et al., 2003d], inhibitors of the cation channel [Huber et al., 2001, Lang et al., 2003d]. Thus, it appears that activation of the cell volume and oxidant sensitive cation channel and subsequent Ca^{2+} entry contribute to the stimulation of erythrocyte scramblase following osmotic shock or oxidative stress. Intriguing evidence points to the role of prostaglandins in the regulation of eryptosis. Hyperosmotic shock and Cl^- removal trigger the release of prostaglandin E_2 (PGE_2) [Lang et al., 2005d]. PGE_2 in turn activates the cation channels [Lang et al., 2005d], increases the cytosolic Ca^{2+} concentration [Lang et al., 2005d] and stimulates phosphatidylserine exposure at the erythrocyte surface [Lang et al., 2005b]. The activation of the cation channels by Cl^- removal is abolished by the cyclooxygenase inhibitor diclophenac [Lang et al., 2005d]. Moreover, phospholipase-A_2 inhibitors quinacrine and

palmitoyl trifluoromethyl ketone and cyclooxygenase inhibitors acetylsalicylic acid and diclophenac blunt the increase of phosphatidylserine exposure following Cl^- removal [Lang et al., 2005d]. PGE_2 further activates the Ca^{2+} dependent cysteine endopeptidase calpain, an effect, however, apparently not required for stimulation of phosphatidylserine exposure [Lang et al., 2005d]. Ca^{2+} entering erythrocyte does not only activate the scramblase but in addition stimulates the Ca^{2+} sensitive "Gardos" K^+ channels in erythrocytes [Bookchin et al., 1987; Franco et al., 1996]. The activation of the channels leads to hyperpolarization of the cell membrane driving Cl^- in parallel to K^+ out of the cell. The cellular loss of KCl and osmotically obliged water results in cell shrinkage. Moreover, the cellular loss of K^+ presumably participates in the triggering of "eryptosis" [Lang et al., 2003b]. Increase of extra cellular K^+ or pharmacological inhibition of the Gardos channels by clotrimazole or charybdotoxin do not only blunt the cell shrinkage but also decrease the phosphatidylserine exposure following exposure to ionomycin [Lang et al., 2003b,c]. Presumably, cellular loss of K^+ somehow stimulates eryptosis as has been shown for apoptosis of nucleated cells [Bortner et al., 1997; Bortner et al., 1999]. As PGE_2 increases cytosolic Ca^{2+} activity [Lang et al., 2005d], it similarly activates the Ca^{2+} sensitive "Gardos" K^+ channels with subsequent cell shrinkage [Allen and Rasmussen 1971; Li et al., 1996]. The effect of osmotic shock on phosphatidylserine exposure is not fully blocked in the absence of calcium [Lang et al., 2003d], pointing to the participation of a mechanism other than nonselective cation and Ca^{2+} sensitive K^+ channels.

Further experiments revealed that C_6-ceramide as well as treatment with bacterial sphingomyelinase triggers eryptosis [Lang et al., 2003d]. Osmotic shock leads to the appearance of ceramide at the erythrocyte surface [Lang et al., 2004b]. Moreover, eryptosis induced by osmotic shock can be inhibited by the sphingomyelinase inhibitor 3,4-dichloroisocoumarin. Ceramides have been reported to form large channels in the outer mitochondrial membrane allowing the release of inter membrane space proteins with a molecular weight cut-off of about 60,000 [Siskind et al., 2002]. However, it was shown here that C_6-ceramide does not enhance Ca^{2+} uptake of erythrocytes. A simple channel-based mechanism of the ceramide effect is therefore rather unlikely. Moreover, the ceramide induced eryptosis is blunted but not abolished in the nominal absence of Ca^{2+}. Thus, C_6-ceramide induced cell death is probably not secondary to increase of cytosolic

Ca^{2+} activity. Instead, the effect of C_6-ceramide adds to or even potentiates the effects of Ca^{2+} entry on phosphatidylserine exposure. This novel activity of ceramide may be due to ceramide-mediated induction of trans bilayer lipid movement as previously demonstrated in large unilamellar vesicles and in erythrocyte ghost membranes [Contreras et al., 2003]. Accordingly, in the presence of C_6-ceramide eryptosis following osmotic shock is accelerated.

Cell shrinkage leads to release of platelet activating factor (PAF), a phospholipid mediator involved in the regulation of inflammation, thrombosis, atherogenesis and cardiovascular function [Goggel et al., 2004; Haynes and Obiako 2002; Montrucchio et al., 1993; Subbanagounder et al., 2002; Zimmerman et al., 2002]. PAF in turn stimulates the breakdown of sphingomyelin and release of ceramide from erythrocytes [Lang et al., 2005b]. PAF further triggers cell shrinkage (decrease of forward scatter) and phosphatidylserine exposure (annexin binding) of erythrocytes. The stimulation of phosphatidylserine exposure is blunted by genetic knockout of PAF receptors (PAF receptor knockout mice), and by the PAF receptor antagonist ABT491 [Lang et al., 2005b]. Thus, PAF participates in the stimulation of sphingomyelinase activation and "eryptosis". PAF further activates Ca^{2+} sensitive K^+ channels (Gardos channels) in the erythrocyte cell membrane [Garay and Braquet 1986] by sensitizing them for the stimulating effects of cytosolic Ca^{2+} [Rivera et al., 2002]. Interestingly, PAF is released from erythrocyte progenitor cells upon increase of cytosolic Ca^{2+} activity [Dupuis et al., 1997].

As summarized in Fig. 3, cellular stress, e.g. osmotic shock, oxidative stress or energy depletion, activate a Ca^{2+} permeable cation channel in the erythrocyte cell membrane presumably via generation of PGE_2. The subsequent entry of Ca^{2+} leads to activation of a Ca^{2+} sensitive scramblase which exposes phosphatidylserine at the surface of the erythrocyte cell membrane. Moreover, activation of the Ca^{2+} sensitive Gardos K^+ channels counteracts cell swelling and prevents hemolysis. Eryptosis is further stimulated by ceramide which is formed in shrunken erythrocytes by PAF-mediated activation of sphingomyelinase. Those mechanisms serve to safely eliminate defective, leaky erythrocytes before they rupture and thus release cytosolic components including hemoglobin. Similar mechanisms may be operative in nucleated cells where they may be

hidden by the more complex apoptotic machinery. Thus, eryptosis may prove to be a valuable model system to analyze mechanisms which are similarly important for the apoptosis of nucleated cells.

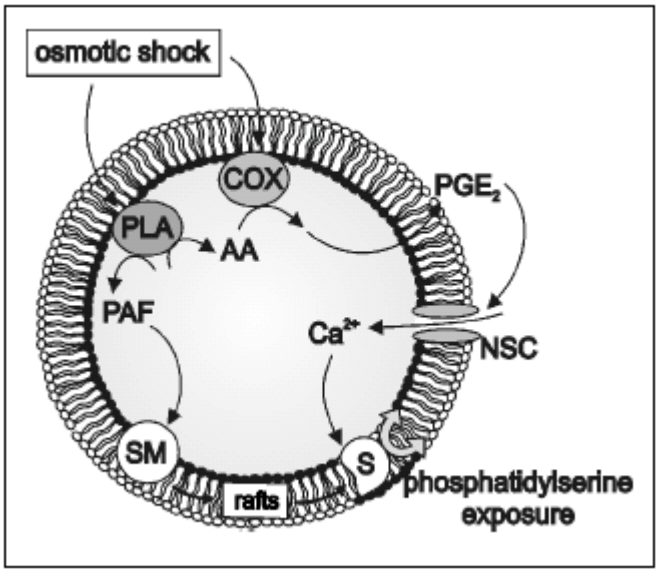

Fig. 3: Mechanisms involved in eryptosis. Note that there are Ca^{2+}- and lipid- (ceramide)-mediated mechanisms. AA: arachidonic acid; COX: cyclooxygenase; NSC: nonselective cation channel; PAF, platelet activating factor; PGE_2: prostaglandin E_2; PLA, phospholipase A_2; S, scramblase; SM, sphingomyelinase.

I. 4. 4. Physiological significance of eryptosis

The mechanisms described here could well participate in the limitation of erythrocyte survival. Erythrocyte ageing is paralleled by increase of cytosolic Ca^{2+} activity [Kiefer and Snyder 2000, Romero and Romero 1999]. Moreover, oxidative stress or defects of antioxidative defense [Damonte et al., 1992] would enhance Ca^{2+} entry via the cation channels and thus accelerate erythrocyte death and clearance from circulating blood. During their daily life, erythrocytes are exposed to several stress situations. In average they pass once a minute in to the lung where they are exposed to oxidative stress. More than once an hour they travel through kidney medulla where they face osmotic shock. Erythrocytes have to squeeze through capillaries which are smaller than themselves. Thus, the integrity of erythrocytes is constantly challenged. Rupture of erythrocyte cell

membranes releases hemoglobin to extracellular fluid which may be filtered at the glomerula of the kidney, precipitate in the acid lumen of the tubules, obliterate the tubules and thus lead to renal failure. To avoid those complications, erythrocytes, as any other cell, require a mechanism allowing them to be disposed without release of intracellular components [Lang et al., 2005c]. Several disorders may decrease the life span of mature erythrocytes by facilitating erythrocyte "apoptosis". As a matter of fact, the sensitivity of sickle cells and of glucose-6- phosphate dehydrogenase deficient cells to osmotic shock and of sickle cells, thalassemic cells and glucose-6- phosphate dehydrogenase deficient cells to oxidative stress and to glucose depletion was significantly higher than that of control cells [Lang et al., 2002b: Lang et al., 2005c]. This enhanced susceptibility most likely contributes to the decrease of erythrocyte life span in those genetic disorders.

I. 4. 5. *Plasmodium* infected erythrocytes

Human erythrocytes maintain cell integrity, stability despite the effect of shear forces of blood circulation. This maintenance is achieved through a set of transporters and channels which include Band 3 protein, which enhances the blood CO_2 – carrying capacity and assists in acid base homeostasis [Cabantchick 1999; Gunn et al., 1973; LaCelle and Rothsteto 1966], membrane transporters of glucose, nucleoside and purine for fuelling the Na/K and Ca pumps via ATP production and of aminoacid and oxidized glutathione transport for maintaining the cell redox status [Brugnara 1997]. Before infection, the substrate turnover of erythrocytes is low [Kirk 2001]. Since they do not synthesize proteins, DNA or membranes, they have no need for amino acids, nucleic acids, lipids or vitamins etc. A prerequisite for intracellular survival of pathogens is an adequate supply of nutrients and disposal of waste products [Gulbins and Lang 2001]. Additional transport systems for nutrient supply and disposal of waste products are needed particularly during infection of erythrocytes with the malaria pathogen *Plasmodium* sp. [Kirk 2001]. The replicating *Plasmodium*, however, has extensive requirements for all the nutrients [Kirk 2001] and, to fuel the replication process, *Plasmodium* parasites consume extensive amounts of glucose. An infected erythrocyte takes up 40–100 times more glucose than a noninfected cell [Kirk 2001] and releases the corresponding amounts of lactic acid [Poole and Halestrap 1993]. To gain access to the necessary nutrients and to dispose of waste products, *Plasmodium* permeabilizes host-erythrocytes for a variety of solutes [Ginsburg et

al., 1983; Kutner et al., 1982], including organic and inorganic anions [Ginsburg and Kirk 1998; Kirk et al., 1994; Staines et al., 2001], cations [Kirk and Horner, 1995; Staines et al., 2001; Duranton et al., 2003], carbohydrates, amino acids, nucleosides [Ginsburg et al., 1985; Sherman 1979] and small peptides [Ginsburg et al., 1985; El Tahir et al., 2003; Lew 2004]. A parallel increase of the host erythrocyte membrane permeability and of the intraerythrocytic parasite metabolic and biosynthetic activity (from 12-16 h post-invasion onwards) has been observed. The *in vitro* growth of *P. falciparum* in culture indeed requires the presence in the culture medium of a hexose sugar, a purine source [Sherman 1979; Sherman and Greenan 1984], seven amino acids (cysteine, glutamate, glutamine, isoleucine, methionine, proline and tyrosine) [Polet and Conrad 1968] and panthotenate [Divo et al., 1985].

I. 4. 6. Eryptosis in malaria

Erythrocyte "apoptosis" may be relevant for the intraerythrocytic survival of the malaria pathogen *Plasmodium*. The parasite invades erythrocytes to escape the immune system. However, transport across the intact erythrocyte cell membrane is not sufficient to meet the excessive demands of the pathogen. Thus, *Plasmodium* induces novel permeability pathways (NPP) allowing the uptake of nutrients and the disposal of waste products [Kirk 2001]. Most recent experiments revealed that NPP is made up of endogenous host cell channels which are activated by the pathogen through oxidation of the cell membrane [Duranton et al., 2003; Huber et al., 2002]. Activation of the cation channel is required for the cellular accumulation of Na^+ and Ca^{2+} which are both needed by the pathogen. By the same token, however, the activation of the cation channel triggers erythrocyte "apoptosis" [Brand et al., 2003]. Presently, it is not entirely clear whether phosphatidylserine exposure of infected host cells is favourable for the host or the pathogen. In other model systems, host cell apoptosis has proven to be a crucial defence mechanism of the host [Grassme et al., 2000]. In any case, erythrocyte "apoptosis" favours the recognition of the erythrocytes by macrophages and thus limits the life span of the infected cell.

I. 5. Rodent malaria parasites as models for human malaria

A wide range of investigations using rodent parasites have provided knowledge for developing and shaping concepts in major areas of research on the human disease. The interest of rodent malaria parasites is that they are practical models for the experimental study of mammalian malaria. These parasites have proved to be analogous to the malaria of man and other primates in most essential aspects of structure, physiology and life cycle [Carter and Diggs 1977]. Metabolic pathways are conserved between rodent and human malaria parasites. No gross differences in metabolic pathways between mammalian malaria parasites have been reported [Homewood and Neame 1980]. The similarity in sensitivity of mammalian malaria parasites to anti-malarial drugs and other specific inhibitors emphasises the similarities in their metabolic processes. The life cycles and the different developmental stages of all rodent and human malaria parasites are highly comparable [Aikawa and Seed 1980; Sinden 1978]. Rodent parasites are recognised as valuable model parasites for the investigation of the developmental biology of malaria parasites, parasite-host interactions, vaccine development and drug testing. The Relationship of rodent parasites to other mammalian malaria parasites in brief are:

- The basic biology of rodent and human parasites is similar.
- The genome organisation and genetics is conserved between rodent and human parasites. [Lin et al., 2004]
- Housekeeping genes and biochemical processes are conserved between rodent and human parasites.
- Methodologies for genetic modification are available.
- Rodent hosts with extensively characterised genetic backgrounds and transgenic lines are valuable and available tools for immunological studies.
- The structure and function of vaccine candidate target antigens are conserved between rodent and human parasites (for example TRAP and CSP of sporozoites; CTRP, P25 and P28 of ookinetes; AMA1 and MSP1 of merozoites; P45/48, P47 and P230 of gametes).
- The manipulation of the complete lifecycle of rodent parasites, including mosquito infections is simple and safe.

- *In vitro* culture techniques for large-scale production and manipulation of different life cycle stages are available. For example, *in vitro* cultures of liver and mosquito stages provide tools to investigate the less accessible parts of the life cycle of the human
- The molecular basis of drug-sensitivity and resistance show similar characteristics in rodent and human parasites.
- Rodent parasites allow *in vivo* investigations of parasite-host interactions and *in vivo* drug testing.

P. berghei is one of the many species of malaria parasites that infect mammals other than humans. A susceptible mosquito vector for *P.berghei*, which is widely used in the laboratory, is *Anopheles stephensi*. *P. berghei* infects laboratory hamsters, rats and mice. In our studies we have used *P. berghei* as our *in vivo* rodent model of malaria.

I. 6. Pathophysiological and pharmacological modification of eryptosis

Malaria is one of the most devastating diseases with lethal outcome in more than 1 million humans per year [Planche and Krishna 2005]. Targeting the pathogen with antimalarial drugs has only been partially successful because of the spread of drug-resistant parasites and the optimal use of effective drugs has always been a major concern. Hence all possibilities to combat this disease must be explored. The course of the disease is not only a function of the pathogen but is heavily influenced by properties of the host. Hence, studies must be carried out to fight infection by altering host physiology which decimates the problem of drug resistance and if successful, these approaches may prove extremely useful, particularly in the treatment of resistent pathogens. Targeting at the accelerated clearance of the infected host cells is expected to provide protection against malaria. Hence, *in vivo* modulation of eryptosis by therapeutic interventions has to be further elucidated.

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I. 6. 1. Influence of iron deficiency on the course of malaria in *P. berghei* infected mice

Iron deficiency is a common disorder leading to the development of anemia in 500 to 600 million people worldwide [Bothwell 1995; Oppenheimer 2001; Planche and Krishna 2005]. The lack of iron impedes hemoglobin synthesis and the formation of erythrocytes with adequate hemoglobin content. Thus, it is generally accepted that iron deficiency-

induced anemia results at least in part from reduced erythropoiesis [Lang et al., 2004b; Spivak 2002].

Most recent observations disclosed that eryptosis is enhanced by iron deficiency [Kempe et al., 2006]. The anemia of iron deficiency is not only the result of reduced erythropoiesis [Arndt et al., 2005; Brugnara 2003; Rivera et al., 2005; Thomas and Thomas 2005] but is at least partially due to decreased life span of iron deficient erythrocytes [Ramachandran and Iyer 1984]. Infection of erythrocytes with *P. falciparum* has been shown to trigger eryptosis [Brand et al., 2003], at least in part due to activation of host cell channels via oxidation of the cell membrane [Duranton et al., 2003; Huber et al., 2002; Tanneur et al., 2006]. Mechanisms explaining the accelerated clearance of iron-deficient erythrocytes are poorly understood, and it was hitherto assumed that erythrocytes undergo extravascular hemolysis, i.e., necrotic cell death. Compelling evidence indicates that the decreased life span of iron deficient erythrocytes is the result of premature eryptosis [Kempe et al., 2006]. Thus, we hypothesised that the protection against a severe course of malaria may similarly be related to accelerated eryptosis. To test this hypothesis, the course of malaria and eryptosis of infected and noninfected erythrocytes were studied in iron deficient and iron repleted mice following infection with *P. berghei.*

I. 6. 2. Impact of NO synthase inhibitior L-NAME on the course of malaria

The pleotropic effects of nitric oxide include the regulation of cell survival or apoptosis [Dimmeler et al., 1997; Dimmeler and Zeiher 1999]. Depending on the source or concentration of NO and on the influence of additional regulators NO may stimulate or inhibit apoptosis [Boas et al., 1998; Brune 2003; Haendeler et al., 1999; Taylor et al., 2003]. NO is partially effective through S-nitrosylation of target proteins [Dimmeler et al., 1997]. Moreover, effects of NO donors on Ca^{2+}-induced phosphatidylserine exposure could be mimicked by cGMP, pointing to a role of protein kinase G [Das et al., 2006; Li and Billiar 1999; Nagai-Kusuhara et al., 2007]. Intravascular formation of NO may be modified by erythrocytes [Crawford et al., 2006; Dejam et al., 2005; Gladwin et al., 2004; Grubina et al., 2007; McMahon et al., 2002; Mehta et al., 2000], which may take up both, NO and nitrite (NO_2^-) [Dejam et al., 2005]. Oxyhemoglobin may react with NO to form

nitrate (NO_3^-), desoxygenized hemoglobin may react with nitrite to form NO [Cosby et al., 2003; Dejam et al., 2005; Nagababu et al., 2003; Rice and Alfrey 2005; Yang et al., 1996].

Most recent experiments confirmed that similar to apoptosis of nucleated cells [Dimmeler et al., 1997; Dimmeler et al., 2002; Dimmeler and Zeiher 1999; Gotoh et al., 2004; Haendeler et al., 1999; Hoffmann et al., 2001a; Kim et al., 2005; Li and Billiar 1999; Liu and Stamler 1999; Taylor et al., 2003; Traister et al., 2004; Wang et al., 2003], erythrocyte cell death is subject to regulation by nitric oxide (NO) [Nicolay et al., 2007]. *In vitro* experiments disclosed that NO is a potent inhibitor of eryptosis [Nicolay et al., 2007]. *In vivo* experiments indicated that endogeneous formation of NO is required for normal erythrocyte life span [Nicolay et al., 2007].

Accelerated eryptosis may confer some protection against a severe course of malaria [Lang et al., 2004c]. Accelerated aging or eryptosis of *Plasmodium* infected erythrocytes with sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C or glucose-6-phosphate dehydrogenase (G6PD)-deficiency is followed by premature clearance of infected erythrocytes thus blunting the increase of parasitemia [Ayi et al., 2004; Cappadoro et al., 1998; de Jong et al., 2001; Kean et al., 2002; Kuypers et al., 1998; Lang et al., 2002b].

The present study has therefore been performed to explore whether inhibition of NO synthase acclerates the clearance of circulating erythrocytes during malaria. L-NAME (*Nω*-Nitro-L-arginine methyl ester hydrochloride) which is a non specific NO synthase inhibitor was chosen and the mice treated with L-NAME were infected with the malaria pathogen *P. berghei* to explore whether inhibition of NO synthase would indeed influence the course of malaria.

I. 6. 3. Influence of lead treatment on *P. berghei* infected mice

Pb^+ ions adhere to erythrocyte cell membranes [Suwalsky et al., 2003], decrease the erythrocyte ATP concentration [Baranowska-Bosiacka and Hlynczak 2003; Grabowska and Guminska 1996], delay the decline of protoporphyrin concentration in mature erythrocytes [Lamola et al., 1975; Piomelli et al., 1975], and decrease 5'-nucleotidase activity [Valentine et al., 1976]. Pb^+ ions have been demonstrated to directly activate Ca^{2+}-

sensitive Gardos K^+ channels in human erythrocytes [Simons 1985] and similar Ca^{2+}-sensitive K^+ channels in other cell types [Cao and Houamed 1999; Nishizaki 2003].

Recent experiments revealed the ability of lead to accelerate eryptosis [Kempe et al., 2005]. Sequelae of lead intoxication include anemia that may be partially due to a shortened life span of circulating erythrocytes [Osterode et al., 1999]. However, this premature elimination of circulating erythrocytes remains poorly understood.

Therefore, the present experiments were performed to explore whether the treatment with lead influences *Plasmodium* infected erythrocytes. Thus, *in vitro* experiments have been performed in human erythrocytes and *in vivo* experiments in mice to explore whether lead favours phosphatidylserine exposure of infected erythrocytes, accelerates the clearance of circulating erythocytes and thus influences the course of malaria.

I. 6. 4. Influence of chlorpromazine on the course of malaria

Chlorpromazine has been recently shown to trigger eryptosis [Akel et al., 2006], the suicidal erythrocyte death characterized by cell shrinkage and by exposure of phosphatidylserine at the cell surface [Berg et al., 2001; Brand et al., 2003; Bratosin et al., 2001; Daugas et al., 2001; Lang et al., 2002a]. As macrophages are equipped with receptors specific for phosphatidylserine [Fadok et al., 2000; Henson et al., 2001], erythrocytes exposing phosphatidylserine at their surface are rapidly recognized, engulfed and degraded [Boas et al., 1998; Yamanaka et al., 2005].

Side effects of treatment with chlorpromazine include anaemia which could result from decreased formation or accelerated clearance of circulating erythrocytes. Accelerated eryptosis has been suggested to confer some protection against a severe course of malaria [Lang et al., 2004c]. Chlorpromazine has indeed been shown to be effective in the treatment of malaria [Basco and Le Bras 1992; Kalkanidis et al., 2004; Loria et al., 1999]. However, it is poorly understood whether the acclerated clearance of erythrocytes plays a role in the antimalarial effect of chlorpromazine. The acclerated clearance of erythrocytes could well contribute to the known anaemia observed in the treatment with this antipsychotic drug.

The present experiments have been performed to explore whether chlorpromazine triggers phosphatidylserine exposure of infected erythrocytes causing the acclerated clearance thus leading to decreased life span of circulating erythrocytes and thus influences the clinical course of malaria.

II. AIM OF THE STUDY

Human erythrocytes are subjected to eryptosis which is an apoptosis-like cell death characterised by cell shrinkage and breakdown of phospatidyl symmetry. Eryptosis with subsequent phagocytosis leads to the degradation and elimination of defective cells. Eryptosis may as well result in the elimination of intracellular pathogens and may serve as host defense mechanism. Accelerated eryptosis of *Plasmodium*-infected RBCs may lead to rapid *in vivo* clearance of infected erythrocytes and may confer protection against severe course of malaria. Hence, our present study was aimed to address the following questions.

1. What is the functional significance of suicidal death of erythrocytes (eryptosis) in malaria?

2. Does phosphatidylserine exposure play a significant role in the erythrocyte clearance and elimination of infected erythrocytes?

3. Does eryptosis induction by pharmacological interventions effect the *in vitro* amplification and growth of the parasite?

4. Does the iron deficiency, L-Name, lead or chlorpromazine modulate eryptosis of infected cells and influence the course of malaria in *P. berghei* infected mice?

III. METHODS

III. 1. Animals: Animal experiments were performed according to German animal protection law and approved by the local authorities. In the study involving iron deficiency, C57BL/6 mice (female aged 4 months) were fed either on control diet (iron content: 180 mg/kg diet; protein content: 17 %; and fat content: 5 %; Altromin C1000, Altromin, Lage, Germany) or an iron deficient diet (iron content: < 10 mg/kg diet; otherwise identical to control diet; Altromin C1038) for 10 weeks. In all the other studies the mice were fed on control diet (1314, Altromin, Heidenau, Germany). The control mice had free access to tap water while the treated groups received L-NAME (1 mg/ml) or lead in the form of 100 μM $Pb(NO_3)_2$ in drinking water from the 10th day of infection. The chlorpromazine treated group received chlorpromazine (1mM) in drinking water from the 1st day of infection.

The following methods were used in our study where ever applicable.

III. 2. Preparation of human and mouse erythrocytes: Human erythrocytes were drawn from healthy volunteers. They were either used without purification or after separation by centrifugation for 25 min; 2000 g over Ficoll (Biochrom KG, Berlin, Germany). Experiments with non-purified or Ficoll-separated erythrocytes yielded the same results. After washing, the buffy coat and upper 10-20% of the red blood cells were discarded and the remaining pellet was used for experiments. The RBCs were then resuspended at 10% hematocrit and stored at 4°C until use (2-5 days). Experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 $CaCl_2$. For the study involving iron deficiency, erythrocyte concentrates were obtained by centrifugation/filtration procedures from the blood of healthy volunteers and patients with iron deficiency. Patients and healthy donors gave their informed consent and the study was approved by the Ethic Committee of the University of Tübingen (project number: 184/2003V). Erythrocytes were washed three times in Ringer solution, stored in SAG mannitol (Haemonetics, Munich, Germany) at 4°C, and washed twice in Ringer solution before use. For incubation, the final hematocrit was adjusted to 0.3 %. Mouse erythrocytes were drawn from the animals by retroorbital venopuncture. Experiments on mouse

erythrocytes were performed at 37°C in Ringer solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 mM Glucose, and 1 mM $CaCl_2$, pH 7.4.

III. 3. *In vitro* culture of *plasmodium falcifarum* infected human erythrocytes: For infection of human erythrocytes, the human pathogen *P. falciparum* strain BinH [Binh et al., 1997] was grown *in vitro* [Huber et al., 2002] in human erythrocytes. Cultures were mainained continuously by routine passage in fresh and stored human erythrocytes. Parasites were cultured as described earlier [Jensen and Trager 1978; Trager and Jensen 1976] at a hematocrit of 2-5% and a parasitemia of 2-10% in RPMI 1640 medium supplemented with 0.5% Albumax II (Gibco, Karlsruhe, Germany), 0.13 µM hypoxanthine, 2 mM L-glutamine (Gibco, Karlsruhe, Germany), 25 mM HEPES/NaOH pH 7.4 (Sigma-Aldrich, Schnelldorf, Germany), 20 µg/ml gentamycin (Gibco, Karlsruhe, Germany) in an atmosphere of 90 % N_2, 5 % CO_2, 5 % O_2.
The dilution factors (Df) for splitting the infected RBCs suspension in order to maintain the *P. falciparum in vitro* culture were calculated as follows:

$$Df = mP * V(RBC) / dP * dV(RBC)$$

Fresh blood was added according to the following formula:

$$dV(RBC) - (V(RBC)\ )/\ Df$$

mP: measured parasitemia; V(RBC): volume of packed erythrocytes in the culture flask; dP:desired parasitemia; dV(RBC): desired volume of packed erythrocytes in the flask. The volume of the medium was adjusted according to the degree of parasitemia and the time until the next medium change [Schlichtherle et al., 2000].

III. 4. Freezing and defreezing of parasites: From time to time samples with high, *i.e.* ≥ 20 % parasitemia, predominantly containing ring stages were deep frozen in liquid nitrogen for stock purposes. The cell pellet obtained after centrifugation (450g / 8 min at RT) was mixed with an equal volume of freezing solution, sterile transferred to a 2.0 ml cryotube vial and directly deep frozen in liquid nitrogen. For defreezing the parasites four cryotubes containing infected RBCs (> 20 % parasitemia) were thawed quickly at 37°C in a water bath. The contents were transferred to a 15 ml falcon tube and centrifuged (450g / 4 min at RT). The supernatant was discarded. A volume of sterile filtered 3.5 % NaCl

solution equal to $V_{(RBC)}$ was added at a rate of 1 - 2 drops per second while gently shaking the tube. Finally the liquid was mixed with a pipette. After centrifugation at 450g for 4 min the supernatant was discarded. The RBC pellet was carefully suspended in original RPMI 1640 medium ($2*V_{(RBC)}$). Washing was repeated until the supernatant became clear. The remaining cell pellet was resuspended in complete medium and transferred to a culture flask. Fresh RBCs were added (amount depending on $V_{(RBC)}$). The culture flask was filled with 90% N_2 /5% O_2 / 5% CO_2.

III. 5. Isoosmotic sorbital synchronization of *P. falciparum* infected human erythrocytes: The *P. falciparum* BinH strain was cultured and synchronized to the ring stage by sorbitol treatment as described previously [Binh et al. 1997]. Briefly, the infected RBCs (>5% parasitemia) were spinned down at 600×*g* and resuspended in isosmotic sorbitol solution (in mM: 290 sorbitol, 5 glucose, 5 HEPES/NaOH, pH 7.4) for 20 min at 21°C in continous shaking. Then the cells were washed twice in malaria culture medium and subculutred for further experiments.

III. 6. *In vitro P. falciparum* growth assay: For the *in vitro* growth assay, synchronized parasitized erythrocytes were aliquoted in 96-well plates (200 µl aliquots,0.5- 1% hematocrit, 0.5 –2 % parasitemia) and grown for 48 h in serum-free Albumax II (0.5%)-supplemented RPMI medium as described earlier [Tanneur et al., 2006]. The erythrocytes were grown in the presence or absence of (0.1 µM- 100 µM) L-NAME or Pb^{2+} ions or chlorpromazine. The parasitemia was assessed at time 0 and after 48 h of culture by flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg, Germany). Parasitemia was defined by the percentage of erythrocytes stained with the DNA/RNA specific fluorescence dye Syto16 (20 nM final concentration, Molecular Probes, Göttingen, Germany). Briefly, RBCs were incubated with Syto16, diluted in PBS or annexin binding buffer at 37°C. The staining procedure was performed for around 30 – 40 min for infected human RBCs. Syto16 green fluorescent nucleic acid stain bound to DNA has a maximum excitation/absorption wavelength of 488 nm, which corresponds to the argon line of the single-laser of the FACS Calibur used, and a maximum emission wavelength of 518 nm. Bound to RNA the absorption maximum is at 494 nm, the emission maximum at 525 nm. This green emission is detected in the Fluorescence 1 (FL-1) channel with a detector for an

emission wavelength of 530 ± 15nm. FACS analysis proved a more sensitive technique for determining parasitemia than either Giemsa or Field´s rapid staining.

III. 7. Intraerythrocytic DNA amplification of *P. falciparum:* To estimate the DNA/RNA amplification in a further series of experiments, the culture was ring stage-synchronized and re-synchronized after 6 h of culture (to narrow the developmental parasite stage), aliquoted (200 µl aliquots, 2 % hematocrit and 10 % parasitemia) and cultured for further 16 h. The erythrocytes were cultured in the presence or absence of (0.1 µM- 100 µM) L-NAME or Pb^{2+} ions or chlorpromazine. Thereafter, the DNA/RNA amount of the parasitized erythrocytes was determined by Syto16 fluorescence as a measure of intraerythrocytic parasite copies.

III. 8. *In vivo* proliferation of *P. berghei* ANKA: For infection of mice *P. berghei* ANKA-parasitized mouse erythrocytes ($1x10^6$) were injected intraperitoneally [Huber et al., 2004] into sex- and age-matched mice fed on control diet and with free access to drinking water or L-NAME (1 mg/ml) or lead (100µM $Pb(NO_3)_2$) containing drinking water from the 10th day of infection. In the group involving the treatment with chlorpromazine, mice were fed on control diet and had free access to drinking water or chlorpromazine (1mM) containing drinking water from 1st day of infection. In the study involving iron deficiency, the mice were infected after there were fed on control or iron deficient diet for 10 weeks and had free access to drinking water through out the study. Parasitemia was determined daily form the 8th day of infection by flow cytometry. The blood samples were stained with the DNA/RNA-specific fluorescence dye Syto-16 and the Syto-16 fluorescence was analysed with a FACS Calibur cytometer (Becton Dickinson , Heidelberg, Germany) at fluorescence channel 1 (FL1 488 nm excitation and 530 nm emission as described above.

III. 9. Fluorescence staining and flourescence microscopy: The Pb^{2+} effect on annexin binding in erythrocytes was further studied using immunofluorescence as described previously [Kempe et al., 2005]. After 24-h exposure (37°C) to Pb^{2+} (0, 0.3, 1, and 3 µM in NaCl-Ringer solution), the erythrocytes were suspended for 20 min in annexin-binding buffer containing annexin-V-fluos (1:50 dilution), centrifuged, resuspended in NaCl-Ringer, and postincubated (37°C) for 10 min in a modified Ringer solution containing (in

mM) 145 NaCl, 5 KCl, 5 glucose, 1.6 $CaCl_2$, 0.8 $MgCl_2$, and 5 HEPES-NaOH (pH 7.4) to wash the cells and to maintain an isosmotic bath condition (298 mosM). Finally, 10 µl of the cell suspension were analyzed under a fluorescence microscope (440/480-nm excitation and 535/50-nm emission wavelength, Q505LP beamsplitter, AHF Analysentechnik, Tübingen, Germany, combined with a Nikon microscope; Düsseldorf, Germany), and digital pictures were taken using a digital imaging system (Visitron Systems, Puchheim, Germany) equipped with Metaview software.

III. 10. Determination of phosphatidylserine exposure: FACS analysis was performed as described [Kempe et al., 2006]. Suspensions of non infected erythrocytes were stained with annexinV-fluos (Roche, Mannheim, Germany), suspensions of *P. falciparum* infected erythrocytes were stained with annexin V-568 (Roche, Germany) and/or with the DNA dye Syto16 (Molecular Probes) to depict phosphatidylserine exposing and infected erythrocytes, respectively. For annexin binding, erythrocytes were washed, resuspended in annexin-binding buffer (140 mM NaCl, 10 mM HEPES, 5 mM Glucose, 5 mM $CaCl_2$. pH 7.4), stained with annexin V-568 (dilution 1:50) or annexin V-fluos (dilution 1:100), incubated for 20 min at room temperature, and diluted 1:5 with annexin binding buffer. Syto16 (final concentration of 20 nM) was either directly added to the diluted erythrocyte suspension (or incubated for 30 min at 37°C) or co-incubated in the annexin binding buffer. Cells were analyzed by flow cytometry (FACS-Calibur, Becton Dickinson) using FL-1 for Syto16 or annexin V-fluos fluorescence intensity and with FL-2 for annexin V-568 fluorescence intensity.

III. 11. Determination of intracellular Ca^{2+} influx in the erythrocytes: Intracellular Ca^{2+} measurements were performed as described previously [Andrews et al., 2002]. Erythrocytes were loaded with fluo-3 AM (Calbiochem, Bad Soden, Germany) by addition of fluo-3 AM stock solution (2 mM diluted in DMSO) to 1 ml of erythrocyte suspension (0.16% hematocrit in Ringer solution; 4 µM fluo-3 AM final concentration). The cells were incubated at 37°C for 15 min under protection from light. An additional 2-µl aliquot of fluo-3 AM was added, and then the mixture was incubated for 25 min. Fluo-3-loaded erythrocytes were centrifuged at 1,000 *g* for 5 min at 22°C and then washed twice with Ringer solution containing 0.5% bovine serum albumin (Sigma) and once with Ringer

solution and incubated. For flow cytometry, fluo-3-loaded erythrocytes were resuspended in 1 ml of Ringer solution (0.16% hematocrit) containing 1 µM $Pb(NO_3)_2$ and incubated at 37°C for 30 min. As a positive control, erythrocytes were stimulated with 1 µM Ca^{2+} ionophore ionomycin (Sigma) for 3 min prior to analysis to increase intracellular Ca^{2+} activity. For negative control, cells were incubated for 30 min at 37°C with vehicle alone. Subsequently, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

III. 12. Measurement of the *in vivo* clearance of fluorescence-labelled erythrocytes: Fluorescence-labelled erythrocytes from mice were obtained by staining erythrocytes with carboxyfluorescein diacetate, succinimidyl ester (CFSE) from Molecular Probes (Leiden, The Netherlands). The labelling solution was prepared by addition of adequate amounts of a CFSE stock solution (10 mM in DMSO) to phosphate-buffered saline (PBS) to yield a final concentration of 5 µM. Then, the cells were incubated with labelling solution for 30 min at 37°C. The cells were pelleted at 1000 x *g* for 5 min, washed and resuspended in fresh, prewarmed PBS. The fluorescence-labelled erythrocytes were injected into the tail veins of mice [Kempe et al., 2006]. At indicated time points, blood was taken from the mice and CFSE-dependent fluorescence intensity of the erythrocytes was measured in the fluorescence channel FL-1. The percentage of CFSE-positive erythrocytes was calculated in % of the whole population.

III. 13. Measurement of the fluorescence-labelled erythrocytes in the spleens of mice: The fluorescence-labelled erythrocytes were injected into the tail veins of mice. After 2 hours the mice were sacrificed and the spleens were dissected and carefully mashed through a net. Finally, CFSE-dependent fluorescence intensity of the erythrocytes in spleen suspensions was measured in the fluorescence channel FL-1.

III. 14. Analysis of blood cell numbers : Erythrocyte density, packed cell volume, mean erythrocyte volume, white blood cell counts, and hemoglobin content were determined using an electronic hematology particle counter (type MDM 905) from Medical

Diagnostics Marx (Butzbach, Germany), equipped with a photometric unit for hemoglobin determinations.

III. 15. Mouse reticulocyte numbers: For determination of relative reticulocyte numbers, 5 µl of whole blood were added to 1 ml Retic-COUNT (Thiazole orange) reagent from Becton Dickinson. Samples were stained for 30 min at room temperature in the dark. Then, FACS analysis of stained samples was performed according to the manufacturer's instructions. Forward scatter (FSC), side scatter (SSC), and thiazole orange-fluorescence intensity (in the fluorescence channel FL-1) of the blood cells were measured on a FACS-Calibur from Becton Dickinson. Finally, the numbers of Retic-COUNT positive reticulocytes were determined and relative reticulocyte numbers were expressed as percentages of the total, gated erythrocyte populations. Gating of erythrocytes was achieved by analysis of FSC vs. SSC dot plots using the CellQuest™ software.

III. 16. Data analysis and statistics: Data are provided as means ± standard error of mean (SEM), n represents the number of independent experiments. Statistical analysis was made by paired or unpaired *t* test or by ANOVA using Dunnett's or Tukey's test as post hoc test, where appropriate. $P \leq 0.05$ was considered statistically significant.

IV. RESULTS

IV. 1. Influence of iron deficiency on the course of malaria in *P. berghei* infected mice

IV. 1. 1. Annexin binding of noninfected and infected erythrocytes from control and iron deficient patients: To elucidate whether iron deficiency or infection with *P. falciparum* modifies the percentage of cells exposing phosphatidylserine at the cell surface, we determined annexin binding in FACS analysis. The percentage of annexin-positive erythrocytes in freshly drawn blood was low in both, patients with iron deficiency (0.42 ± 0.02 %, n = 6) and healthy individuals (0.46 ± 0.16 %, n = 8). A 24 hour incubation in RPMI 1640 medium increased the percentage of annexin binding cells in blood from patients with iron deficiency to values (5.75 ± 0.07 %, n = 12) significantly ($p \leq 0.05$, two-tailed t-test) higher than in blood from healthy individuals (3.94 ± 0.08 %, n = 12). As illustrated in Fig. 4, 48 h *in vitro* infection significantly enhanced the percentage of annexin binding infected erythrocytes as compared to the co-cultured uninfected erythrocytes. The increase was significantly higher in erythrocytes from iron deficient patients than in erythrocytes from healthy individuals (Fig. 4B). Thus, iron deficiency increases the effect of infection on eryptosis.

IV. 1. 2. Intra erythrocytic DNA amplification: To test for intraerythrocytic amplification infected erythrocytes were ring-stage synchronized and cultured for further 24h. As a result, iron deficiency slightly but significantly decreased the DNA/RNA content (Fig.5). This points to a delayed intraerythrocytic growth of the parasite in iron-deficient erythrocytes as compared to control erythrocytes.

IV. 1. 3. *In vitro* growth of *P. falciparum* in erythrocytes of control and iron deficient patients: Moreover, the number of infected erythrocytes was markedly decreased following 48 h of incubation (Fig. 6). This decrease of parasite growth might be due to impaired intraerythrocytic parasite amplification and/or due to an impaired reinvasion. Comparison of the Fig. 6 (DNA/RNA content prior to parasite release and re-invasion) and Fig. 7 (number of parasitized cells after parasite re-invasion) suggests that in addition to

intraerythrocytic parasite amplification the invasion into iron deficient erythrocytes was impaired.

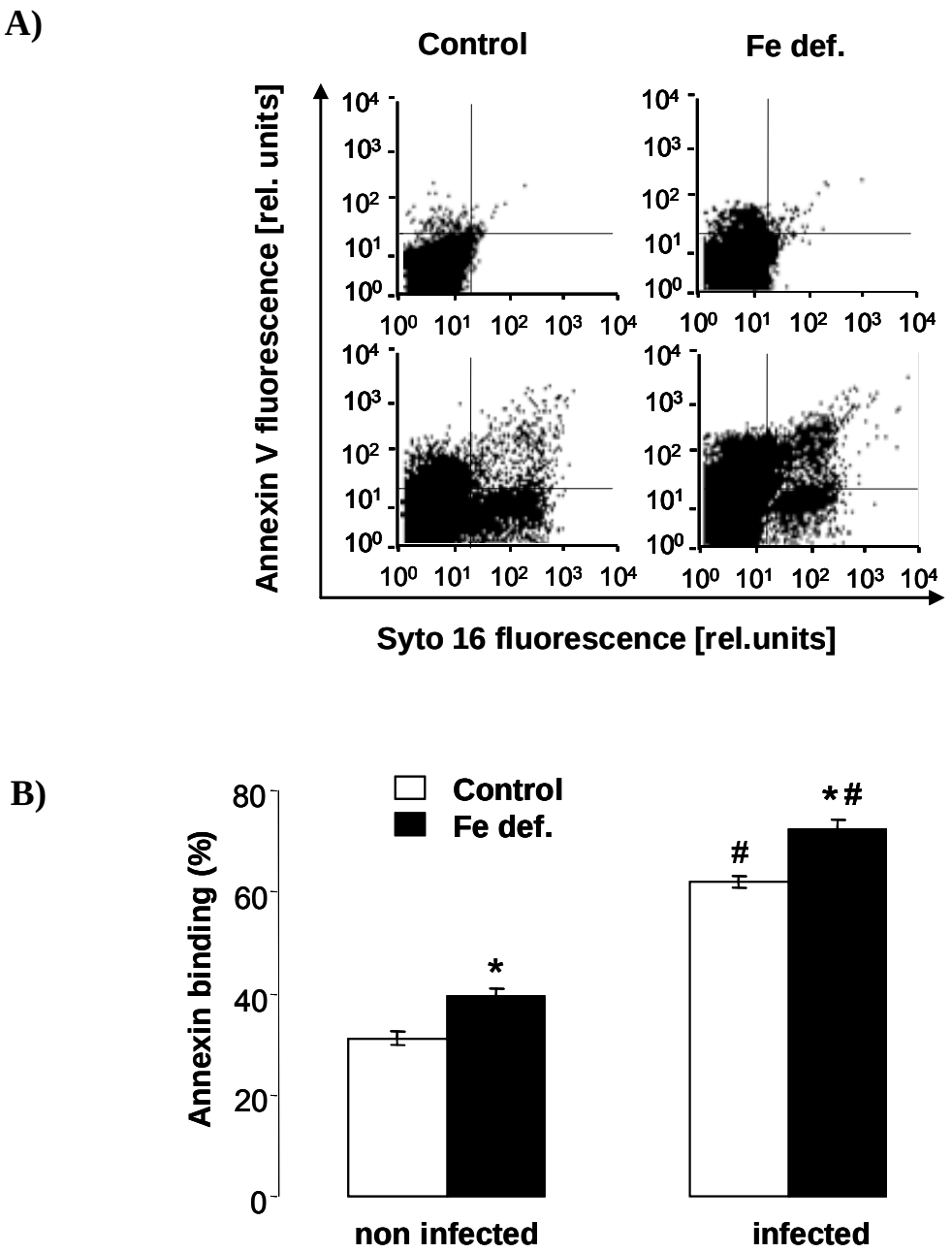


Fig. 4: Effects of iron deficiency on phosphatidylserine exposure of infected and non infected human erythrocytes

A. Dot blots of annexin binding and Syto16 fluorescence of human erythrocytes from healthy individuals (left panels) and iron deficient patients (right panels) prior to (upper panels) and 48 hours after (lower panels) infection with *P. falciparum.*

B. Arithmetic means ± SEM (n = 6) of annexin binding after 48 h incubation of noninfected (left bars) and infected (right bars) erythrocytes from healthy individuals (open bars) or iron deficient individuals (closed bars).* indicates significant difference ($p \leq 0.05$) to erythrocytes from healthy individuals, # indicates significant difference between infected and noninfected erythrocytes.

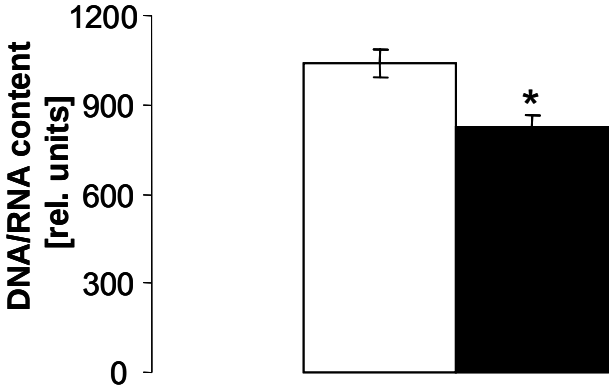


Fig. 5: Effects of iron deficiency on intra erythrocytic DNA amplification of infected and noninfected human erythrocytes

Arithmetic means ± SEM (n = 8) of DNA amplification in erythrocytes from healthy individuals (open bars) or iron deficient individuals (closed bars). * indicates significant difference ($p \leq 0.05$) to erythrocytes from healthy individuals.

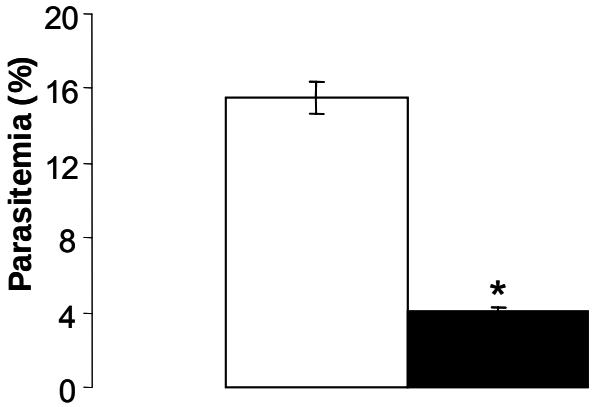


Fig. 6: Effects of iron deficiency on *in vitro* growth of *P. falcifarum* in erythrocytes of control and iron deficient patients.

Arithmetic means ± SEM (n = 8) of parasitemia after *in vitro* growth of *P. falciparum* in erythrocytes of healthy individuals (open bars) and iron deficient patients (closed bars). * indicates significant difference ($p \leq 0.05$) to erythrocytes from healthy individuals.

IV. 1. 4. Forward Scatter of control and iron deficient erythrocytes: Infection of human erythrocytes with *P. falciparum* induces a biphasic change of the host erythrocyte volume. In the early trophozoite stage infected cells loose KCl and water leading to cell shrinkage. In the present study, ring-stage-synchronized erythrocytes when grown for 48h re-invaded and were in the early trophozoite stages. Accordingly, infected cells had a lower forward scatter than uninfected erythrocytes (Fig. 7). Noninfected and infected iron-deficient erythrocytes exhibited a lower forward scatter than uninfected and infected control erythrocytes, respectively (Fig. 7B), indicating lower cell volumes of the iron-deficient human erythrocytes.

IV. 1. 5. *In vivo* clearance of fluorescence labelled erythrocytes: Further experiments have been performed to explore whether the reported enhanced phosphatidylserine exposure in erythrocytes from iron deficient mice leads to enhanced clearance of erythrocytes from circulating blood *in vivo*. To this end erythrocytes from iron-deficient and iron replete mice were labelled with the dye CFSE. As illustrated in Fig. 8, the decay of CFSE-labelled erythrocytes was significantly more rapid in iron deficient than in iron replete animals. Most importantly, the decline was particularly fast in infected CFSE-labelled erythrocytes as shown in Fig. 8B. Along those lines, the number of labelled erythrocytes recovered in the spleen was significantly enhanced in iron deficient animals (Fig. 8C).

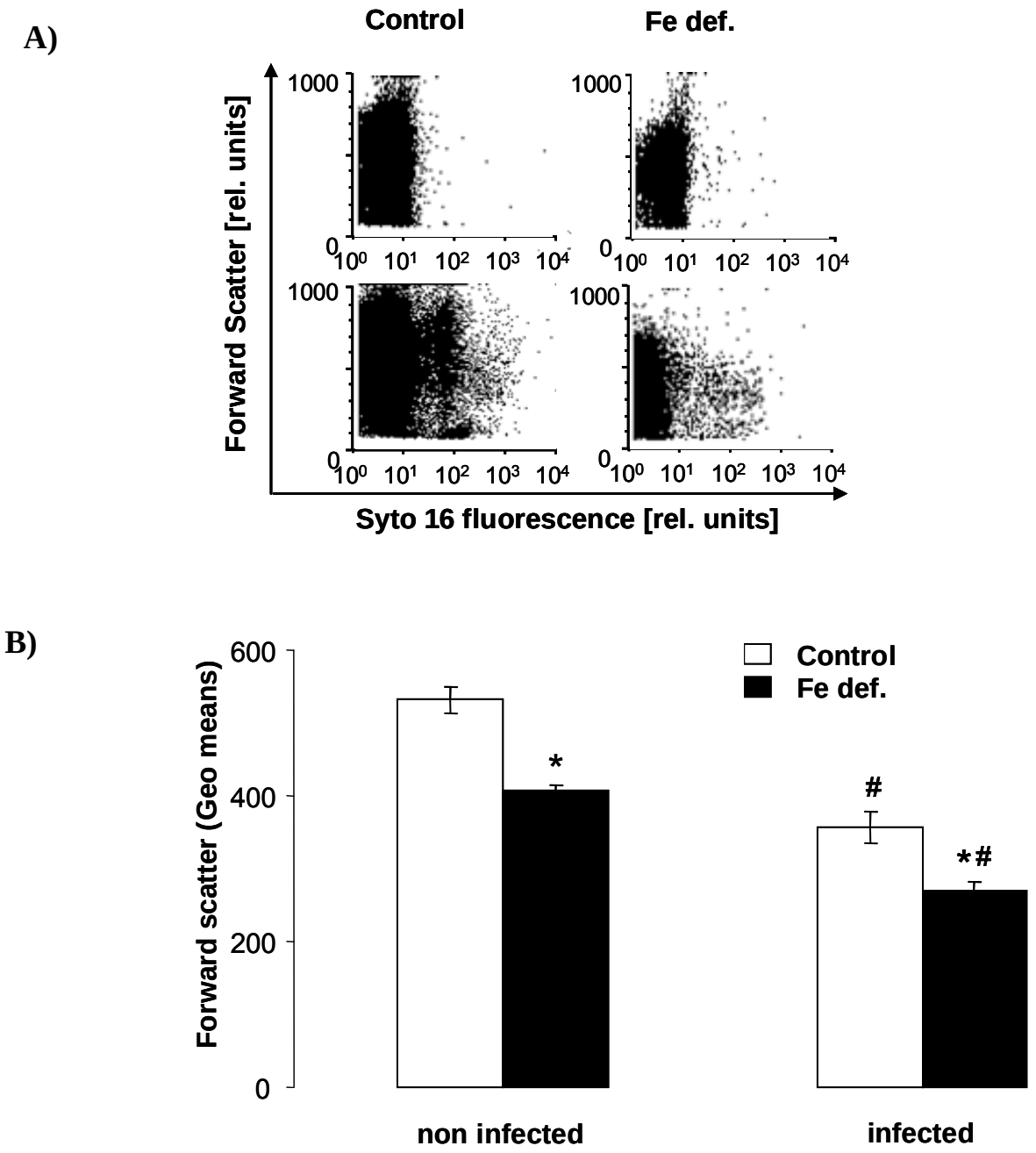


Fig. 7: Effects of iron deficiency on forward scatter of infected and noninfected erythrocytes.

A. Dot blots of forward scatter and syto16 fluorescence of human erythrocytes from healthy individuals (left panels) and iron deficient patients (right panels) prior to (upper panels) and 48 hours after (lower panel) infection with *P. falciparum.*

B. Arithmetic means ± SEM (n = 8) of forward scatter of noninfected (left bars) and infected (right bars) erythrocytes from healthy individuals (open bars) or iron deficient

individuals (closed bars). * indicates significant difference ($p \leq 0.05$) to erythrocytes from healthy individuals, # indicates significant difference between infected and noninfected erythrocytes.

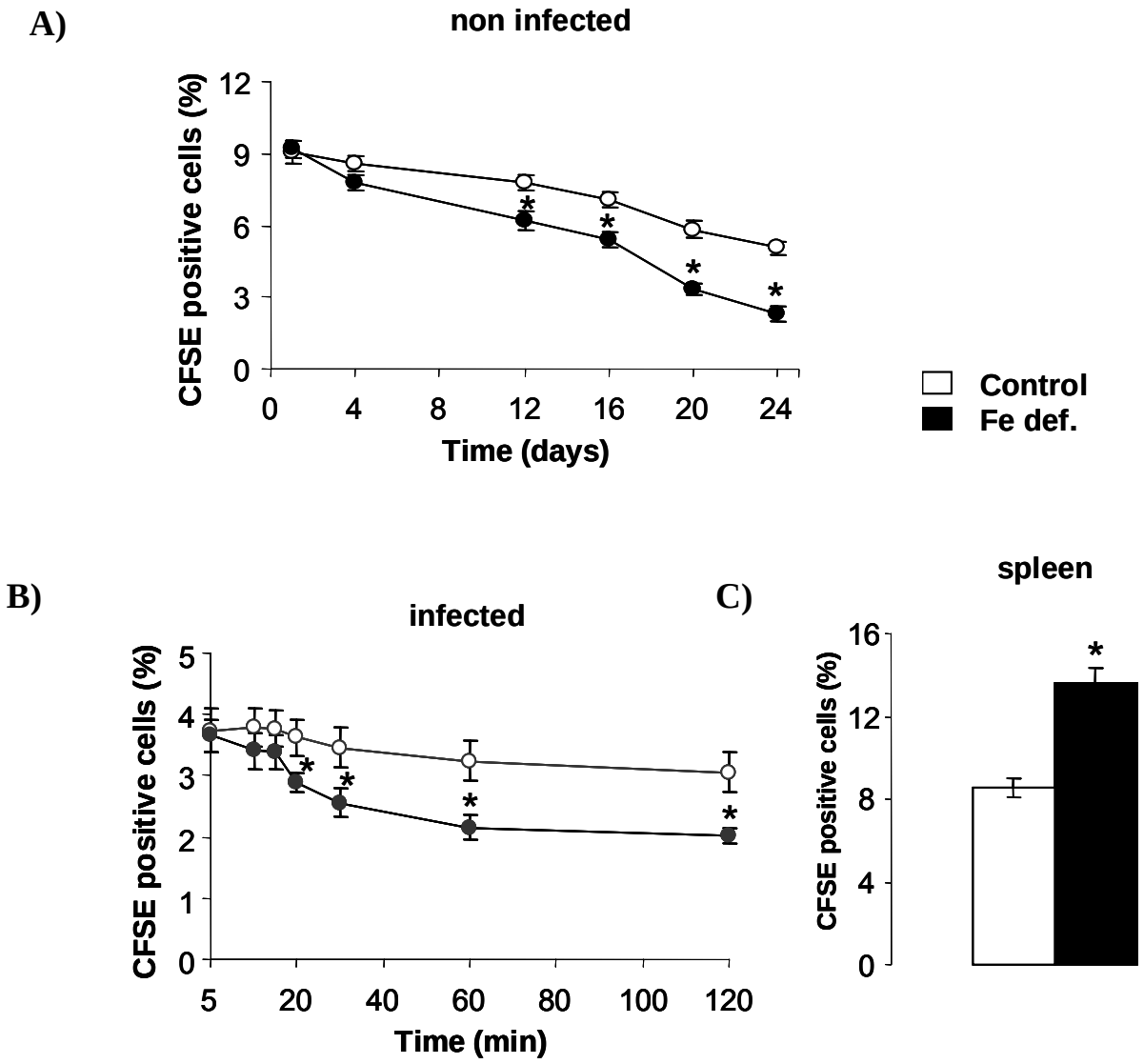


Fig. 8: Clearance of erythrocytes from circulating blood.

A: Arithmetic means ± SEM (n = 6) of the percentage of CFSE labelled non infected erythrocytes in circulating blood of iron deficient (closed symbols) or iron replete (open symbols) animals. * indicates significant difference ($p \leq 0.05$) from iron replete animals.

B: Arithmetic means ± SEM (n = 6) of the percentage of CFSE labelled erythrocytes infected with *P. berghei* in circulating blood of iron deficient (closed symbols) or iron

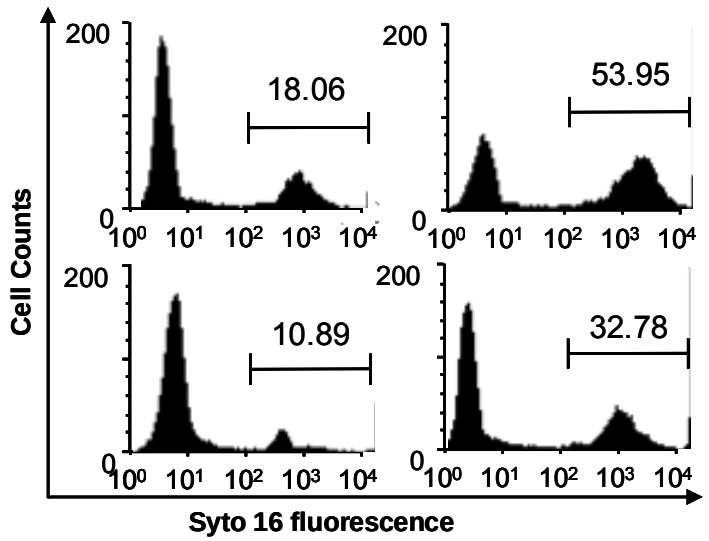
replete (open symbols) animals (33 % parasitemia each). * indicates significant difference ($p<0.05$) from iron replete animals.

C: Abundance of CFSE labelled erythrocytes in spleens from iron replete (open bar) or iron deficient (closed bar) mice. Arithmetic means ± SEM (n = 6). * indicates significant difference ($p<0.05$) from iron replete animals.

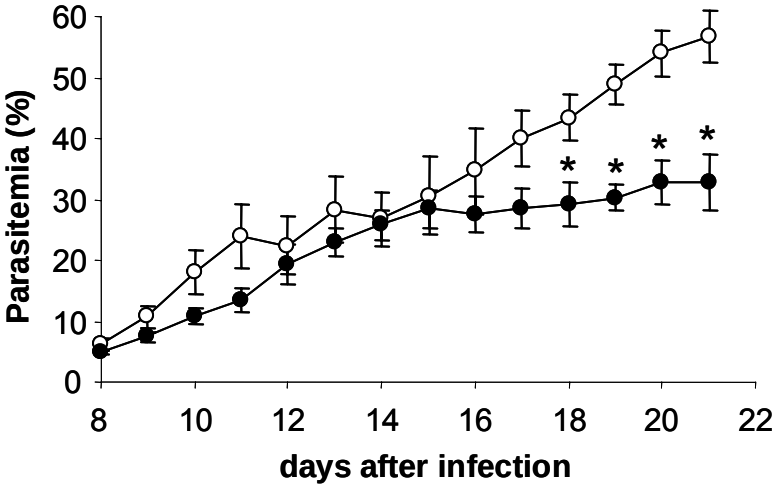
IV. 1. 6. *In vivo* proliferation of *P. berghei* in control and iron deficient mice: Further experiments have been performed to determine whether the effect of iron deficiency on survival of circulating erythrocytes would affect the course of malaria. To this end wild type mice were infected with *P. berghei* and the parasitemia monitored daily. As illustrated in Fig. 9, parasitized erythrocytes appeared in the circulating blood approximately 8 days after infection. In iron replete animals, the percentage of parasitized erythrocytes increased gradually up to 57 % within the following two weeks. The increase of parasitemia was significantly blunted in iron deficient animals (Fig. 9).

The effects of iron deficiency on the parasitemia were paralleled by enhanced survival of the infected mice. Whereas all iron replete animals died within 30 days after the infection, some 20 % of the iron-deficient animals survived the infection for more than 30 days. Thus, iron deficiency indeed significantly modified the course of malaria.

A)



B)



C)

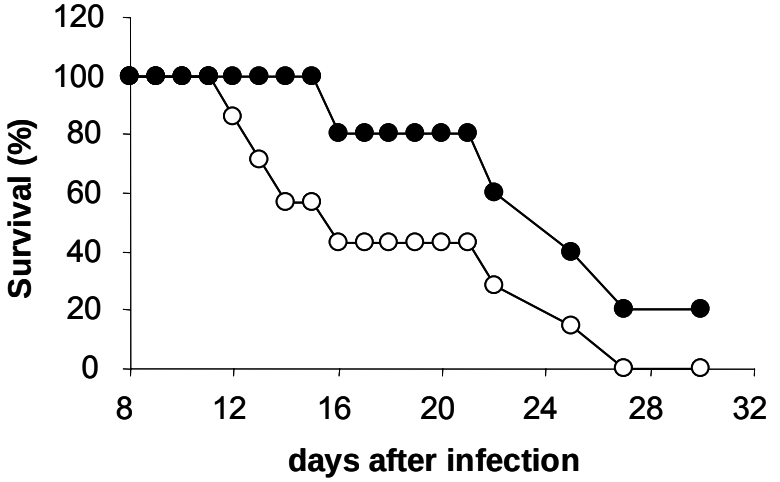


Fig. 9: Parasitemia and survival of *P. berghei* infected mice.

A: Original histograms of parasitemia in iron replete animals (upper panels) and iron deficient animals (lower panels) 10 (left panels) and 20 (right panels) days after infection with *P. berghei.*

B: Arithmetic means ± SEM (n = 6) of parasitemia in iron replete mice (open circles) or in iron deficient mice (closed circles) as a function of days after infection with *P. berghei.* * indicates significant difference ($p \leq 0.05$) from iron replete animals.

C: Survival of iron replete (open circles) or iron deficient (closed circles) mice as a function of days after infection with *P. berghei.*

IV. 1. 7. Haematological parameters: A mouse model of iron deficiency was accomplished by feeding the mice either a control diet containing 180 mg/Kg iron or a diet containing<10 mg/kg for 10 weeks. Indeed, dietary iron depletion led to the expected alterations of blood parameters, i.e.., increase of relative reticulocyte numbers, decrease of the mean corpuscular volume, decrease of the haemoglobin concentration and decrease of haematocrit in the peripheral blood of iron-deficient mice as compared with the respective control animals (Table 1).

	Control	**Iron deficient**	**P**
Erythrocytes	8.2 ± 0.3	8.3 ± 0.7	n.s
Hematocrit	45 ± 2	36 ± 3	0.04
MCV	49.9 ± 1.6	45.5 ± 0.5	0.03
RTC	4.7 ± 0.1	6.6 ± 0.1	0.04
HBG	21.7 ± 1.3	14.2 ± 1.6	0.01
WBC	13.4± 1.1	12.8 ± 1.6	n.s.

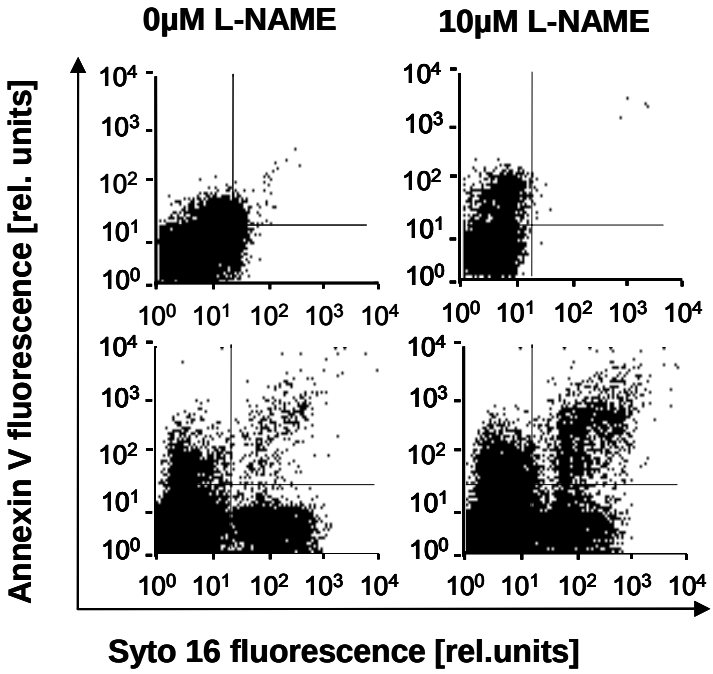
Table 1: Erythrocyte number (10^6/µl), packed cell volume (hematocrit, %), mean corpuscular volume (MCV, fl), reticulocyte count (RTC, %), hemoglobin (HBG, g/100 ml), white blood cell count (WBC 1000/µl) in control and iron-deficient mice (all values arithmetic means ± SEM, n = 8). Erythrocyte and leukocyte counts were determined using MDM 905 (Medical Diagnostics Marx, Butzbach, Germany). Data are provided for control and iron-deficient mice. The P value indicates significant difference between control and iron deficient mice; n. s. is not significantly different.

IV. 2. Impact of NO synthase inhibitior L-NAME on the course of malaria

IV. 2. 1. Annexin binding of noninfected and infected erythrocytes of control and L-NAME treated erythrocytes: To elucidate, whether inhibition of NO synthase or infection with *P. falciparum* modifies the percentage of cells exposing phosphatidylserine at the cell surface, we determined annexin binding in FACS analysis. The percentage of annexin-positive erythrocytes in freshly drawn human blood was low (1.7 ± 0.2 %, n = 8). A 24 hour incubation in Ringer solution increased the percentage of annexin binding cells to (4.5 ± 0.2 %, n = 8), a 48 hour incubation in Ringer solution increased the percentage of annexin binding noninfected erythrocytes to (17 ± 1 %, n = 10). As illustrated in Fig. 10, infection significantly enhanced the percentage of annexin binding erythrocytes. The presence of L-NAME significantly increased the percentage of annexin binding erythrocytes (Fig. 10B).

IV. 2. 2. *In vitro* growth of *P. falciparum* in erythrocytes of control and L-NAME treated erythrocytes: L-NAME did not significqntly modify the DNA/RNA amplification or the *in vitro* percentage of infected erythrocytes. Thus, L-NAME did apparently not influence the intraerythrocytic growth of the pathogen and the *in vitro* infection of erythrocytes (Fig. 11).

A)



B)

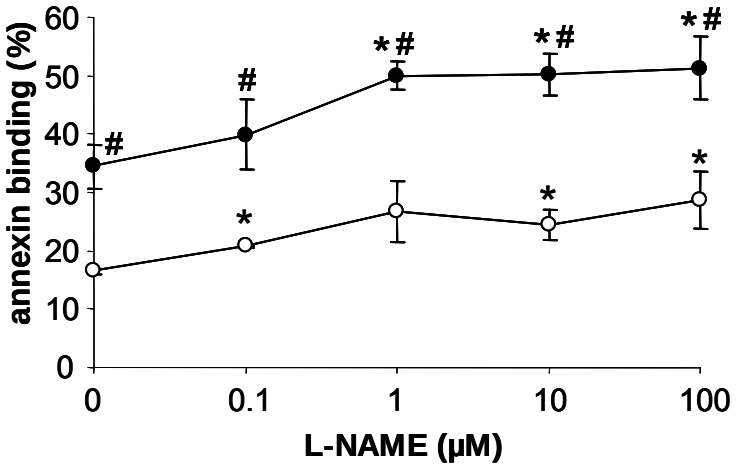


Fig. 10: Effects of NO-synthase inhibitor L-NAME on phosphatidylserine exposure of infected and noninfected erythrocytes.

A. Dot blots of annexin binding and Syto16 fluorescence of human erythrocytes in noninfected cells (upper panels) and in cells infected with *P. falciparum* (lower panels) following a 48 hours incubation in the absence (*left panels*), or presence (right panels) of 10 µM L-NAME.

B. Arithmetic means ± SEM (n = 6) of annexin binding of infected (closed symbols) and noninfected (open symbols) erythrocytes as a function of L-NAME concentration (arithmetic means ± SEM, n = 6). * indicates significant difference ($p \leq 0.05$) from control, # indicates significant difference to noninfected erythrocytes.

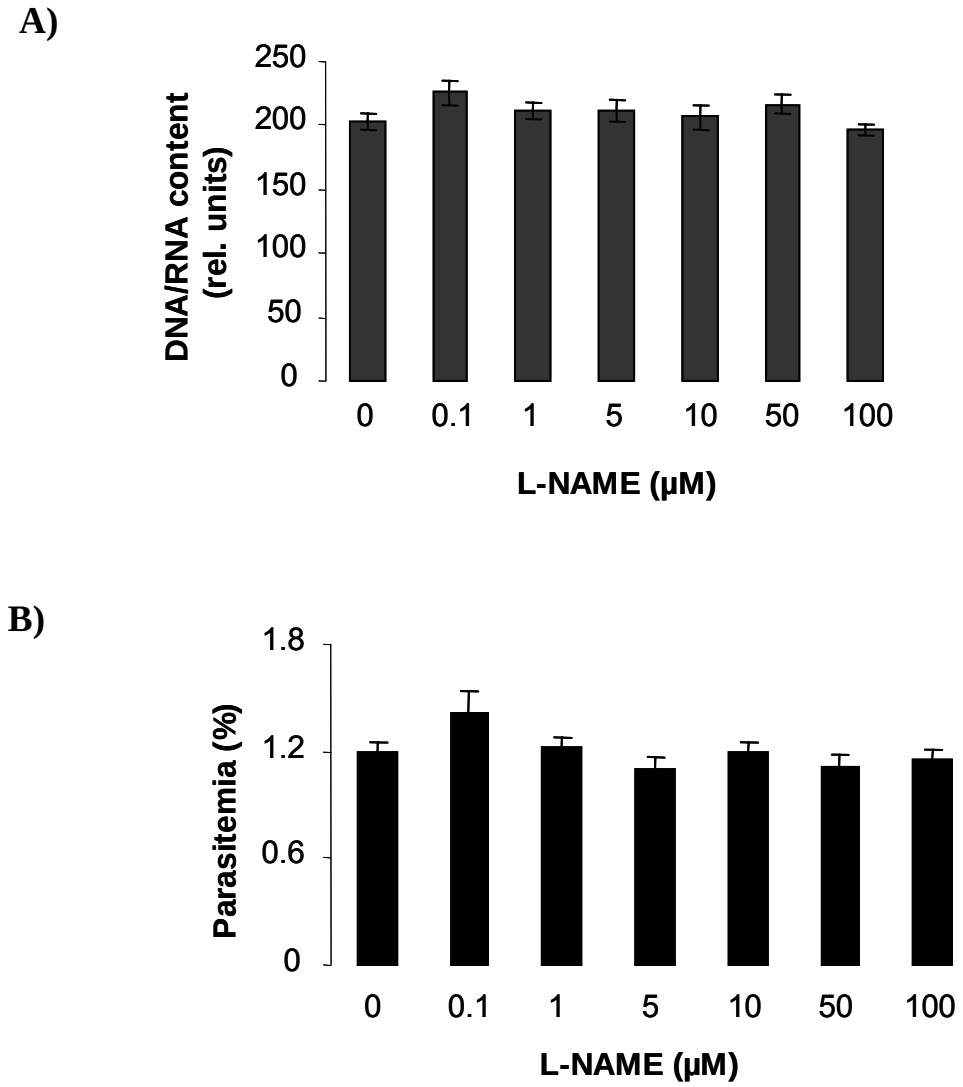


Fig. 11: Effects of NO-synthase inhibitor L-NAME on parasite growth.

A. Intraerythrocytic DNA amplification in erythrocytes as a function of L-NAME concentration (arithmetic means ± SEM, n = 8-10).

B. *In vitro* growth of *P. falciparum* in human erythrocytes as a function of L-NAME concentration (arithmetic means ± SEM, n = 8-10).

IV. 2. 3. Forward Scatter of control and L-NAME treated erythrocytes: The effect of L-NAME on phosphatidylserine exposure was paralleled by a decrease of forward scatter of both infected and noninfected erythrocytes (Fig. 12).

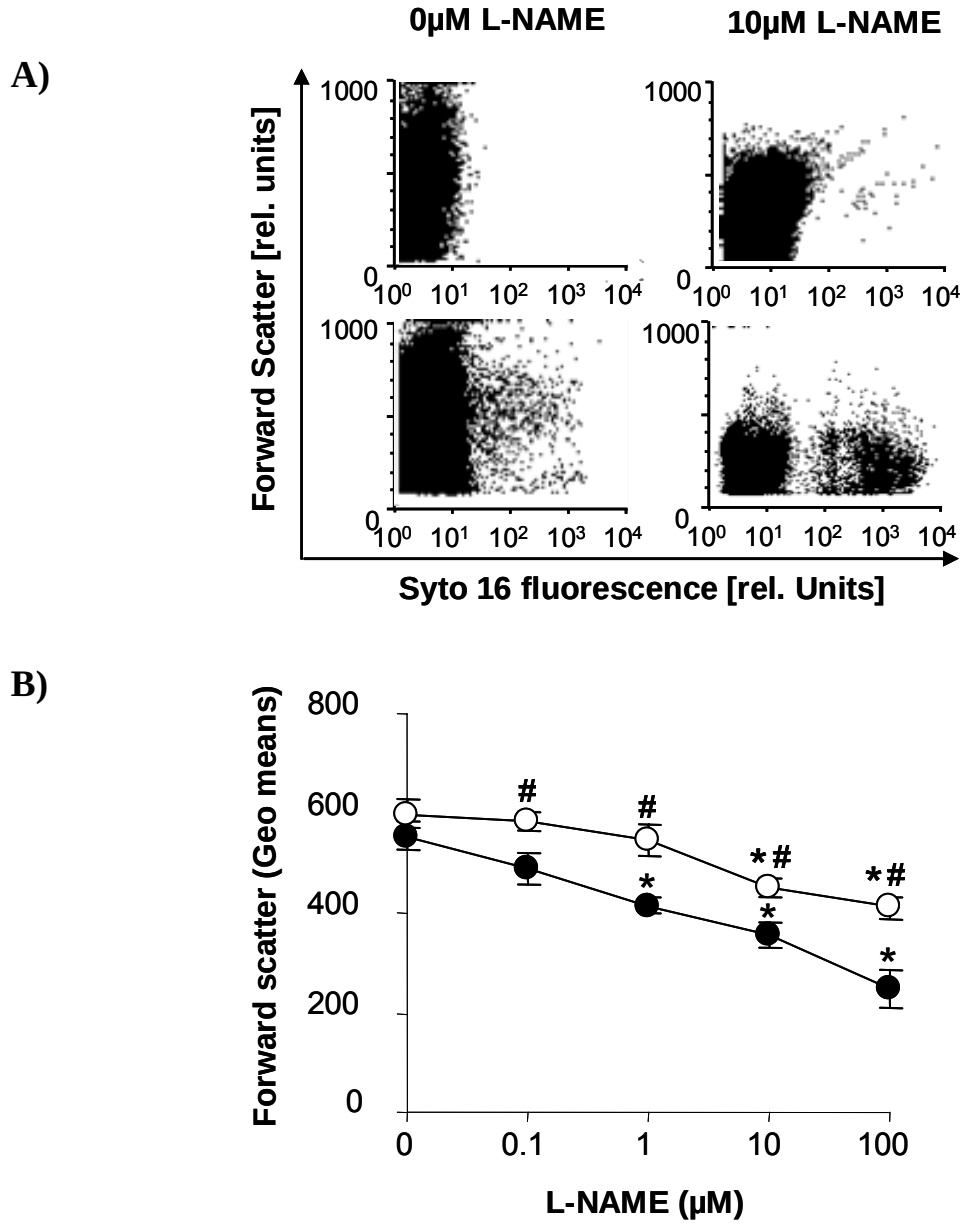


Fig. 12: Effects of L-NAME on forward scatter of infected and noninfected erythrocytes.

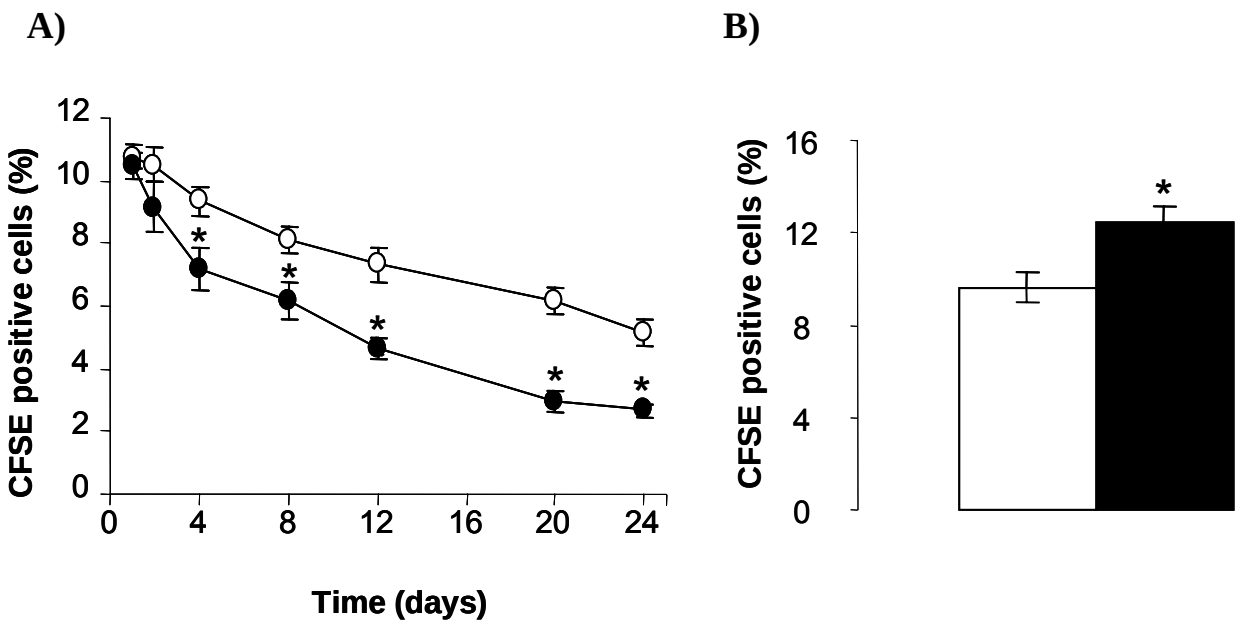
A. Dot blots of forward scatter and syto16 fluorescence of human erythrocytes in noninfected cells (upper panels) and in cells infected with *P. falciparum* (lower panels)

following a 48 hours incubation in the absence (*left panels*), or presence (right panels) of 10 μM L-NAME.

B. Arithmetic means ± SEM (n = 6) of forward scatter of infected (closed symbols) and noninfected (open symbols) erythrocytes before and after treatment with L-NAME, as a function of L-NAME concentration (arithmetic means ± SEM, n = 6). * indicates significant difference ($p \leq 0.05$) from control, # indicates significant difference to noninfected erythrocytes.

IV. 2. 4. *In vivo* clearance of fluorescence labelled erythrocytes: Further experiments have been performed to explore whether the enhanced phosphatidylserine exposure leads to enhanced clearance of erythrocytes from circulating blood *in vivo*. To this end erythrocytes have been labelled with the dye CFSE and were injected into the tail vein of untreated and L-NAME treated mice. As illustrated in Fig. 13A, clearance of erythrocytes was significantly faster in L-NAME treated mice than in untreated animals. Conversely, the number of labelled erythrocytes recovered in the spleen was significantly enhanced by L-NAME treatment (Fig. 13B).

As illustrated in Fig. 13C, only a small portion of labelled infected erythrocytes were cleared within 120 minutes in untreated mice, but a significantly larger percentage of labelled infected erythrocytes disappeared within 120 minutes after injection in L-NAME treated mice. Conversely, the number of labelled erythrocytes recovered in the spleen was significantly enhanced by L-NAME treatment (Fig. 13D).



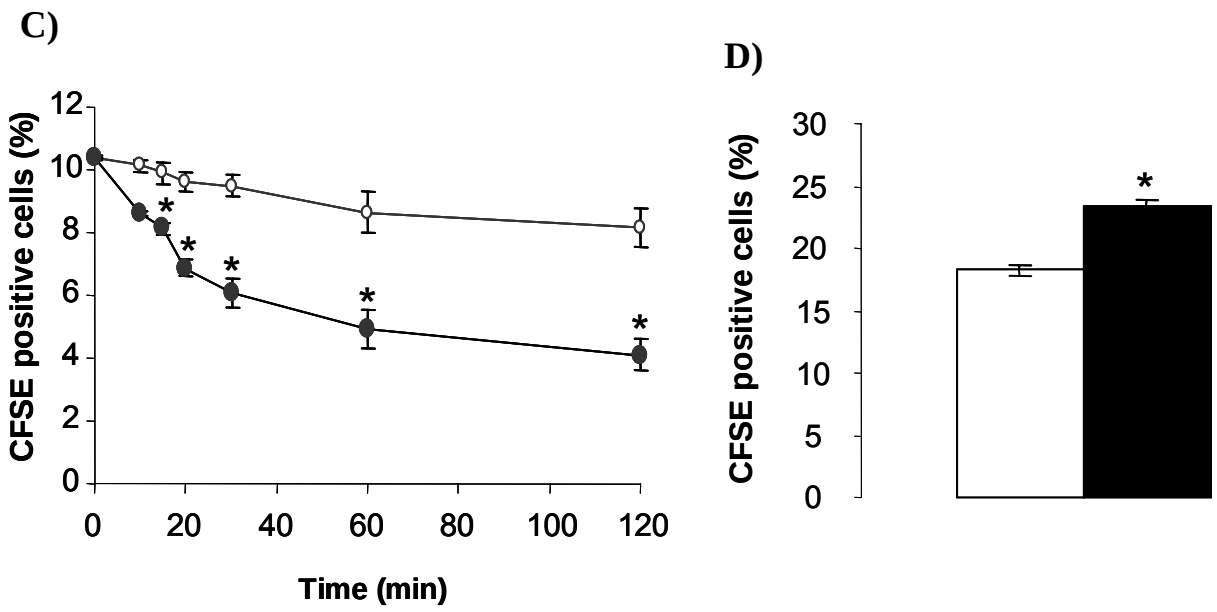


Fig. 13: Clearance of erythrocytes from circulating blood of noninfected and infected mice.

Disappearance of CFSE-labelled erythrocytes from circulating blood following injection into the tail vein of untreated and L-NAME treated mice (1mg/ml in drinking water).

A: Arithmetic means ± SEM (n = 8) of the percentage of CFSE-labelled noninfected erythrocytes in circulating blood of control mice (open symbols) and L-NAME treated mice (closed symbols). * indicates significant difference ($p \leq 0.05$) from control mice.

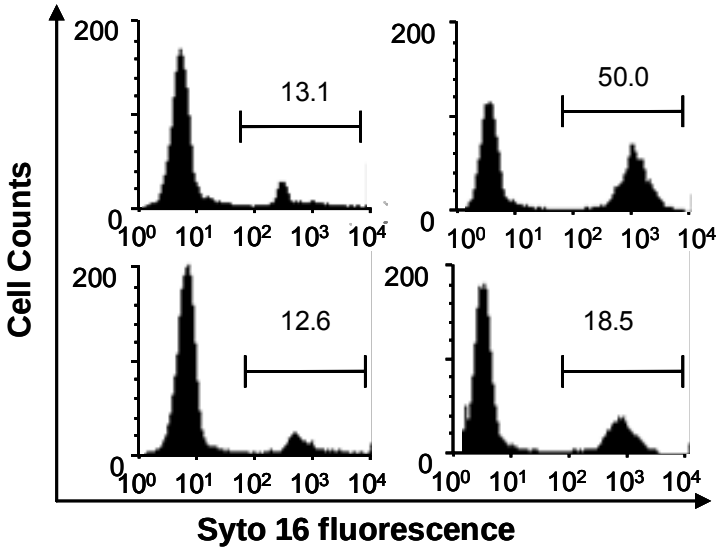
B: Abundance of CFSE-labelled noninfected erythrocytes in spleen of untreated (open bar) and L-NAME treated (closed bar) mice. Arithmetic means ± SEM (n = 8). * indicates significant difference ($p \leq 0.05$) from control mice.

C: Arithmetic means ± SEM (n = 6) of the percentage of CFSE-labelled infected erythrocytes in control mice (open symbols) and L-NAME treated mice (closed symbols). * indicates significant difference ($p \leq 0.05$) from control mice.

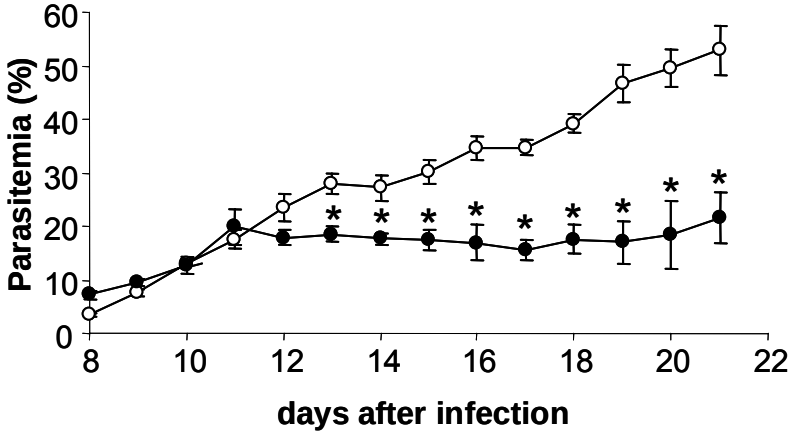
D: Abundance of CFSE-labelled infected erythrocytes in spleen of untreated (open bar) and L-NAME treated (closed bar) mice. Arithmetic means ± SEM (n = 6). * indicates significant difference ($p \leq 0.05$) from control mice.

IV. 2. 5. *In vivo* proliferation of *P. berghei* in control and L-NAME treated mice: Further experiments have been performed to determine whether the effect of L-NAME on phosphatidylserine exposure and survival of circulating erythrocytes would affect the course of malaria. To this end, wild type mice were infected with *P. berghei* and the parasitemia monitored daily. As illustrated in Fig. 14, parasitized erythrocytes appeared in the circulating blood approximately 8 days after infection. In untreated animals, the percentage of parasitized erythrocytes increased gradually up to 53% within the following two weeks. In contrast, the addition of L-NAME (1 mg/ml) to the drinking water significantly blunted the increase of parasitemia (up to 21%). Thus, L-NAME treatment indeed significantly modified the increase of parasitemia. The favourable influence of L-NAME on parasitemia was, however, not paralleled by significantly enhanced survival of infected animals.

A)



B)



C)

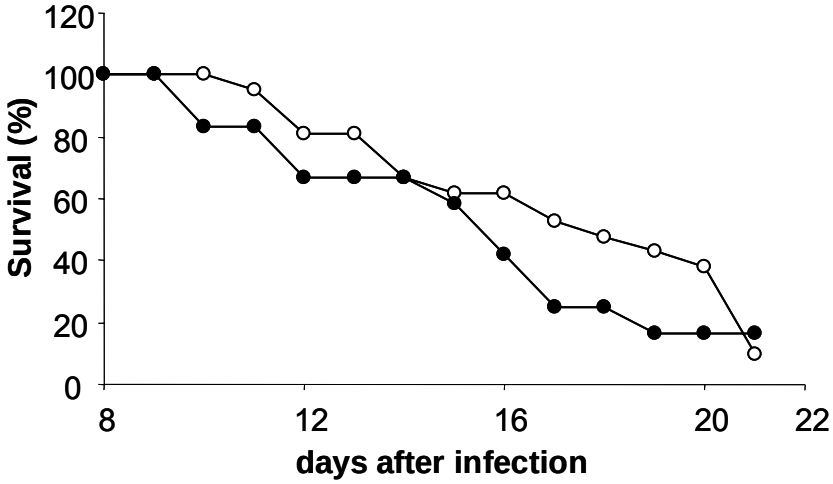


Fig. 14: Parasitemia and survival of *P. berghei* infected mice.

A: Original histograms of parasitemia in untreated animals (upper panels) and animals treated from day 10 until day 20 with L-NAME (1mg/ml in drinking water, lower panels) 10 (left panels) and 20 (right panels) days after infection with *P. berghei*.

B: Arithmetic means ± SEM (n = 15-21) of parasitemia in mice without treatment (open circles) or treated with L-NAME (closed circles) as a function of days after infection with *P. berghei.*

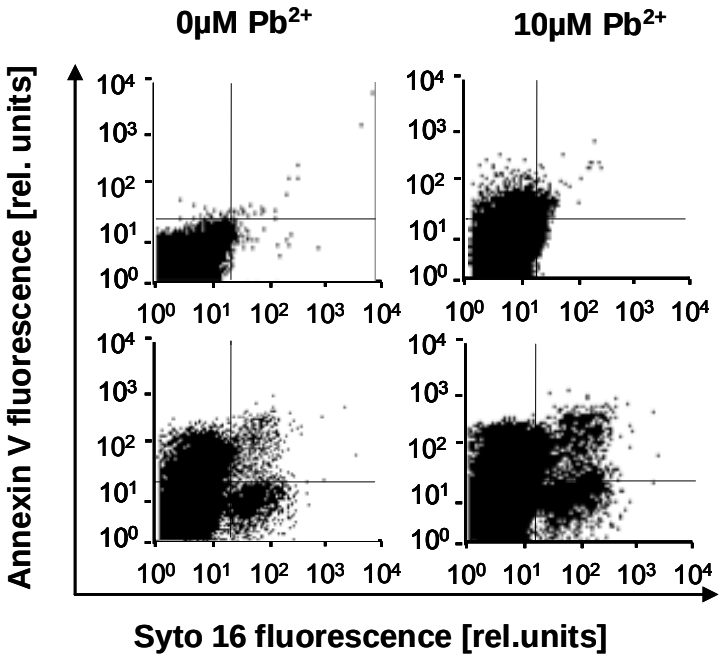
C: Survival of mice without treatment (open circles) or treated with L-NAME (closed circles) as a function of days after infection with *P. berghei.*

IV. 3. Influence of lead treatment on *P. berghei* infected mice

IV. 3. 1. Annexin binding of noninfected and infected control and lead treated erythrocytes: To elucidate, whether $Pb(NO_3)_2$ and/or infection with *P. falciparum* modifies the percentage of cells exposing phosphatidylserine at the cell surface, we determined annexin binding in FACS analysis. The percentage of annexin-positive erythrocytes in freshly drawn blood was low (1.5 ± 0.1 %, n = 8). A 24 hour incubation in Ringer solution increased the percentage of annexin binding cells to (5.9 ± 0.1 %, (n = 8), a 48 hour incubation in Ringer solution increased the percentage of annexin binding of noninfected erythrocytes to 13.7 ± 2.8 % (n = 8). As illustrated in Fig. 15, infection significantly enhanced the percentage of annexin binding erythrocytes. The presence of $Pb(NO_3)_2$ significantly increased the percentage of annexin binding erythrocytes. The effect was particularly prominent in infected erythrocytes (Fig. 15B). Thus, $Pb(NO_3)_2$ preferably triggered phosphatidylserine exposure of infected cells.

IV. 3. 2. Fluorescence staining and flourescence microscopy: The Pb^{2+}-stimulated phosphatidylserine exposure of the erythrocytes was confirmed using immunofluorescence microscopy (Fig. 16), further demonstrating that phosphatidylserine exposure was accompanied by a change in erythrocyte morphology. Exposure to Pb^{2+} stimulated the transition from the biconcave to an echinocyte morphology suggesting Pb^{2+}-induced erythrocyte shrinkage.

A)



B)

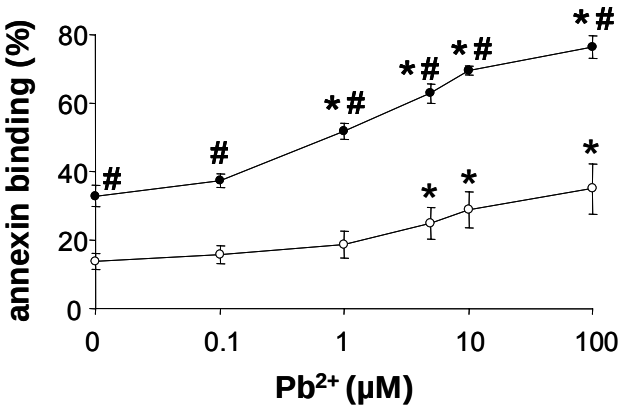


Fig. 15: Effects of $Pb(NO_3)_2$ on phosphatidylserine exposure of infected and noninfected erythrocytes.

A. Dot blots of annexin binding and Syto16 fluorescence of human erythrocytes in noninfected cells (upper panels) and in cells infected with *P. falciparum* (lower panels) following a 48 hours incubation in the absence (*left panels*), or presence (right panels) of 10 µM Pb^{2+} ($Pb(NO_3)_2$).

B. Arithmetic means ± SEM of annexin binding of infected (closed symbols) and noninfected (open symbols) erythrocytes treated with lead as a function of the Pb^{2+} concentration (arithmetic means ± SEM, n = 8). * indicates significant difference ($p \leq 0.05$) from absence of Pb^{2+}, # indicates significant difference to noninfected erythrocytes.

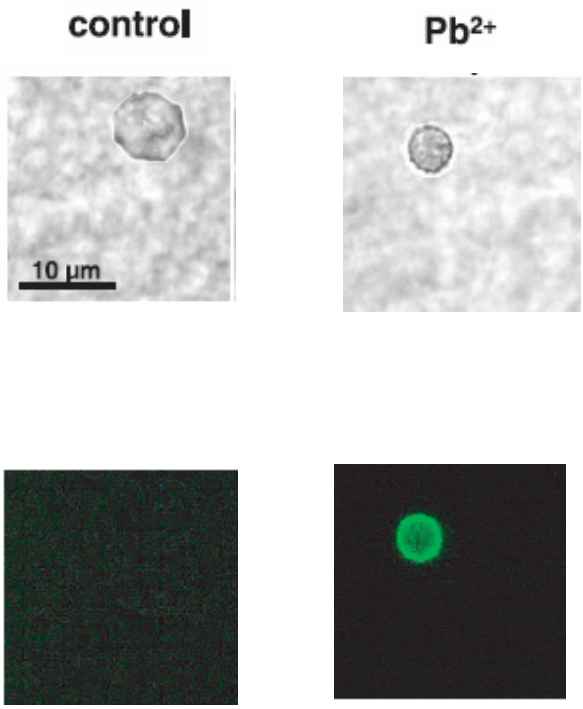
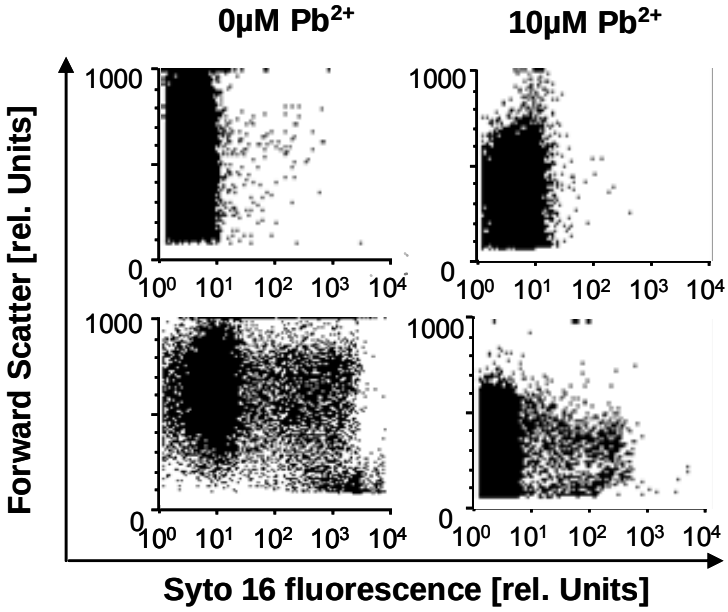


Fig. 16: Erythrocyte shrinkage after exposure to Pb^{+} ions.

Light transmission (*top*) and fluorescence (annexin-V-fluos) photomicrographs (*bottom*) of erythrocytes exposed for 24 h at 37°C to Pb^{2+}. Binding of annexin-V-fluos is accompanied by a change from biconcave to echinocyte erythrocyte morphology.

IV. 3. 3. Forward Scatter of control and lead treated erythrocytes: The effect of $Pb(NO_3)_2$ on phosphatidylserine exposure was paralleled by a decrease of forward scatter of both infected and noninfected erythrocytes reflecting a decrease of cell volume (Fig. 17).

A)



B)

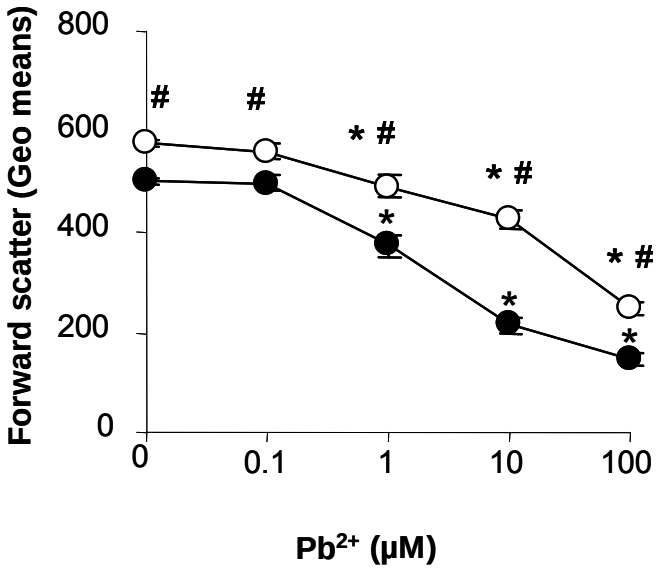


Fig. 17: Effects of $Pb(NO_3)_2$ on forward scatter of infected and noninfected erythrocytes.

A. Dot blots of forward scatter and Syto16 fluorescence of human erythrocytes in noninfected cells (upper panels) and in cells infected with *P. falciparum* (lower panels) following a 48 hours incubation in the absence (*left panels*), or presence (right panels) of 10 µM Pb^{2+} ($Pb(NO_3)_2$).

B. Arithmetic means ± SEM (n=8) of forward scatter of infected (closed symbols) and noninfected (open symbols) erythrocytes treated with lead as a function of the Pb^{2+} concentration (arithmetic means ± SEM, n = 8). * indicates significant difference ($p \leq 0.05$) from absence of Pb^{2+}, # indicates significant difference from noninfected erythrocytes.

IV. 3. 4. Intracellular Ca^{2+} influx in control and lead treated erythrocytes: Reportedly, erythrocyte shrinkage induces activation of Ca^{2+}-permeable nonselective cation channels [Huber et al., 2001]. Thus erythrocytes were loaded with the Ca^{2+}-sensitive fluorescence dye fluo-3 (2 µM fluo-3 AM) to determine the effect of Pb^{2+} on cytosolic Ca^{2+} activity. Cells were incubated in the absence or presence of Pb^{2+} (1 µM for 30 min) or the Ca^{2+} ionophore ionomycin (1 µM), and fluo-3 fluorescence intensity was monitored using FACS. As illustrated in Fig. 18, Pb^{2+} indeed increased fluo-3 fluorescence intensity. As a positive control, the Ca^{2+} ionophore ionomycin (1 µM) similarly enhanced the fluo-3

fluorescence intensity (Fig. 18B). The data strongly suggest that the observed Pb^{2+} effect on fluo-3-loaded erythrocytes indeed reflect an increase in cytosolic free Ca^{2+} concentration.

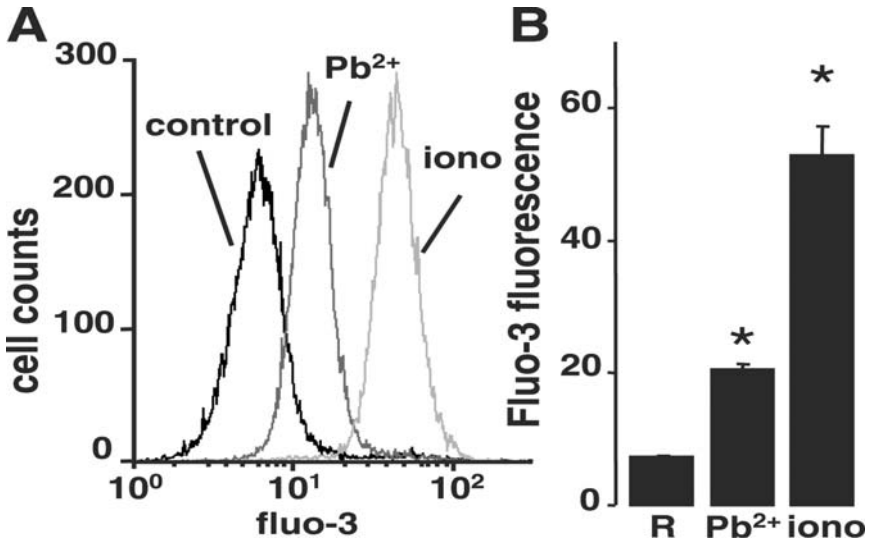


Fig. 18: Pb^{2+}-induced increase in cytosolic free Ca^{2+} concentration.

A: Fluorescence-activated cell sorting (FACS) histograms showing the Ca^{2+}-sensitive fluorescence of fluo-3-loaded erythrocytes incubated in NaCl-Ringer solution (control), in NaCl-Ringer solution containing 1 µM Pb^{2+} (Pb^{2+}), or in NaCl-Ringer solution containing 1 µM Ca^{2+} ionophore ionomycin (iono).

B: Mean fluo-3 fluorescence (arithmetic means ± SE; n = 6) of erythrocytes incubated as in *A* either in Ringer solution (R) or in Ringer solution containing Pb^{2+} (1 µM; 20-min incubation) or ionomycin (1 µM; 5-min incubation).

IV. 3. 5. *In vitro* growth of *P. falciparum* in erythrocytes of control and lead treated erythrocytes: Lead did not significantly modify the DNA/RNA content or the *in vitro* percentage of infected erythrocytes. Thus, lead did not apparently influence the intraerythrocytic growth of the pathogen and the *in vitro* infection of erythrocytes (Fig. 19).

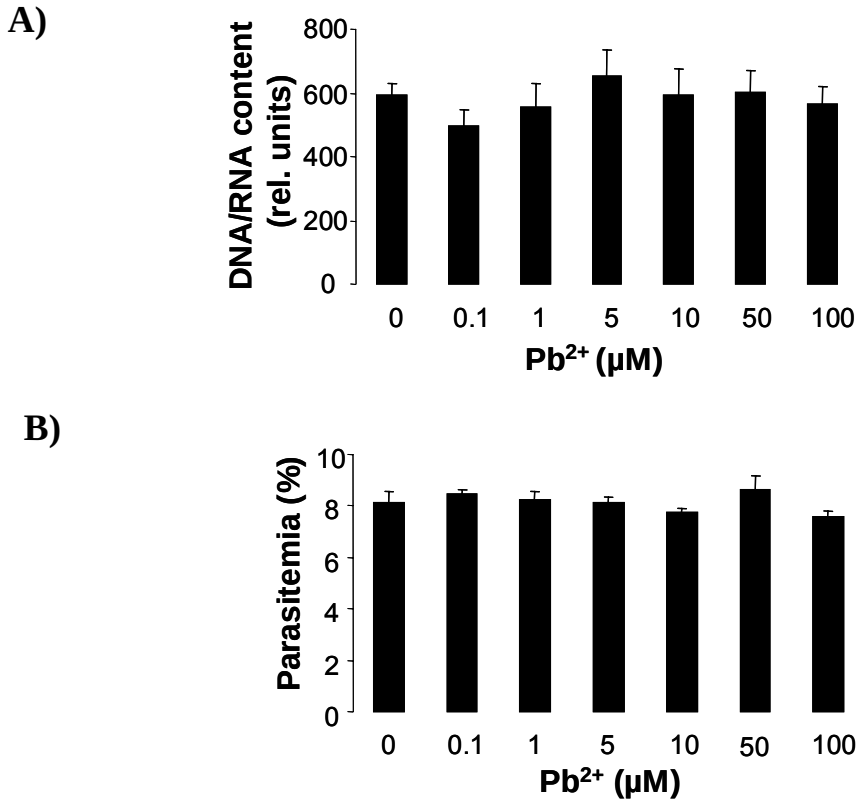


Fig. 19: Effects of $Pb(NO_3)_2$ on parasite growth.

A. Intraerythrocytic DNA amplification in erythrocytes as a function of the Pb^{2+} concentration (arithmetic means ± SEM, n = 8-10).

B. *In vitro* growth of *P. falciparum* in human erythrocytes as a function of Pb^{2+} concentration (arithmetic means ± SEM, n = 8-10).

IV. 3. 6. *In vivo* clearance of fluorescence labelled erythrocytes: Further experiments were performed to explore whether the enhanced phosphatidylserine exposure leads to enhanced clearance of erythrocytes from circulating blood *in vivo*. To this end, erythrocytes were either left untreated or treated with 10 µM $Pb(NO_3)_2$ and were labelled with the dye CFSE. As illustrated in Fig. 20, the clearance of labelled untreated erythrocytes was slow but significantly accelerated by prior treatment of the labelled erythrocytes with $Pb(NO_3)_2$. Along those lines, the number of labelled erythrocytes recovered in the spleen was significantly enhanced by $Pb(NO_3)_2$ treatment.

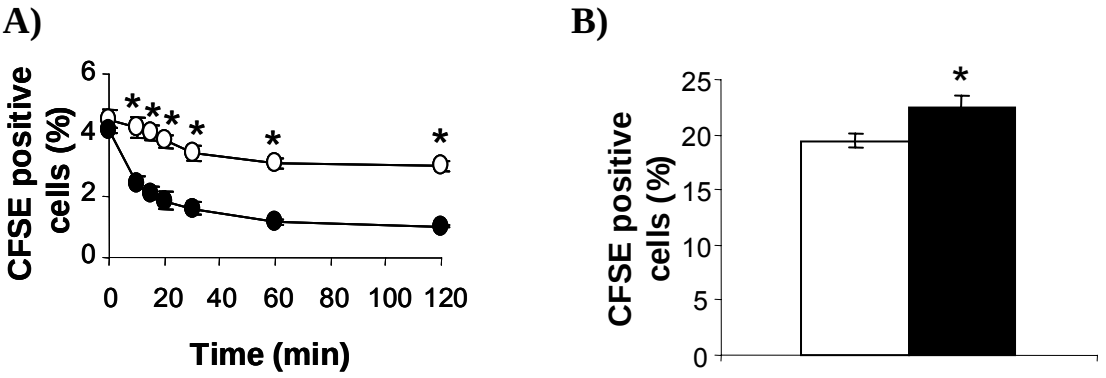


Fig. 20: Clearance of $Pb(NO_3)_2$ treated erythrocytes from circulating blood.

A: Disappearance of untreated and $Pb(NO_3)_2$ treated CFSE-labelled erythrocytes from circulating blood following injection into the tail vein. Arithmetic means ± SEM (n = 6) of the percentage CFSE-labelled untreated (open symbols) or $Pb(NO_3)_2$ treated (closed symbols) erythrocytes after injection. * indicates significant difference from the untreated erythrocytes.

B: Abundance of CFSE-labelled untreated (open bar) or $Pb(NO_3)_2$ treated (closed bar) erythrocytes in spleen. Arithmetic means ± SEM (n =6). * indicates significant difference from the untreated erythrocytes.

IV. 3. 7. *In vivo* proliferation of *P. berghei* in control and lead treated mice: Further experiments were performed to determine whether the effect of lead on phosphatidylserine exposure and survival of circulating erythrocytes would affect the course of malaria. To this end, wild type mice were infected with *P. berghei* and the parasitemia monitored daily. As illustrated in Fig. 21, parasitized erythrocytes appeared in the circulating blood approximately 8 days after infection. In untreated animals, the percentage of parasitized erythrocytes increased gradually up to 52% within the following two weeks. The addition of 100 µM $Pb(NO_3)_2$ to the drinking water significantly blunted the increase of parasitemia. The effects of $Pb(NO_3)_2$ on the parasitemia was paralleled by enhanced survival of the $Pb(NO_3)_2$ treated animals. Whereas all nontreated animals died within 30 days after the infection, approximately 38% of the lead treated animals survived the infection for more than 30 days (Fig. 21C). Thus, lead treatment indeed significantly modified the course of malaria.

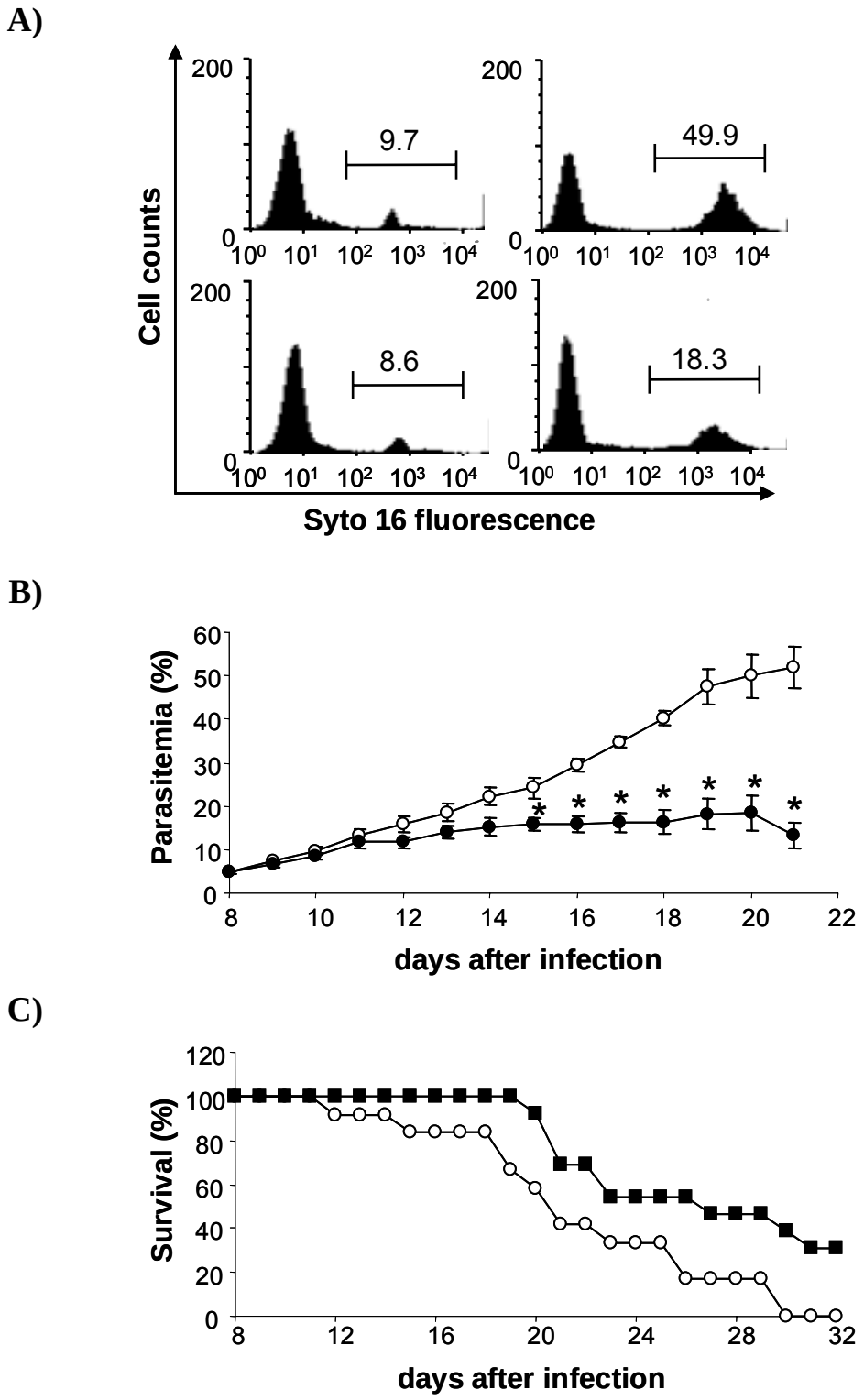


Fig. 21: Parasitemia and survival of *P. berghei* infected mice.

A: Original histograms of parasitemia in untreated animals (upper panels) and animals treated from day 10 until day 20 with 100 µM $Pb(NO_3)_2$ (lower panels) 10 (left panels) and 20 (right panels) days after infection with *P. berghei*.

B: Arithmetic means ± SEM (n = 12) of parasitemia in mice without treatment (open circles) or with 100 µM $Pb(NO_3)_2$ (closed circles) as a function of days after infection with *P. berghei.* * indicates significant difference from the untreated animals.

C: Survival of mice without treatment (open circles) or treated with 100 µM $Pb(NO_3)_2$ (closed squares) as a function of days after infection with *P. berghei*.

IV. 3. 8. Haematological parameters: To explore the toxicity of the $Pb(NO_3)_2$ treatment, the blood count has been analysed in noninfected mice treated with 100µM $Pb(NO_3)_2$. As listed in table 2, the lead treatment was followed by a slight decrease of erythrocyte number, hematocrit and haemoglobin concentration.

	control	10 days	20 days	30 days
Erythrocytes	9.25 ± 0.10	9.35 ± 0.02	9.32 ± 0.5	9.05 ± 0.27
Hematocrit	45.07 ± 0.60	44.05 ± 1.34	44.2 ± 3	40.87 ± 1.59
MCV	48.27 ± 0.42	47.12 ± 0.59	47.27 ± 0.83	45.07 ± 0.65*
RTC	1.29 ± 0.15	1.35 ± 0.19	1.89 ± 0.32	3.33 ± 0.34*
HBG	23.11 ± 0.75	22.36 ± 1.2	21.43 ± 0.7	21.58 ± 0.76
WBC	10.36 ± 0.64	10.01 ± 0.57	9.9 ± 0.8	11.18 ± 1.64

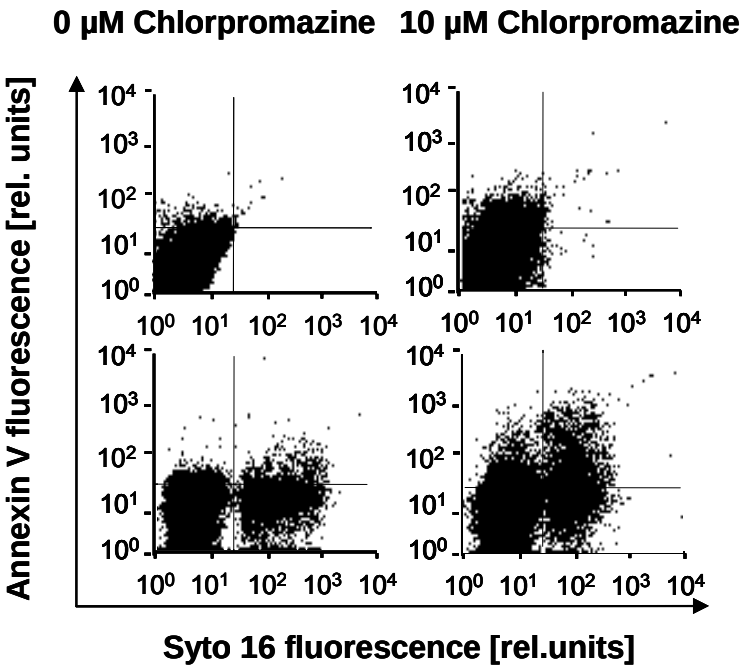
Table 2: Erythrocyte number (10^6/µl), packed cell volume (hematocrit, %), mean corpuscular volume (fl), reticulocyte count (RTC, %), , hemoglobin (HBG, g/100 ml), white blood cell count (WBC 1000/µl) in mice prior to (control) and following a 10, 20 or 30 days treatment with 100 µM $Pb(NO_3)_2$ (all values arithmetic means ± SEM, n = 6). Erythrocyte and leukocyte counts were determined using MDM 905 (Medical Diagnostics Marx, Butzbach, Germany). * indicates significant difference from the control animals.

IV. 4. Influence of chlorpromazine on the course of malaria

IV. 4. 1. Annexin binding of noninfected and infected control and chlorpromazine treated erythrocytes: Phosphatidylserine at the cell surface was estimated from annexin V binding in FACS analysis. The percentage of annexin-binding erythrocytes in freshly drawn blood was low 1.48 ± 0.05 %, n=6). A 24 hour incubation in Ringer solution increased the percentage of annexin binding cells to 5.28 ± 0.29 % (n=6), a 48 hour incubation in Ringer solution increased the percentage of annexin binding noninfected erythrocytes to 14.93 ± 0.39 % (n=6), As shown in Fig. 22, infection significantly enhanced the percentage of annexin binding erythrocytes. The effect of infection was significantly enhanced in the presence of chlorpromazine (Fig. 22B). The effect reached statistical significance at 10 µM chlorpromazine. Thus, chlorpromazine potentiated the scrambling effect of infection on phosphatidylserine exposure.

IV. 4. 2. *In vitro* growth of *P. falciparum* in control and chlorpromazine treated erythrocytes: Intraerythrocytic DNA amplification in erythrocytes was not significantly influenced by chlorpromazine at the concentrations employed. *In vitro* growth of *P. falciparum* in human erythrocytes was, however, significantly decreased at chlorpromazine concentrations ≥ 5 µM (Fig. 23).

A)



B)

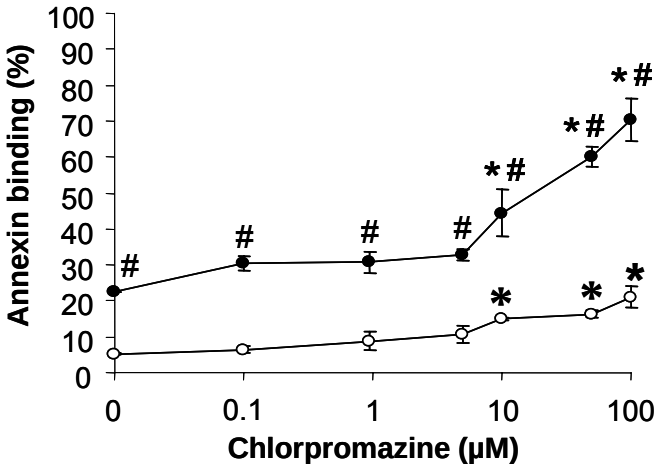


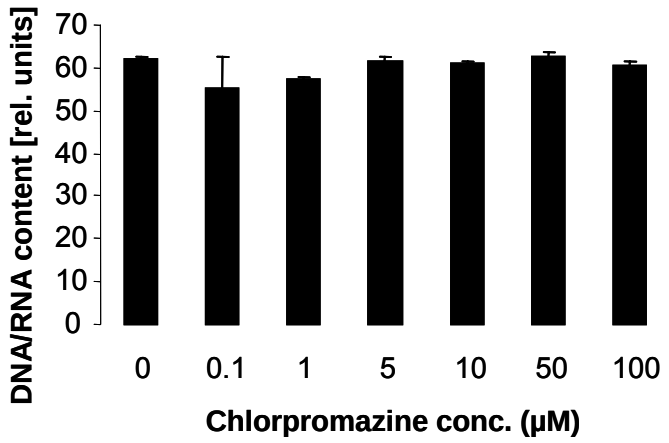
Fig. 22: Effects of Chlorpromazine on phosphatidylserine exposure of infected and noninfected erythrocytes.

A. Dot blots of annexin binding and Syto16 fluorescence of human erythrocytes in noninfected cells (upper panels) and in cells infected with *P. falciparum* (lower panels) following a 48 hours incubation in the absence (*left panels*), or presence (right panels) of 10 µM chlorpromazine.

B. Arithmetic means ± SEM (n=6) of annexin binding of infected (closed symbols) and noninfected (open symbols) erythrocytes as a function of the chlorpromazine

concentration. * indicates significant difference ($p<0.05$) from absence of chlorpromazine, # indicates significant difference from noninfected erythrocytes.

A)



B)

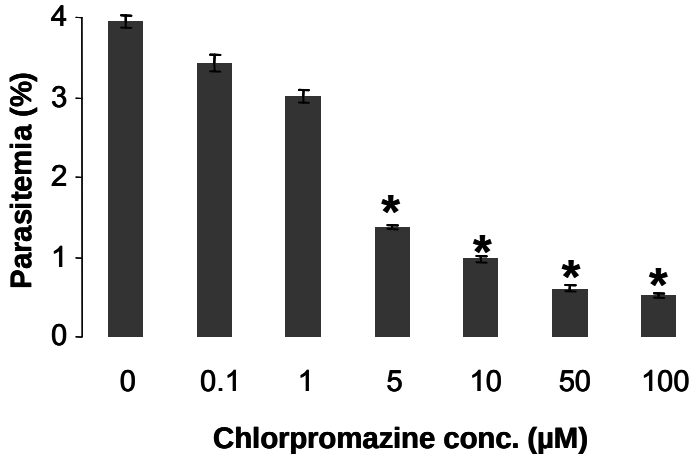


Fig. 23: Effects of chlorpromazine on parasite growth.

A. Intraerythrocytic DNA amplification in erythrocytes as a function of the chlorpromazine concentration (arithmetic means ± SEM, n=6).

B. *In vitro* growth of *P. falciparum* in human erythrocytes as a function of chlorpromazine concentration (arithmetic means ± SEM, n=6). * indicates significant difference ($p<0.05$) from absence of chlorpromazine.

IV. 4. 3. Forward scatter of control and chlorpromazine treated erythrocytes: Infection further decreased erythrocyte forward scatter. Chlorpromazine led to a moderate but significant further decrease of forward scatter, pointing to chlorpromazine induced cell shrinkage (Fig. 24).

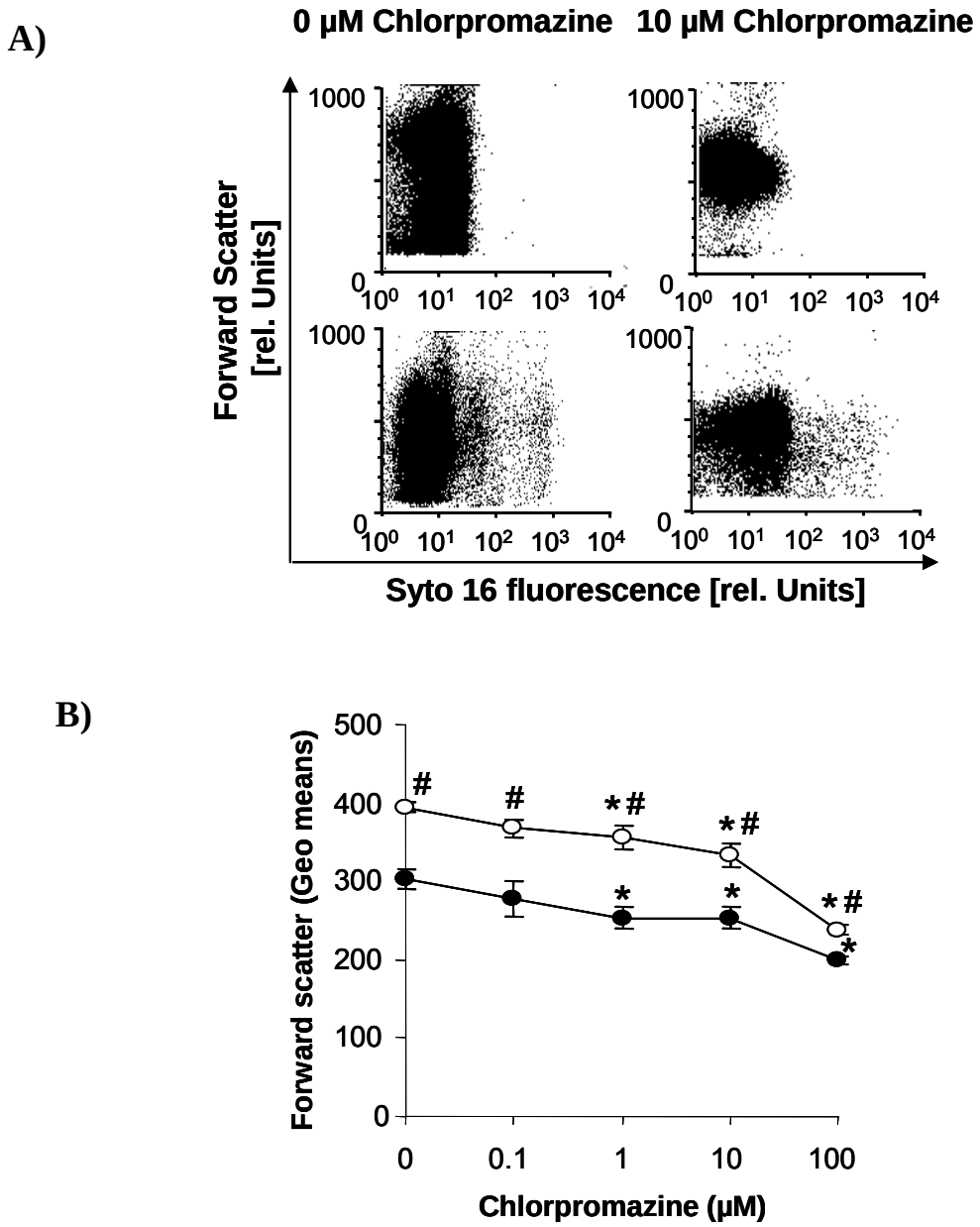


Fig. 24: Effects of chlorpromazine on forward scatter of infected and noninfected erythrocytes.

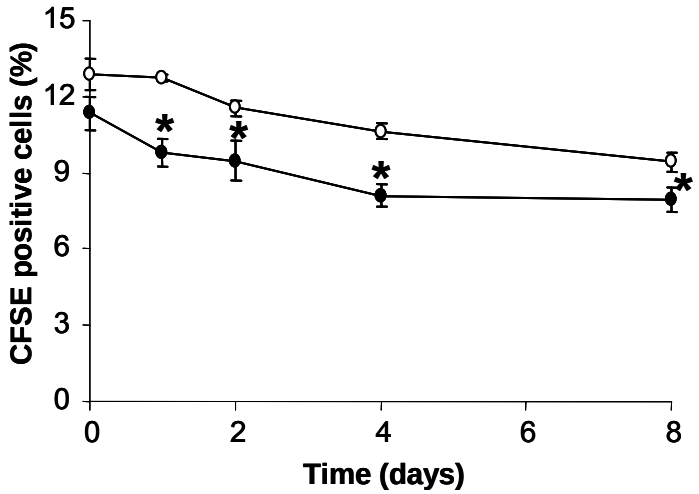
A. Dot blots of forward scatter and syto16 fluorescence of human erythrocytes in noninfected cells (upper panels) and in cells infected with *P. falciparum* (lower panels)

following a 48 hours incubation in the absence (*left panels*), or presence (right panels) of 10 µM chlorpromazine.

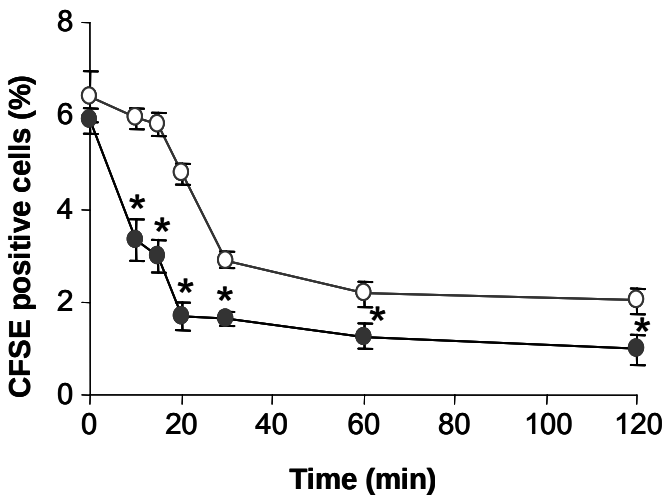
B. Arithmetic means ± SEM (n=6) of forward scatter of infected (closed symbols) and noninfected (open symbols) erythrocytes as a function of the chlorpromazine concentration. * indicates significant difference ($p<0.05$) from absence of chlorpromazine, # indicates significant difference to noninfected erythrocytes.

IV. 4. 4. Clearance of chlorpromazine treated erythrocytes from circulating blood: A next series of experiments explored, whether the enhanced phosphatidylserine exposure accelerates clearance of erythrocytes from circulating blood *in vivo*. For this purpose mice were either left untreated or treated with 100 µM chlorpromazine in drinking water, their erythrocytes were labelled with the dye CFSE and injected into the tail vein. As illustrated in Fig. 25B, the clearance of erythrocytes in chlorpromazine treated mice was slightly, but significantly faster than erythrocyte clearance in untreated mice. Most importantly, the decline was particularly fast in infected CFSE-labelled erythrocytes (Fig. 25B). On the other hand, the fraction of labelled erythrocytes recovered in the spleen was significantly enhanced by chlorpromazine treatment (Fig. 25C).

A)



B)



C)

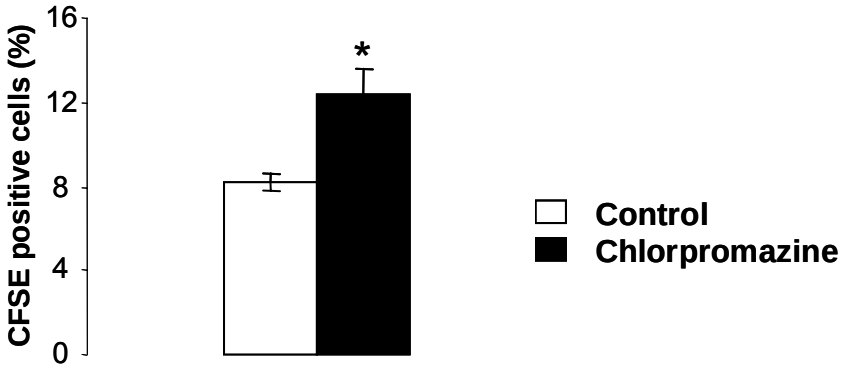


Fig. 25: Clearance of chlorpromazine treated erythrocytes from circulating blood. Disappearance of CFSE-labelled noninfected and infected erythrocytes from circulating blood following injection into the tail vein of untreated and chlorpromazine treated (100 µM) mice in drinking water.

A: Arithmetic means ± SEM (n = 6) of the percentage of CFSE-labelled noninfected erythrocytes in circulating blood of untreated (open symbols) or chlorpromazine (open symbols) treated animals. * indicates significant difference ($p<0.05$) from untreated animals.

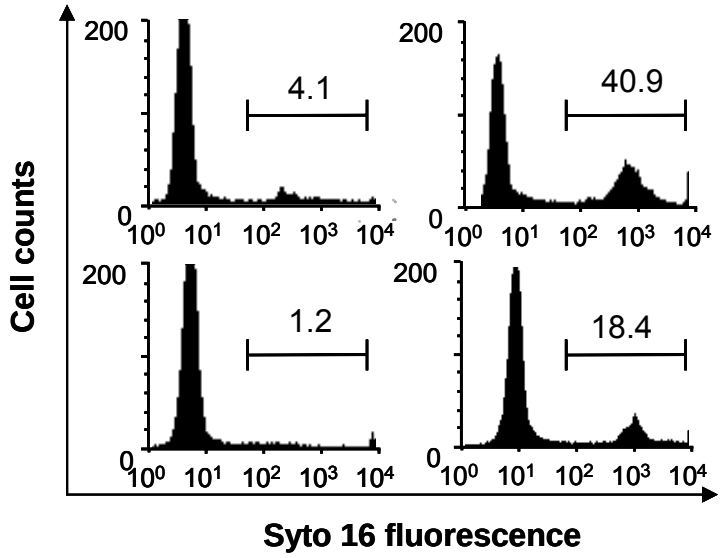
B: Arithmetic means ± SEM (n=6) of the percentage CFSE labelled infected erythrocytes in circulating blood of untreated (open symbols) or chlorpromazine treated (closed symbols) animals. * indicates significant difference from the untreated animals.

C: Abundance of CFSE-labelled untreated (open bar) or chlorpromazine treated (closed bar) erythrocytes in spleen. Arithmetic means ± SEM (n=6). * indicates significant difference from the untreated animals.

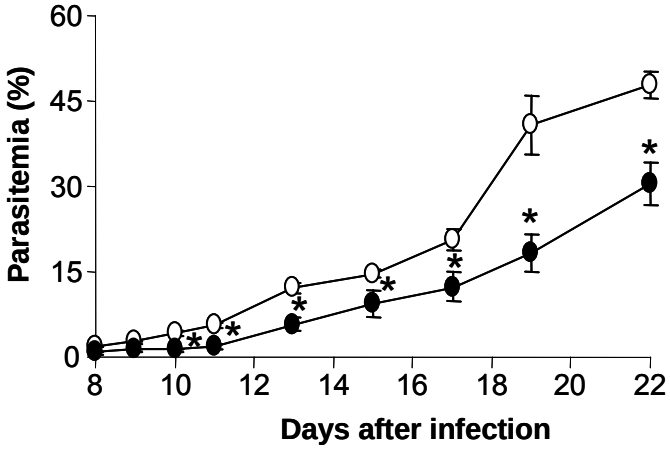
IV. 4. 5. *In vivo* proliferation of *P. berghei* in control and chlorpromazine treated mice: Further experiments were performed on the impact of chlorpromazine treatment on the course of malaria infection *in vivo.* To this end mice were infected with *P. berghei.* As shown in Fig. 26, parasitemia was less than 10% 8 days after infection but gradually increased the next 14 days. In untreated animals, the percentage of parasitized erythrocytes increased up to 48%. The addition of 100 µM chlorpromazine had only a slight effect on parasitemia (data not shown). Thus, the concentration of chlorpromazine was increased to 1 mM chlorpromazine, which indeed significantly blunted the increase of parasitemia.

The effect of chlorpromazine on the parasitemia was paralleled by enhanced survival of the chlorpromazine treated animals. Whereas all untreated animals died within 25 days after the infection, 80% of the chlorpromazine treated animals survived the infection for more than 30 days (Fig. 26C). Thus, chlorpromazine treatment indeed significantly modified the course of malaria.

A)



B)



C)

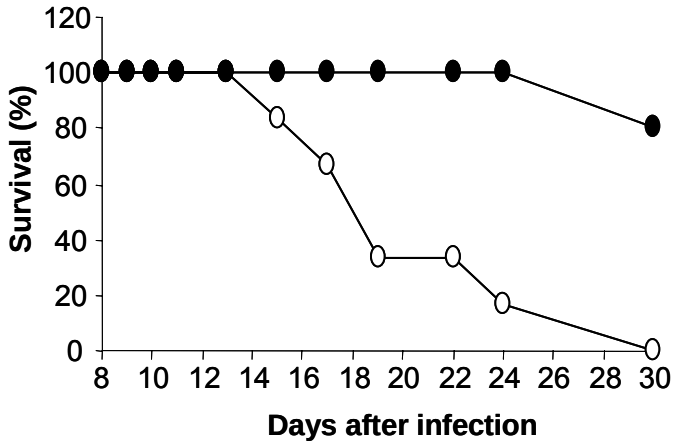


Fig. 26: Parasitemia and survival of *P. berghei* infected mice.

A: Original histograms of parasitemia in untreated animals (upper panels) and animals treated from day 10 until day 19 with 1 mM chlorpromazine (lower panels) 10 (left panels) and 19 (right panels) days after infection with *P. berghei*.

B: Arithmetic means ± SEM (n=10-11) of parasitemia in mice without treatment (open circles) or with 1 mM chlorpromazine (closed circles) as a function of days after infection with *P. berghei.* * indicates significant difference from the untreated animals.

C: Survival of mice without treatment (open circles) or with 1 mM chlorpromazine (closed circles) as a function of days after infection with *P. berghei*.

V. DISCUSSION

Our observations indicate that *Plasmodium* infected eryhtrocytes can be targeted for induction of eryptosis *in vivo*. This acclerated eryptosis of *Plasmodium*-infected RBCs results in rapid clearance of infected erythrocytes and confer protection against severe course of malaria. *Plasmodium* imposes oxidative stress to the host cell [Duranton et al., 2003; Duranton et al., 2004; Huber et al., 2002; Huber et al., 2004] leading to cation channel activiation [Duranton et al., 2004; Huber et al., 2002; Huber et al., 2004] and Ca^{2+} entry [Duranton et al., 2004]. Thus, infection was expected to trigger phosphatidylserine exposure. Infection of erythrocytes with *Plasmodium* was indeed found to triggers eryptosis [Brand et al., 2003]. If the stimulation of eryptosis remains anabated, it may proceed too fast for the pathogen to allow intraerythrocytic maturation. It has been observed that accelerated aging or eryptosis of *Plasmodium*-infected erythrocytes in sickle-cell trait, beta-thalassemia-trait, homozygous Hb-C and G6PD-deficiency leads to the clearance of ring stage-infected erythrocytes and has thus been attributed to the partial malaria resistance conferred by these genetic disorders [Ayi et al., 2004; Cappadoro et al., 1998; de Jong et al., 2001; Kean et al., 2002; Kuypers et al., 1998; Lang et al., 2002a]. Early clearance of infected erythrocytes prevents on the one hand tissue sequestration of the late stage-infected erythrocytes and on the other hand hemozoin-intoxication of trophozoite-engulfing macrophages which impairs macrophage function [Schwarzer et al., 1992].

The manipulation of eryptosis of the *Plasmodium*-infected erythrocytes by diet modifications or pharmacological interventions may provide some protection during malaria. Therefore, we have chosen to study the influence of iron deficiency, NO synthase inhibition, $Pb(NO_3)_2$ and chlorpromazine on eryptosis, parasitemia and survival during the course of malraia.

V. 1. Influence of iron deficiency on the course of malaria

The present studies clearly demonstrate that iron deficiency confers some protection following infection with the malaria pathogen *P. berghei*. Moreover, they provide evidence that the protection correlates with accelerated clearance of infected erythrocytes from circulating blood. As shown previously [Kempe et al. 2006], iron deficiency leads to

activation of non-selective cation channels with subsequent Ca^{2+} entry and increase of cytosolic Ca^{2+} activity. Moreover, calcium uptake into the red blood cells was shown to be markedly enhanced in patients with iron deficiency [Shimoda and Yawata 1985]. The increase of cytosolic Ca^{2+} activity in turn leads to scrambling of the erythrocyte cell membrane [Dekkers et al., 2002; Woon et al., 1999], which is enhanced in iron deficient erythrocytes [Kempe et al. 2006]. The breakdown of phosphatidylserine asymmetry leads to exposure of phosphatidylserine at the erythrocyte surface and subsequent binding to phosphatidylserine receptors at macrophages [Fadok et al., 2000; Henson et al., 2001; Messmer and Pfeilschifter 2000]. The enhanced phosphatidylserine exposure may further allow adhesion of erythrocytes to endothelial cells [Boas et al., 1998; Eda and Sherman 2002]. This adhesion may impede the microcirculation and it is thus tempting to speculate that it participates in the generation of ischemic complications of iron deficiency [Hartfield et al., 1997; Pereira and Sarnak 2003].

The enhanced susceptibility of iron deficient erythrocytes to eryptosis may relate to altered cell volume regulation. The reduced hemoglobin content may decrease the colloid osmotic pressure and thus cell volume [Schwarzer et al., 1992]. Since the non-selective cation channel is activated by cell shrinkage [Huber et al., 2001], the decreased cell volume could account for the enhanced activity of the channel. Entry of Na^{+} through this channel serves to increase cell volume, the entry of Ca^{2+}, however, activates the Ca^{2+} sensitive K^{+} channel (GARDOS channel) [Bookchin et al., 1987; Brugnara et al., 1993]. The subsequent hyperpolarisation of the cell membrane drives Cl^{-} out of the cell and the cellular loss of KCl with osmotically obliged water leads to subsequent further cell shrinkage.

In addition, iron deficient erythrocytes are more sensitive to oxidative stress [Ramachandran and Iyer 1984; Vives Corrons et al., 1995], which similarly activates the cation channel [Duranton et al., 2003; Lang et al., 2002b; Lang et al., 2003a]. The cellular loss of K^{+} through the GARDOS channel contributes to the stimulation of eryptosis [Lang et al., 2003d]. Loss of cellular K^{+} may participate in the triggering of apoptosis in several cell types [Bortner et al., 1997; Bortner and Cidlowski 1999; Gomez-Angelats et al., 2000; Hughes, Jr. et al., 1997; Hughes, Jr. and Cidlowski 1999; Montague et al., 1999; Perez et al., 2000]. Activation of Ca^{2+} sensitive K^{+} channels similarly participates in the cell

shrinkage and deformation of desoxygenized sickle cells [Bookchin et al., 1987; Brugnara et al., 1993; Franco et al., 1996; Joiner 1993; Lew and Bookchin 1991].

While accelerated eryptosis and clearance of infected erythrocytes provides an explanation for the slower increase of parasitemia in iron deficiency, the *in vitro* decrease of intraerythrocytic growth and the *in vitro* decrease of infected erythrocytes do point to additional mechanisms involved in the decreased parasitemia of iron deficient erythrocytes. Thus, accelerated clearance of infected erythrocytes contributes to but does presumably not fully account for the decreased parasitemia. Iron deficiency enhances the susceptibility of eythrocytes to eryptosis. As a result, iron deficient erythrocytes undergo accelerated eryptosis following infection with *Plasmodium* and are thus rapidly cleared from circulating blood together with the pathogen. The accelerated eryptosis precedes the full intraerythrocytic maturation of the pathogen and thus blunts the increase of parasitemia. Beyond that iron deficiency impairs intraerythrocytic growth and infection of erythrocytes.

V. 2. Impact of NO synthase inhibitor L-NAME on the course of malaria

As reported previously [Nicolay et al., 2007], NO is a potent inhibitor of eryptosis. Thus, inhibition of NO synthase is expected to favour or accelerate eryptosis. Accordingly, the NO synthase inhibitor L-NAME triggers Ca^{2+} sensitive cell membrane scrambling with subsequent exposure of phosphatidylserine at the erythrocytes surface. The treatment of mice with L-NAME accelerated the clearance of erythrocytes from circulating blood. The majority of cleared labelled cells were accumulated in the spleen. L-NAME treatment delayed the development of parasitemia.

The antieryptotic effect of NO donators was mimicked by di-butyryl-cGMP [Nicolay et al., 2007], suggesting the involvement of guanylate cyclase, cGMP and protein kinase G, a well known signalling pathway of NO action [Feil et al., 2003; Fleming et al., 1999; Friebe and Koesling 2003; Geng et al., 1998; Klein 2002; McHugh and Cheek 1998; Munzel et al., 2005; Pohl and Busse 1989; Schlossmann et al., 2003]. cGMP stimulates the Ca^{2+} ATPase [Dedkova and Blatter 2002; Ogurusu et al., 1990; Rashatwar et al., 1987; Uneyama et al., 1998; Vrolix et al., 1988], which contributes to the regulation of erythrocyte Ca^{2+} concentration [Davis et al., 1982; Xu and Roufogalis 1988]. However,

NO donators did not appreciably decrease cytosolic Ca^{2+} activity [Nicolay et al., 2007] and may thus be effective through other signalling pathways. In nucleated cells the antiapoptotic effect of NO is in part due to inhibition of caspases [Dimmeler et al., 1997; Rossig et al., 1999]. However, caspases are apparently not involved in the triggering of eryptosis following increase of cytosolic Ca^{2+} activity [Lang et al., 2003a]. Presumably, NO is effective through nitrosylation [Benhar and Stamler 2005; Haendeler et al., 2002; Haendeler et al., 2004; Hoffmann et al., 2001b; Melino et al., 1997] of enzymes necessary for induction of cell membrane scrambling [Nicolay et al., 2007].

Despite its favourable influence on the development of parasitemia, L-NAME treatment did not significantly decrease or delay the death rate of *Plasmodium* infected mice. Impaired NO formation leads to hypertension, peripheral ischemia and organ damage, which presumably outweighs the decerease of parasitemia. Thus, due to those severe side effects, inhibition of NO synthase is apparently not a therapeutic option in the treatment of malaria.

V. 3. Influence of lead treatment on the course of malaria

The treatment with $Pb(NO_3)_2$ has previously been shown [Kempe et al., 2005] to stimulate Ca^{2+} activated charybdotoxin and clotrimazol sensitive K^+ channels [Bookchin et al., 1987; Brugnara et al., 1993] resulting in erthrocyte shrinkage [Lang et al., 2003b]. Moreover, the activated cation channels may allow the entry of $Pb(NO_3)_2$ thus enhancing the toxicity of $Pb(NO_3)_2$ treatment in infected cells. $Pb(NO_3)_2$ treatment triggers Ca^{2+} sensitive cell membrane scrambling with subsequent exposure of phosphatidylserine at the erythrocytes surface.

The treatment with lead significantly decreased the development of *in vivo* parasitemia. The effect is presumably due to premature phosphatidylserine exposure of infected cells. *In vitro*, phoshpatidylserine exposure does not lead to sequestration and clearance of erythrocytes and thus does not significantly modify the increase of parasitemia. $Pb(NO_3)_2$ treated phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood. The majority of cleared labelled cells were accumulated in the spleen (Fig. 20). Decreased *in vivo* parasitemia following lead treatment may be expected to affect the survival of infected

animals. Similar to previous series [Huber et al., 2004], none of the untreated mice survived the infection with *P. berghei* (Fig. 21). In contrast, some 38 % of the $Pb(NO_3)_2$ treated mice were still alive 30 days after the infection illustrating that $Pb(NO_3)_2$ treatment indeed favourably modifies the course of malaria.

Considering the toxicity of lead, sequelae of lead intoxication include anaemia which is partially due to shortened life span of circulating erythrocytes [Osterode et al., 1999]. Lead ions adhere to erythrocyte cell membranes [Suwalsky et al., 2003], decrease the erythrocyte ATP concentration [Baranowska-Bosiacka and Hlynczak 2003; Grabowska and Guminska 1996], delay the decline of protoporphyrin concentration in mature erythrocytes [Lamola et al., 1975; Piomelli et al., 1975], and decrease 5´ nucleotidase activity [Valentine et al., 1976].

In conclusion, $Pb(NO_3)_2$ treatment may lead to slight anaemia, but is protective against a lethal course of malaria infection. We speculate that a similar mechanism confers protection against a severe course of malaria in carriers of the sickle cell anaemia trait. The observations may not only serve to understand the mechanisms of host defence against the malaria pathogen but open new perspectives for the treatment of malaria.

V.4. Influence of chlorpromazine on the course of malaria

The present experiments confirm the ability of chlorpromazine at therapeutical concentrations [Dorson and Crismon 1988] to induce eryptosis [Akel et al., 2006]. The ability of chlorpromazine to trigger scrambling of the cell membrane is shared by several amphiphiles [Hagerstrand et al., 1998; Rosso et al., 1988; Schneider et al., 1986; Schrier et al., 1983; Schrier et al., 1986; Schrier et al., 1992]. Chlorpromazine further decreased forward scatter pointing to cell shrinkage, which was presumably due to increase of cytosolic Ca^{2+} activity, activation of Ca^{2+} sensitive K^+ (GARDOS) channels [Lang et al., 2003b], K^+ exit, hyperpolarization of the erythrocyte membrane potential and Cl^- exit [Myssina et al., 2004]. The exit of KCl is followed by osmotically obliged water and thus leads to cell shrinkage [Lang et al., 1998a]. Chlorpromazine has further been reported to induce echinocytic shape [Eskelinen 1987] and cell swelling [Thompson et al., 1993].

As shown earlier [Koka et al., 2007a; Koka et al., 2007b], eryptosis is further stimulated by *P. falciparum* infection. Besides its effect on eryptosis, chlorpromazine

exerted a remarkable inhibitory effect on *in vitro* parasitemia. This effect does not correlate with the scrambling effect of chlorpromazine and is not paralleled by decreased intraerythrocytic survival of the pathogen but may be an effect on parasite internalization. Most importantly, chlorpromazine proved to be similarly effective *in vivo*. Following infection with *P. berghei* the parasitemia decreased significantly in chlorpromazine treated mice than in untreated mice.

Decreased *in vivo* parasitemia following chlorpromazine treatment may be expected to affect the survival of infected animals. None of the untreated mice survived the infection with *P. berghe*i. In contrast, 80 % of the chlorpromazine treated mice were still alive 30 days after the infection. Thus chlorpromazine treatment indeed favourably modifies the course of malaria.

The protective effect of chlorpromazine is probably not the result of a direct toxic action on the pathogen, as chlorpromazine, even at excessive concentrations, did not decrease the intracellular DNA/RNA content. Rather chlorpromazine decreases the ability of pathogens to invade noninfected erythrocytes leading to a marked decrease of parasitemia. Moreover, the scrambling effect of chlorpromazine decreased the life span of infected erythrocytes in circulating blood. In conclusion, chlorpromazine triggers eryptosis of infected erythrocytes, an effect, which favourably influences the course of malaria.

V.5. Conclusions

All the observations in our study which include iron deficiency [Koka et al. 2007a], treatment with lead [Koka et al. 2007b], chlorpromazine and NO synthase inhibition by L-NAME accelerate eryptosis of infected erythrocytes and are thus partially protective against malaria. The observations support the view that accelerated suicidal death of infected erythrocytes is a host mechanism to counteract infection with the intracellular pathogen and open new perspectives for pharmacological treatment of this devastating disease.

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VII. PUBLICATIONS OBTAINED DURING Ph.D WORK

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